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Functional Analysis of Unp, a Mammalian Ubiquitin Protease

Paola Blanchette

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Department of Biochemistry
University of Ottawa,
Ottawa, Ontario
Canada

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Abstract

The murine Unp gene encodes a ubiquitously expressed protein that fractionates with the nuclear fraction (hence its name ubiquitous nuclear protein) (Gupta, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Gray, D. A. (1993). *Oncogene* 8, 2307-10). It possesses properties of an oncogene, such as the ability to promote tumours in a nude mouse assay (Gupta, K., Chevrette, M., and Gray, D. A. (1994). *Oncogene* 9, 1729-31), and its human homologue, USP4 (previously known as Unph), was shown to be over expressed in certain types of human lung tumours (Gray, D. A., Inazawa, J., Gupta, K., Wong, A., Ueda, R., and Takahashi, T. (1995). *Oncogene* 10, 2179-83).

Although very little was known about this protein's normal function, even less on its mechanism of tumorigenicity, the predicted protein sequence gave some clues on its function. It possesses the two conserved domains present in all ubiquitin specific proteases, and the two motifs common to viral oncoproteins through which they interact with the retinoblastoma gene product pRb. In addition to these features it also possesses a region that resembles a nuclear localisation signal.

A mutational approach was taken in combination with ubiquitin cleavage assays and binding assays to study Unp's function. With these, it was confirmed that Unp is a ubiquitin specific protease, its ability to cleave ubiquitin dependent on the conserved cysteine, and may be dependent on Unp phosphorylation status. Unp is a phosphoprotein, being phosphorylated on serine residue(s). It is capable of binding to pRb's hypophosphorylated as well as the hyperphosphorylated forms, a binding that is dependent on an intact conserved motif 2 (CR2). It is also capable of binding to the other pocket proteins, p107 and p130,

although with different requirements of conserved regions that for pRb.

With these and other results obtained, a model is proposed linking Unp's activity as a deubiquitinating enzyme and its interactions with the pocket proteins with its role as an oncogene. The data obtained also supports the newer view that ubiquitin specific proteases, have a role in the specific regulation of protein levels and not just as general ubiquitin recycling enzymes as previously believed.

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List of Abbreviations

Amp	Ampicillin
β -gal	Betagalactosidase
C-terminal	Carboxy-terminal
Cdk	Cyclin dependent kinase
cDNA	Complementary DNA
CIP	Calf intestine phosphatase
CMV	Cytomegalovirus
CR1/2	Conserved region 1 or 2
Cys	Cysteine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethyl diaminetetraacetic acid
ER	Endoplasmic Reticulum
ES cells	Embryonic stem cells
FBS	Fetal bovine serum
GST	Glutathione S-transferase
His	Histidine
IPTG	Isopropylthio- β -D-galactosidase
IRES	Internal ribosome entry site
Kb	Kilobase
KDa	Kilodalton
LB	Luria broth
MEM	Minimal essential medium
mRNA	Messenger ribonucleic acid
mut	Mutant
N-terminal	Amino-terminal
NLS	Nuclear localization signal
NSCLC	Non small cell lung cancer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
pRb	Retinoblastoma tumor suppressor protein
pSer	Phospho-serine
pThr	Phospho-threonine
pTyr	Phospho-tyrosine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylimide gel electrophoresis
SCLC	Small cell lung cancer
SV40	Simian virus 40
TBST	Tris buffered saline with Tween
TRIS	Tris (hydrozymethyl) aminomethane
Ub	Ubiquitin
Ubp	Ubiquitin specific protease

USP	Ubiquitin specific protease
WT	Wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactosida

Chapter 1

Introduction

Cancer has been the leading cause of death in Canada surpassing heart disease since 1993 (Statistics_Canada, 1999). In the year 2000 there were an estimated 65,000 deaths due to cancer and an estimated 132,100 new cases of cancers diagnosed, a death to case ratio of 0.49 (Canadian Cancer Statistics, LCDC, Health Canada, 2000). Lung cancer is the most prevalent form of cancer in the human population, although it is second to breast cancer in women and to prostate cancer in men. Lung cancer has one of the highest death/case ratios at 0.85 surpassed only by pancreas and esophagus cancers. In combination with its high prevalence in the population, this high ratio makes lung cancer the most deadly of all cancers in Canada with 17,700 predicted death in 2000 compared with 5,500 for breast cancer and 4,200 for prostate cancer. Lung cancers are classified in 4 major groups: small cell lung carcinoma (SCLC), squamous cell carcinoma, adenocarcinoma and large cell carcinoma with the last three collectively known as non small cell lung carcinomas (NSCLC). The SCLC, accounting for 20-25 % (Travis et al., 1995) of the lung tumours, has a very aggressive clinical course with frequent early metastasis and a poor prognosis (Bast et al., 2000).

Specific chromosomal aberrations have been associated with lung cancer early on. Already in 1982, Whang-Peng et al. found constitutive deletions in chromosome 3p in 16 SCLC (Whang-Peng et al., 1982a, 1982b). A finding which was later confirmed by DNA polymorphism analysis in SCLC (Naylor et al., 1987; Yokota et al., 1987) as well as in NSCLC (Yokota et al., 1987; Kok et al., 1987). This event, the loss of a region of 3p was

reminiscent of the loss of the tumour suppressor pRb in retinoblastomas (Francke et al., 1976) and thus led to a model of a lung specific tumour-suppressor on chromosome 3p. In addition to chromosome 3, Yokota et al. identified two other chromosomes that consistently underwent loss of heterozygosity, chromosomes 13 and 17 (Yokota et al., 1987). While the tumour suppressor affected in chromosome 13 was found to be pRb (Harbour et al., 1988) and p53 in chromosome 17 (Takahashi et al., 1989), the identity of the chromosome 3 tumour suppressor has yet to be identified. There may be three tumour suppressors in chromosome 3 since three distinct regions have been shown to be frequent targets for 3p deletions in lung tumours (Hibi et al., 1992), these regions are 3p25, 3p21.3 and 3p14-cen.

The human homologue (USP4) of the recently cloned mouse gene Unp has been localized to chromosome 3p21.3 (Gupta et al., 1993; Gray et al., 1995). Although, as an oncogene (Gupta et al., 1994; Gray et al., 1995), Unp is unlikely to be the 3p lung tumour suppressor, it has been hypothesized that it could be activated by the chromosomal rearrangement occurring during the loss of heterozygosity event in the 3p21.3 region. Once activated, the Unp oncoprotein would be but one participant in the formation of lung tumours, with other oncogenes including the myc family (Little et al. 1983; Funa et al., 1987), the Ras family (Rodenhuis and Slebos, 1990; Slebos et al., 1990), and c-erbB-2 (Schneider et al., 1989).

In simple terms, the formation of a tumour can be defined as an unregulated growth of cells beyond what is required for the normal maintenance of the organism. Two types of events have been implicated in tumour formation: the loss of tumour suppressors and the activation of oncogenes, although oncogenesis may require a combination of many such

events (Vogelstein and Kinzler, 1993) (Hartwell and Kastan, 1994). Both of these types of events disrupt the control of the cell cycle. Tumour suppressor proteins normally act to provide a negative influence on proliferation while the oncogene products act to promote proliferation, when required. Disruption of these proteins eliminates the regulation that they provide on the cell cycle.

1.1 Cell Cycle

The cell cycle is an ordered progression through specific phases allowing a cell to grow (G1 phase), duplicate its DNA (S phase) and eventually to divide into two cells (M phase), all in the appropriate order. Progression through the cycle is mediated by the temporally regulated cyclin-dependent kinases (Cdks) (Simanis and Nurse, 1986; Draetta et al., 1989; Taya et al., 1989; DeCaprio et al., 1992). Although present throughout the cycle, these kinases are active only in specific phases (Hagan et al., 1988; Draetta et al., 1989). This temporal regulation of the Cdks is achieved by their required association with a cyclin partner whose abundance oscillates during the cell cycle (Evans et al., 1983). This oscillation is the result of their rapid, phase specific proteolytic degradation (Evans et al., 1983) which assures directionality in the cycle (Amon et al., 1994; Amon et al., 1993). A degraded cyclin cannot be accidentally reactivated as might occur if inactivation were achieved by a reversible modification. A Cdk whose activity is dependent on that cyclin will only be reactivated in the next cycle when the cyclin is reexpressed. The specific degradation of the cyclins (the Cdk partners are unaffected) is mediated by the ubiquitin pathway of protein degradation (Glotzer et al., 1991; Deshaies et al., 1995; Yaglom et al., 1995) (see section 1.2). The discovery of the ubiquitin pathway of protein degradation has

had a strong impact on the understanding of the cell cycle control, since it is involved at many levels of regulation. In addition to regulating the core of the cell cycle machinery (ie the activity of the cdks) it is also involved in the fine tuning of its regulation. Inhibitors of G1-S transition must be degraded following mitogenic signals. The activity of positively acting proteins (proto-oncogenes) must be tightly regulated, partly by limiting their half-life in order to prevent deregulated growth. These are but just examples of the importance of the ubiquitin pathway in the control of the cell cycle.

1.1.1 Checkpoints

Smooth progression through the cell cycle is achieved by the action of many checkpoints, in all phases of the cycle. These checkpoints assure that all the required events are completed before continuing to the next step in the cycle. Of these, two major checkpoints involved in the G1-S transition are usually deregulated in tumors; the pRb pathway and the p53 checkpoint.

pRb pathway

The pRb pathway is a major checkpoint that links the extracellular signals (thus the conditions outside the cell) to the control of the initiation of another round of division (Polyak et al., 1994; Day et al., 1997; Alesse et al., 1998). If the conditions do not support proliferation, the pRb pathway will favor entry into a quiescent state. If, on the other hand, the conditions favor proliferation, then it will regulate the proper transition from G1 to S phase. The critical aspect in this control is the ability of pRb to bind and functionally inactivate the transactivational activity of the E2F transcription factors (Chellappan et al.,

1991). In quiescent or early G1 cells pRb is in its active, hypophosphorylated form. It interacts with the E2F transcription factors and converts their transactivation potential into an active repression of E2F responsive genes (Ferreira et al., 1998; Ross et al., 1999). Near the end of the G1 phase, the G1 Cdks (Cdk2, 4 and 6) become activated and they phosphorylate their substrates which includes the pRb protein (Hinds et al., 1992; Kato et al., 1993; Ewen et al., 1993). Hyperphosphorylation of pRb results in its functional inactivation and the release of free E2F (Brown et al., 1999; Harbour et al., 1999). With this event, the cell passes the restriction point and is committed to another round of division (Pardee, 1989). The pRb-free form of E2F, as a heterodimer with DP-1 binds the DNA at specific E2F binding sites and transactivates the genes necessary for passage to S phase. These genes include regulatory molecules such as cyclin A, cyclin E, p107, E2F, B-myb, MCM2-7 and cdc2 and proteins involved in DNA replication including thymidine kinase, DHFR, DNA pol α and ckc6 (DeGregori et al., 1995; Leone et al., 1998).

pRb is part of a family with p107 and p130, and are collectively called the pocket proteins. They share 30-35% amino acid identity, mostly concentrated in a region termed the pocket (Ewen et al., 1991; Li et al., 1993; Hannon et al., 1993). This region, composed of two domains, box A and B separated by a spacer sequence, is crucial for binding and regulating many cellular proteins, including the E2F family members (Chow and Dean, 1996; Chow et al., 1996; Shao et al., 1997).

Despite the similarity between p107 and p130 with pRb, only pRb has been demonstrated to have tumor suppressor properties (Lipinski and Jacks, 1999). Unlike what has been observed for pRb, very few mutations in p107 or p130 have been found in human tumors. This may be due to an additional role of pRb not performed by p107 and p130. pRb

actively participates in promoting differentiation in certain lineages. pRb null mice die between 13-15 days of gestation with pronounced defects in erythroid, neuronal and lens development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In contrast to pRb, both p107 and p130 null mice are normal and fertile (Cobrinik et al., 1996; Lee et al., 1996), at least in a 129/sv background. More severe defects have been observed in a different background (LeCouter et al., 1998a; LeCouter et al., 1998b). Mice null for both p107 and p130, survive to birth but die perinatally, showing pronounced defects in endochondrial bone development with excessive chondrocyte proliferation (Cobrinik et al., 1996). The strain-dependent variations in the p107 and p130 phenotypes suggest the existence of yet unidentified modifier genes that are able to alter their function.

E2F was originally identified as a factor that bound specifically to an element in the adenovirus E2 promoter (Kovesdi et al., 1987; Yee et al., 1987). There are currently 6 members in the family (E2F-1 to E2F-6). All six contain highly conserved DNA binding domains and dimerization domains (to dimerize with their DP partners) (Helin, 1998). The C-terminal region of E2F-1 to 5 also contains a potent transactivation domain and embedded in it is a highly conserved domain that mediates binding to pRb-family of proteins. E2F-6, which does not bind to pRb family members or possess a transactivation domain, is thought to be involved in repression of E2F-dependent transcription (Morkel et al., 1997). Although pRb can bind all E2Fs (Ikeda et al., 1996; Moberg et al., 1996), it preferentially interacts with E2F-1 to 3 (Lee et al., 1993). E2F-4 binds with high affinity to both p107 and p130 (Beijersbergen et al., 1994a; Ginsberg et al., 1994; Vairo et al., 1995) while E2F-5 is seen to interact mostly with p130 (Sardet et al., 1995). The different complexes appear in different conditions and seem to have drastically different effects. For example, during

quiescence, the predominant complex is p130/E2F-4, and is involved in actively repressing E2F responsive genes. Free E2F-4 has not been shown to be very active in transactivation (Humbert et al., 2000; Rempel et al., 2000). In cycling cells the complexes mostly involve pRb in combination with E2F 1-3 (DeGregori et al., 1997; Lukas et al., 1997). These complexes function in part in the proper regulation of the transactivation potential of these E2Fs.

p53 checkpoint

The p53 checkpoint is the other major checkpoint to be disrupted in tumors, occurring in over half of all tumors (Sherr, 1998; Hollstein et al., 1994). It is activated by irregularities in the cell cycle, whether caused by DNA damage (Fritsche et al., 1993; Hall et al., 1993; Zhan et al., 1993) or by the presence of oncogenes (Debbas and White, 1993; Lowe and Ruley, 1993; Hermeking and Eick, 1994; Wagner et al., 1994; Qin et al., 1994; Wu and Levine, 1994; Serrano et al., 1997). Activation results in increased levels of the p53 protein which is normally kept low. The increase in protein levels is mostly the consequence of a change of its half life. In normal growing cells, the p53 protein has a very short half life due to its constitutive degradation by the ubiquitin pathway (Fuchs et al., 1998; Haupt et al., 1997; Honda et al., 1997). When the checkpoint is activated, the p53 protein becomes phosphorylated and this form is not recognized by the degradation machinery (Fuchs et al., 1998). The kinases involved in phosphorylating p53 are dependent on the type of the activating stress (Giaccia and Kastan, 1998). The activation of the p53 pathway results in one of two possibilities. Induction of cell cycle arrest, usually following the induction of p21, an inhibitor of G1 Cdks, or the induction of apoptosis. Induction of a cell cycle arrest

prevents the replication of damaged DNA while apoptosis eliminates aberrant cells (Stewart et al., 1999; Sionov and Haupt, 1999). Thus both alternatives function in preventing the transmission of damage to the cell's progeny. The choice between these two options depends on many factors including the cell type, the oncogenic status of the cell, the extracellular growth and survival stimuli, the intensity of the stress signals, the level of p53 expression and the interaction of p53 with specific proteins.

1.1.2 Oncogenic viruses

Some DNA viruses are dependent on host cell DNA replicative machinery and as such require cycling cells for replication. These viruses are able to push the host cells to cycle uncontrollably, thus promoting the formation of tumors. To accomplish that task, these viruses must be able to overcome the checkpoints that protect the cell cycle and thus, have evolved proteins to interfere with these checkpoints. The large T antigen of the SV40 virus (DeCaprio et al., 1988), the E7 of papillomavirus (Dyson et al., 1989), and E1A of adenovirus (Fattaey et al., 1993) are all able to bind the pRb protein and functionally inactivate it. The binding occurs through two conserved domains that they share, termed CR1 and CR2. Binding of these viral proteins to pRb through these domains causes pRb to release E2F thus inducing the cell cycle (Watanabe et al., 1992) (Fattaey et al., 1993). This improper induction of the cell cycle would normally activate the p53 checkpoint (Debbas and White, 1993), but another set of proteins also inactivate the p53 protein. The SV40 large T antigen (Levine et al., 1991) and the adenovirus E1B (Debbas and White, 1993; Yew and Berk, 1992) proteins bind to p53 and inactivate its transactivational potential. The papillomavirus E6 protein action also results in the inactivation of the p53 protein, but is

doing so by a different strategy. It is able to recruit a cellular protein, E6AP and use it to induce the ubiquitin-mediated degradation of p53 (Scheffner et al., 1993).

1.2 The Ubiquitin Pathway

The concept of intracellular protein degradation has drastically changed over the years. It has evolved from an unregulated, nonspecific terminal scavenger process assumed by the lysosome, to a highly complex, tightly regulated process termed the ubiquitin pathway. It plays important roles in a broad range of basic cellular processes, including cell cycle, differentiation and development, response to stress and extracellular modulators, modulation of cell surface receptor, DNA repair, signal transduction, regulation of transcription, chromosomal silencing, regulation of the immune and inflammatory responses, biogenesis of organelles, and apoptosis. Thus, to assume these functions, the ubiquitin pathway must be highly specific. It must be able to distinguish between the short-lived proteins and the many stable proteins that are present in the cell and sometimes even in the same complex, and to discriminate between mutated or misfolded proteins from their normal counterparts (Laney and Hochstrasser, 1999). A classical example that demonstrates the specificity involved, is the degradation of cyclin B1. Cyclin B1 is a short-lived protein that is essential in the G2-M phase transition of the cell cycle. It is the regulatory component of a cyclin-dependent kinase complex created by binding with Cdc2. In order for the cell to complete its M-phase, the cyclin B1 subunit must be irreversibly inactivated (Glutzer et al., 1991). The cell accomplishes this inactivation by selectively degrading the cyclin component from the complex, while the Cdc2 partner remains unaffected.

One key determinant for this specificity is that the short-lived proteins are first tagged

by covalent attachment of ubiquitin molecules before being directed to the degradation machinery, the proteasome. It is this feature that is lacking in the prokaryotic ATP dependent proteases, Clp and Lon, which have only limited specificity (Gottesman et al., 1997).

Degradation by the ubiquitin pathway involves 2 major steps: first, the recognition and conjugation of the substrates (see Figure 1.1) and second, the degradation by the proteasome.

1.2.1 Components of the Ubiquitin Pathway

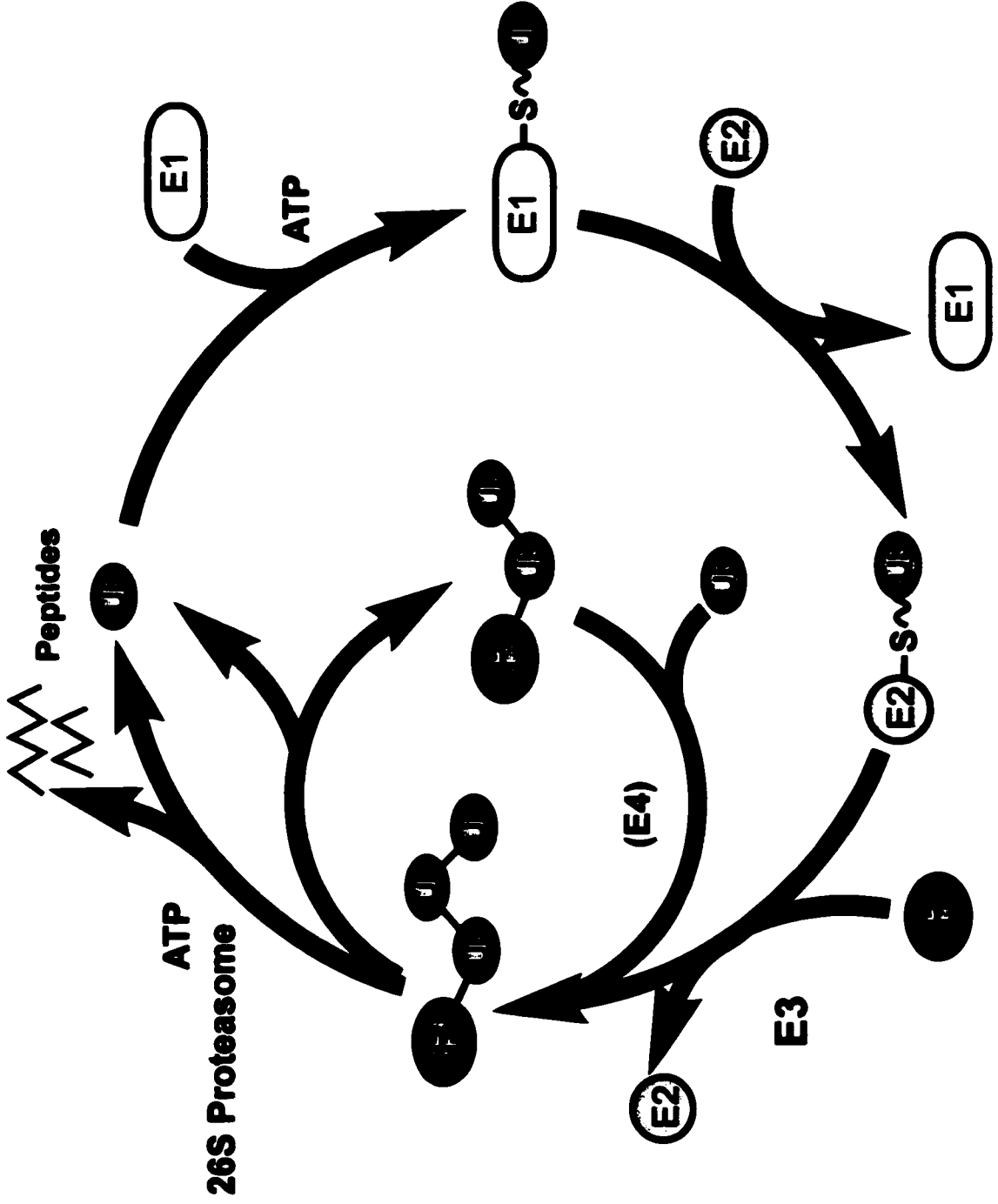
Ubiquitin

Ubiquitin, named for its expression in all cells and tissues, and in most species (with the exception of prokaryotes) is a small, highly conserved and extremely stable 76 amino acid polypeptide. The primary sequence among higher eukaryotes is absolutely conserved, and only a few (up to 3) residues substitutions are observed in lower organisms (Haas and Siepmann, 1997). Part of this unusual sequence conservation derives from structural requirements for correct folding and stability (Lazar et al., 1997), and the selective pressures imposed by interactions with multiple components of the ubiquitin/proteasome pathway.

The great stability of ubiquitin reflects its tightly folded structure which consists of a globular domain made of a 3.5 turn amphipathic α -helix intercalated into a five-strand mixed β -sheet (Vijay-Kumar et al., 1987). From this is protruding a flexible 4 amino acid c-terminal tail. This structure is often depicted as a stick and ball model in diagrams. The observation that more mutations occurs in lower eukaryotes, suggest that ubiquitin is involved in fewer functions (perhaps in fewer protein interactions), implying reduced

Figure 1.1 Ubiquitin pathway

Ubiquitin is first activated on its C-terminal glycine by an E1 activating enzyme, a reaction requiring ATP. This molecule of ubiquitin is transferred to the active site cysteine present in the E2 conjugating enzyme. The E2, with the E3 ligase enzyme then conjugates the ubiquitin onto an internal lysine residue of the substrate. Some classes of E3 will first transfer the ubiquitin to themselves before conjugation to the substrate. This cycle is repeated until a polyubiquitin chain is formed, with the participation of another enzyme, E4 with some substrates. Once the polyubiquitin chain has attained sufficient length, it is recognized by the proteasome, the substrate degraded and the ubiquitin recycled.



selective pressure (Haas and Siepmann, 1997).

Ubiquitin Conjugating Enzymes

Conjugation of ubiquitin to the substrate is achieved by a cascade of enzymatic reactions involving up to 4 classes of enzymes designated E1 to E4. Prior to conjugation to a substrate the ubiquitin molecule must be activated. This role is assumed by the E1 enzyme (also termed ubiquitin activating enzyme) a single gene copy in most cells (with the exception of wheat which contains 3 related genes (Hatfield and Vierstra, 1992)). The human gene, Hsub1 is alternatively spliced to produce a short cytoplasmic form as well as a longer nuclear form, E1a and E1b respectively (Hatfield et al., 1990; Stephen et al., 1997). The E1 enzyme has an essential role since deletion of the E1 in yeast is lethal (McGrath et al., 1991).

The activation of ubiquitin by the E1 enzyme requires the hydrolysis of ATP thus forming PPi in addition to a tightly bound (to the E1) ubiquitin-adenylate at its C-terminal glycine 76. The adenylate then becomes the donor of ubiquitin to an active-site sulfhydryl group of E1 forming a thiol-ester bond. This E1 (with one ubiquitin bound) is capable of undergoing another round of ATP-dependent AMP-ubiquitin formation resulting in a ternary complex of one E1 with two activated ubiquitin molecules (Haas et al., 1982). The activated ubiquitin is then transferred from the E1 to a conserved cysteine of a ubiquitin conjugating enzyme (E2, also called ubiquitin carrier protein), in a transthioation reaction. Unlike E1, there are many members of the E2 family (11 members in yeast), functionally characterized by their absolutely conserved cysteine residue and their ability to receive ubiquitin molecules from E1.

In the last step of ubiquitin conjugation, the E2 catalyses the transfer of the ubiquitin molecule from itself to the ϵ -amino group of a lysine residue in the substrate to generate an isopeptide bond. In some cases, the ubiquitin can be conjugated in a linear fashion on the N-terminal residue of the substrate (Breitschopf et al., 1998). Usually a member from a third group of enzymes, the ubiquitin ligases (E3), is required in order for this transfer to occur. The critical function of the E3s is substrate recognition. Each E3 will recognize specific motifs in their substrates whether a short sequence, as in the destruction box recognized by the APC complex (Glotzer et al., 1991), or specific phosphorylation events on substrates of the SCF complexes (Chen et al., 1995; Diehl et al., 1997). These motifs are termed degradation signals. For many substrates, these signals are still unknown.

The currently known E3s have been divided into 6 subgroups with regard to their structures and the type of degradation signals they recognize. In three of these subgroups, the E3s are in the form of a complex (the APC, the SCF and the VHL subgroups). Each of these 3 subgroups, the members are almost identical. The complexes remains mostly the same except for one component, the substrate recognition protein (call F-box protein in SCF), which varies depending on the substrate it conjugates. This allows a greater variety of substrates to be conjugated with a limited number of proteins involved. For example, in the SCF complex (for Skp/Cullin/F box) the identity of the F box protein will change according to the identity of the substrate. Proteins targeted by these SCF complexes are mostly although not all, involved in the degradation of proteins implicated in the G1/S transition of the cell cycle and tend to be recognized in a phosphorylation dependent manner. These E3s often also serve as scaffold proteins that bring together the E2 and the substrate.

Hect domain proteins, another subclass of E3s, are involved in an additional step in the conjugation cascade. The ubiquitin molecule is first transferred from the E2 to the E3 enzyme before it catalyses the transfer to the substrate.

Once the first ubiquitin is attached on an internal lysine residue of the substrate, a polyubiquitin chain is formed by subsequent rounds of ubiquitination, involving the same cascade, except that with these, the ubiquitin is conjugated on an internal lysine of the previous ubiquitin molecule, usually on lysine 48. In some cases, yet another enzyme, termed E4, is required for the conjugation of a chain of more than three ubiquitins (Koegl et al., 1999). Since it is not required for cell viability in yeast (Koegl et al., 1999), this enzyme seems to be required only for a subset of substrates.

In addition to lysine 48, six other lysines are present in ubiquitin and although they can be utilized for polyubiquitination, chains linked through lysine 48 bonds are the principal signal for proteasomal proteolysis (Chau et al., 1989; Finley et al., 1994). In fact, of all the lysine mutants, only the mutation of lysine 48 is lethal when expressed as the sole source of ubiquitin in yeast. In addition, there has been no evidence to date demonstrating that conjugation through other lysines serves as a target for proteolysis. On the contrary, it has been shown that lysine 63 conjugates have a role in DNA repair (Hofmann and Pickart, 1999) that is independent from proteolysis, as seen by two lines of evidence: first, it has been shown that the inability to assemble lysine 63-linked chains does not inhibit the turnover of short-lived and abnormal proteins (Spence et al., 1995) and second, the conjugation of a lysine 63-linked chain to a specific yeast protein does not destabilize that protein (Hofmann and Pickart, 1999). These observations suggest that the proteasome does not recognize lysine 63-linked polyubiquitin chains as a degradation signal.

Proteasome

The proteasome is a large, 26S, multicatalytic protease that degrades poly-ubiquitinated proteins into small peptides, ranging from 3 to 22 residues in length (Kisselev et al., 1999). The proteasome is a 2.1MDa complex composed of approximately 65 subunits (Baumeister et al., 1998; Rechsteiner, 1998). It can be separated into two subcomplexes, a catalytic 20S subunit, to which is attached a regulatory 19S particle on each end (see Figure 1.2).

20S proteasome

The 20S proteasome is made of 14 different subunits, classified as α and β subunits on the basis of their homology to the simpler version of the proteasome found in the archaeobacterium *Thermoplasma acidophilum* (Lowe et al., 1995). Electron microscopy and X-ray crystallography of these proteasomes, as well as the 20S proteasomes from *Saccharomyces cerevisiae* (Groll et al., 1997) reveals a cylindrical structure made up of 4 stacked rings, $\alpha, \beta, \beta, \alpha$, with a narrow channel in the centre. Each ring is made up of seven subunits and whereas in the primitive archaeobacteria proteasome these subunits are identical, in the yeast (and higher eukaryotes) the rings are made up of seven different subunits giving the general structure of $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$. Three catalytic sites: the trypsin-, chymotrypsin- and post-glutamyl peptidyl hydrolytic-like sites are located in the β -subunits, facing the interior of the barrel, shielded from the cytoplasm (Groll et al., 1997). The sites are only active when the two β -rings are attached and the β -subunits from the two rings are able to interact. The catalytically inactive α rings play an essential role in stabilizing the two β -rings as well as interacting with the 19S regulatory subcomplexes.

Figure 1.2 Representation of the proteasome

A The 26S proteasome consisting of the core 20S proteasome and capped at each end by a 19S regulatory subunit. Figure modified from Voges et al., 1999.

B The structure of the 20S proteasome showing the degradative chamber formed by the assembly of the 2 β rings and the narrow entrance to the tunnel created by the α rings. The active sites formed at the interface of the 2 β rings are shown. Figure modified from Stock et al., 1996

A



B



19S Regulatory Complex

Electron microscopy has also shown that both ends of the 20S proteasome are capped by a 19S particle. This complex can be further divided in two domains: a base, consisting of six ATPases and three non-ATPases subunits, in addition to the lid which consists of eight non ATPase subunits (Glickman et al., 1998).

Since folded proteins cannot reach the small chamber inside the barrel shape of the 20S proteasome, this subunit by itself hydrolyses only small peptides and denatured proteins in an ATP independent process. To enable the proteasome to degrade folded proteins specifically marked for degradation by a ubiquitin chain, the 19S complex is thought to carry out a number of different biochemical functions. First, it can recognize polyubiquitinated substrates. The subunit Rpn10/S5A has been shown to bind polyubiquitin chains. Since it is not essential in yeast (van Nocker et al., 1996), there must be additional ubiquitin binding component(s). It is possible that this function could be achieved by a complex of two or more proteins in the 19S particle. Second, binding of the 19S complex to the 20S proteasome may function to create a channel for the substrates to enter the proteasome. Unlike the *Thermoplasma* 20S proteasome, the yeast 20S does not have the narrow opening at each end of the particle (Groll et al., 1997). Third, since the chamber inside the 20S proteasome is not big enough to accommodate folded proteins, it is assumed that the 19S particle plays a crucial role in unfolding the targeted polypeptide chain and in inserting it into the 20S particle. This function is thought to be accomplished by a reverse chaperone activity of the six ATPases at the base of the 19S complex. There is some evidence to suggest that this type of activity is possible from the study of the *Escherichia coli* clpA-clpP protease (Kessel et al., 1995). ClpA is the regulatory subunit of the clpP protease and like the six

ATPases in the base of the 19S, it has homology to the AAA family of ATPases. In the presence of clpP, clpA promotes unfolding and degradation of the substrates, but in the absence of clpP, it has been shown to function as a chaperone (ie. in promoting proper folding) (Wickner et al., 1994). The base of the 19S particle which contains the 6 ATPase subunits has also been demonstrated to exhibit chaperone-like activity (Braun et al., 1999). This suggests that in the presence of the 20S, it may also be capable of reversing its activity. And fourth, the 19S complex possesses deubiquitinating activity provided by the UCH37 deubiquitinating enzyme in the complex (Lam et al., 1997) (deubiquitinating enzymes will be discussed in a subsequent section).

Proteasomes in Antigen Presentation

When comparing the 20S subunits of the proteasome from mammalian cells with yeast, three additional β -type subunits are found in the mammalian cells. These subunits, LMP2, LMP7 and MECL1, are inducible by γ -interferon. In replacing three other closely related β -type subunits in the normal 20S, they help form the immunoproteasome (also called immunosome). These immunoproteasomes have been implicated in antigen processing, supported by the observation of a deficit in presentation of certain antigens in cells from mice carrying a deletion of either LMP2 or LMP7 (Fehling et al., 1994; Van Kaer et al., 1994).

Two other proteasome interacting polypeptides, the REG α and REG β , are induced by γ -interferon. These subunits, assembling in an alternating pattern, form a ring-shaped hexamer called the 11S regulator or PA28 (Song et al., 1997). This regulator binds and activates the 20S proteasome in a similar manner as the 19S regulator with the exception that it does not require ATP for its assembly. A PA28-20S-PA28 complex can only digest

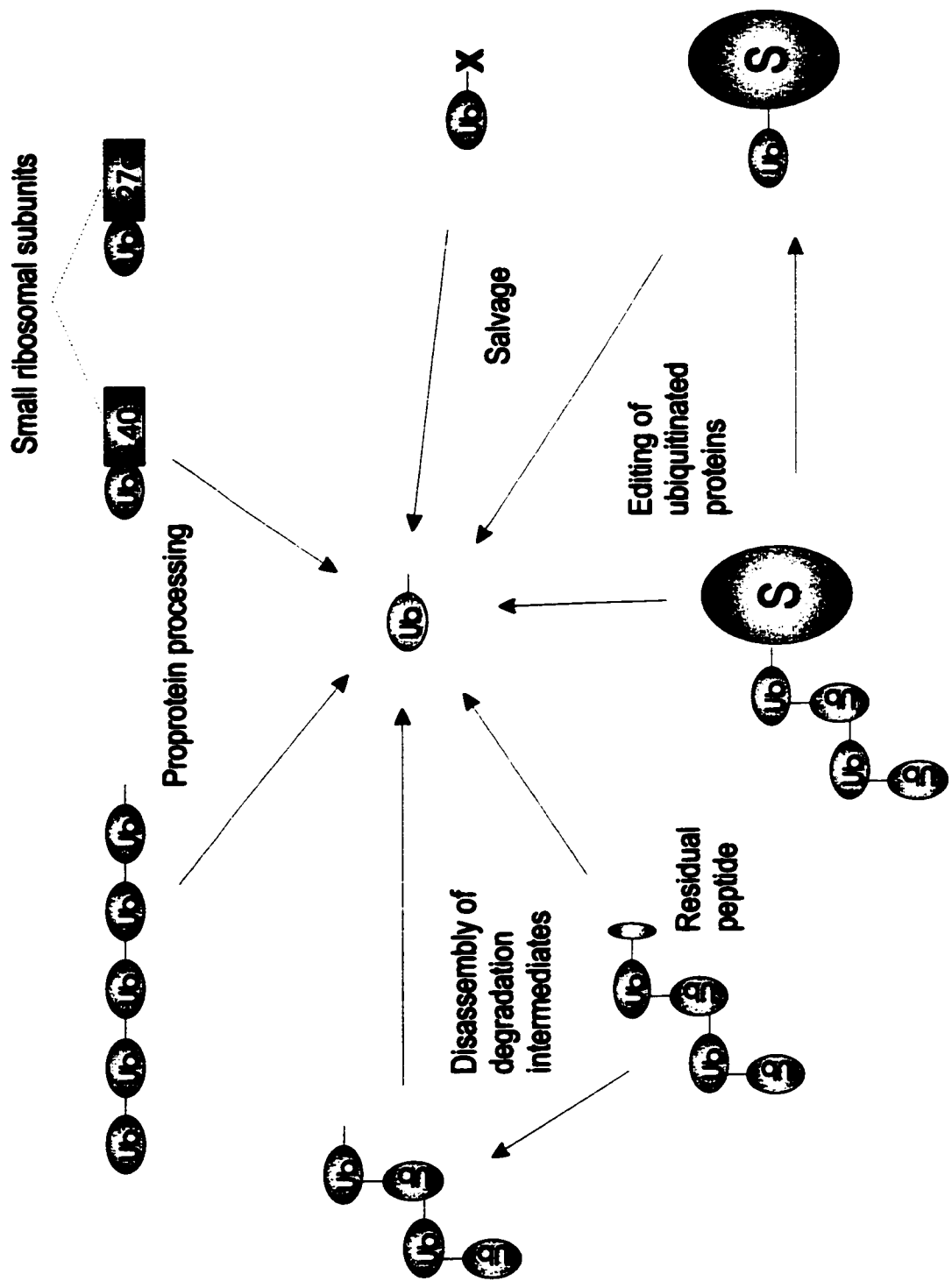
peptides and not intact ubiquitin-conjugated proteins. Combined with the fact that the subunits are induced by γ -interferon it also suggests a role in antigen presentation. Indeed, it has been shown that overexpression of PA28a (REG α) in cell lines that also expressed viral protein antigens results in an enhanced presentation of peptides derived from these proteins (Groettrup et al., 1996). This type of complex is probably acting downstream of the 26S proteasome by trimming larger peptides that were generated by the 26S complex. This may assure that peptides of the proper length are generated to be utilized in antigen presentation (usually around 9 amino acid long). Interestingly, the existence of an asymmetrical 19S-20S-PA28 proteasome has been reported (Hendil et al., 1998) suggesting that this process may occur in a consecutive manner.

Deubiquitinating Enzymes

Possessing opposite actions to the ubiquitin conjugating enzymes, are the deubiquitinating enzymes (also called ubiquitin specific proteases). They specifically hydrolyse ester, thiol ester, and amide bonds to the carboxyl-terminal glycine (G76) of ubiquitin (Rose and Warms, 1983; Pickart and Rose, 1985; Tobias and Varshavsky, 1991). These enzymes have roles at many levels in the ubiquitin pathway (see Figure 1.3). The first of them being the processing of precursors of ubiquitin. Unlike most other proteins, ubiquitin is not translated in a “ready to use” form, but instead it is produced as a fusion to either other ubiquitin molecules (polyubiquitin fusion gene) or to ribosomal subunits (fused with L40 and S27a). These precursors are then processed by the deubiquitinating enzymes (the identities of which are still unknown) to produce monomeric free ubiquitin ready to be conjugated on substrates. As an interesting note, fusion of ubiquitin to the ribosomal subunit

Figure 1.3 The multiple function of deubiquitinating enzymes

Ubiquitin is synthesized as precursor fusions to either itself or small ribosomal subunits that must be processed by cleavage at the C-terminal glycine. Following degradation of the substrate, the polyubiquitin chain is cleaved from the residual peptide and then disassembled into monomers. Deubiquitinating enzymes can counterbalance the effects of the conjugating machinery by editing polyubiquitinated substrates. Ubiquitin can also be trapped by reactions with cellular thiols or amines and must be salvaged (mostly the function of the UCH). Figure modified from Wilkinson, 1997.



is required for efficient assembly of the ribosomes, the ubiquitin moiety assuming a chaperone-like function (Finley et al., 1989). Deubiquitinating enzymes also function to salvage ubiquitin that has been trapped by reaction with small cellular nucleophiles, to recycle the ubiquitin from the polyubiquitin chains of a degraded substrate, and to edit polyubiquitin chains on a substrate. The deubiquitinating enzymes are divided in two families, the ubiquitin carboxyl-terminal hydrolases (UCH, also called type 1 UCH) and the ubiquitin-specific proteases (UBP, also called type 2 UCH) (Wilkinson, 1997).

UCH

This family was the first to be identified. Members were originally purified on the basis of their ability to bind ubiquitin affinity columns (Miller et al., 1989). They exhibit a preference for cleaving small adducts from ubiquitin such as peptides and amino acids. They tend to be small proteins (25 to 30 KDa) with the exception of human BAP1, which has a molecular mass of 81KDa (Jensen et al., 1998). The crystal structure of a human UCH, UchL3, has been published (Johnston et al., 1997). The catalytic core domain strongly resembles that of cathepsin B, a member of the papain family of thiol proteases (Johnston, et al., 1997). In the active site is located the catalytic triad consisting of a cysteine, a histidine and an aspartic acid, all of which had been predicted by mutational analysis (Larsen et al., 1996). The specificity of UCHs is enforced by substrate-induced conformational changes induced by binding to ubiquitin (Johnston, et al., 1997).

Ubp

The first three Ubps were originally cloned using an assay to detect yeast enzymes that could deubiquitinate a ubiquitin- β -galactosidase fusion protein (Baker et al., 1992; Tobias and Varshavsky, 1991). This assay has since become the traditional test for Ubp

activity. Members of the Ubp family are extremely diverse (unlike UCHs that have up to 40% conservation across species (Chung and Baek, 1999). They are homologous only in 2 regions: the Cys box and the His box (Papa and Hochstrasser, 1993; Wilkinson et al., 1995). They are bigger and more numerous than the UCH (16 Ubps in yeast compared with 1 UCH). They can cleave at the C-terminal of ubiquitin in natural and engineered fusions irrespective of their sizes. In addition to their ability in cleaving linear fusions, many of the Ubps have been shown capable of cleaving ubiquitin conjugated by isopeptide linkages (the form of ubiquitin in the chains) (Lam et al., 1997; Papa et Hochstrasser, 1993; Amerik et al., 1997; Layfield et al., 1999). Presently, more than 70 Ubps have been cloned in different species (and entered in GenBank), but for a very few exceptions, their substrate specificities are unknown.

1.2.2 Regulation of the Ubiquitination Pathway

Traditionally, regulation of the ubiquitination pathway has been thought to be accomplished exclusively at the level of ubiquitin conjugation, with the role of deubiquitinating enzymes limited to the recycling of ubiquitin, a view still held in the recent review by Ciechanover et al. (2000). But it is becoming increasingly evident that regulation is also achieved by the action of deubiquitinating enzymes. An interesting parallel can be made between the ubiquitin conjugating/deubiquitinating enzymes and the kinases/phosphatases enzymes. Originally, only the kinases were viewed as key regulators of protein phosphorylation *in vivo*, while the small number of phosphatases were thought to play only housekeeping roles. In the current view, the important regulatory functions can be performed by either the kinase or the phosphatase depending on which pathway is

involved (D'Andrea and Pellman, 1998; Denu et al., 1996; Klingmuller et al., 1995).

Recognition of Ubiquitin Chain by the Proteasome

In an *in vitro* system it was determined that a substrate needs to be ubiquitinated by 4 moieties to be efficiently recognized by the proteasome (Cook et al., 1994). Four consecutive ubiquitin molecules on the chain have been shown to form a tetramer structure, and it is this structure that is recognized by the proteasome, not the individual ubiquitin moieties (Cook et al., 1994). In fact, the affinity of the proteasome for the tetramer is about 100 fold that with only two ubiquitin molecules present (Thrower et al., 2000). All ubiquitin moieties in the chain are equivalent in forming the tetramer binding unit. However, inside the unit all ubiquitins are not equivalent. Ubiquitin 1 (the proximal) and 3 are the most directly involved in the actual binding, although the ubiquitin 2 and 4 are necessary for the formation of the proper conformation (Thrower et al., 2000). Binding of the substrate to the proteasome is mediated entirely through the polyubiquitin chains. The substrate itself does not bind the proteasome (Thrower et al., 2000). Contrary to the previous belief that the chain participates in the unfolding of the substrate (Ghislain et al., 1996; Pickart, 1997), there is no indication that the chain performs any functions besides targeting to the proteasome (Thrower et al., 2000).

In an *in vivo* system more than four ubiquitin molecules are required for efficient degradation (Thrower et al., 2000; Hochstrasser, 1996). This discrepancy can be attributed to the function of the deubiquitinating enzyme UCH 37, present in the 19S subunit. It cleaves ubiquitin from a proteasome bound substrate starting from its distal end. This results in gradually decreasing the length of the chain (Lam et al., 1997). Since all the experiments

to determine proteasomal affinity were performed in the presence of ubiquitin aldehyde, an inhibitor of deubiquitinating enzymes, its effect was not observed. This suggests a model in which a longer polyubiquitin chain than four moieties is required so that the substrate will be bound to the proteasome sufficiently long to allow time for the protein to be unfolded, probably the limiting step in degradation (Thrower et al., 2000), and fed to the 20S proteasome. If the chain is not sufficiently long, the action of the proteasome-bound UCH will result in a chain shorter than four ubiquitins before the protein is unfolded and degraded. Since a chain this length cannot stably bind to the proteasome it might escape degradation.

The Monoubiquitin Signal

A consequence for the requirement of polyubiquitin chains for recognition and degradation is that monoubiquitin conjugated on proteins can serve as a different type of signal.

Some proteins in the cell are tagged with a single ubiquitin molecule. However, unlike the polyubiquitin chain, monoubiquitination does not serve as a signal for degradation. The functions of this signal is starting to emerge, although they are far from being fully understood.

Histones

Normally, between 5 and 10% of nuclear histones H2A and H2B are monoubiquitinated during interphase. In synchronized cells experiments, it was found that both types of histones were completely deubiquitinated during metaphase, which coincides with complete condensation of the mitotic chromatin (Mueller et al., 1985). The histones were reubiquitinated by the time the cells had progressed to anaphase, a time frame of only

minutes (Matsui et al., 1979). It is still unknown what the function of these modifications are but it has been speculated that they must be removed, possibly by Ubp-M (Cai et al., 1999) for the cell to undergo mitotic condensation and mitosis. Ubp-M was shown to be able to deubiquitinate histone H2A *in vitro* and an inactive mutant transfected in cells was observed to associate closely with mitotic chromosomes followed by apoptosis of the cells.

Endocytosis

Another recently found role for monoubiquitin is in the endocytosis of plasma membrane proteins. This function is mostly studied in yeast where the proteins, the pheromone receptors Ste2p (Hicke and Riezman 1996; Terrel et al. 1998) and Ste3p (Davis et al. 1993; Roth and Davis 1996), the uracil permease FUR4 (Gallan et al. 1996) and the maltose transporter (Lucero et al. 2000) were found to be ubiquitinated on the cytoplasmic tail followed by endocytosis and degradation in lysosome-like vacuoles. In these cases, the function of the ubiquitin molecule appears to be a direct signal for internalization of the plasma membrane protein. It has recently been shown that ubiquitination of the cytoplasmic tail does not promote internalization by inducing a structural change to expose a previously masked signal, rather, the ubiquitin molecule itself is the signal (Shih et al., 2000). Two patches on the three-dimensional surface of ubiquitin are required for this function. Ubiquitination, or at least the ubiquitination machinery is also required for endocytosis of mammalian hormone receptors including the growth hormone receptor (GHR)(Govers et al., 1999), the epithelial growth factor receptor (EGFR)(Opresko et al., 1995; Waterman et al., 1999) and the epithelial Na⁺ channel (EnaC) (Staub et al., 1997). In these cases the proteins can be polyubiquitinated.

Site of degradation

Ubiquitin mediated degradation clearly occurs in the cytosol. In addition there is strong evidence to suggest that it occurs at the cytosolic side of the endoplasmic reticulum (ER) membranes. Proteins to be degraded from the ER must first be retrotransported into the cytoplasm (Plempner and Wolf, 1999; Sommer and Wolf, 1997). It is still unclear if degradation occurs in the nucleus. Although components of the system have been localized to the nucleus (Brooks et al., 2000), conjugation and degradation have yet to be demonstrated in this compartment. In addition, drugs that inhibit nuclear export almost completely prevent Mdm2- and E6-AP/E6- dependent degradation of p53 (Freedman and Levine, 1998). There is also evidence that suggests the degradation of p27 also occurs in the cytosol following nuclear export (Tomoda et al., 1999). The relationship between nuclear export and degradation is still not understood.

Deubiquitination

Depending on the level of action, whether before or after the proteasomal degradation step, a deubiquitinating enzyme may promote either the degradation or the stability of a given protein substrate. Two well-characterized Ubps that promote degradation are Isopeptidase T (Ubp14 in yeast) and Doa4. IsoT's role in the cell is to disassemble unanchored polyubiquitin chains. It sequentially degrades these chains into free monomeric ubiquitin starting from the free proximal end of the branch chain (the end that was conjugated to the substrate) (Wilkinson et al., 1995), having for an effect the recycling of the ubiquitin pool. An Ubp14 null yeast strain accumulates unanchored polyubiquitin chains (Amerik et al., 1997). The half-life of several ubiquitin pathway substrates were also

prolonged, probably due to excess ubiquitin chains binding to the proteasome and interfering with its activity.

Doa4's role appears to be the cleavage of the peptide remnant on the chain after proteasomal degradation (Amerik et al., 1997; Wilkinson et al., 1995) thus producing free polyubiquitin chains that become substrates for Ubp14. Doa4 mutants exhibit growth defects, hypersensitivity to DNA damage (Singer et al., 1996), and impaired degradation of several ubiquitin substrates (Papa and Hochstrasser, 1993). Small peptides bound to ubiquitin polymers also specifically accumulate in these mutants (Papa and Hochstrasser, 1993). It is still unresolved whether Doa4 is an actual component of the proteasome.

On the other hand, deubiquitinating enzymes may promote the stability of substrates by specifically removing ubiquitin moieties from the chains, and preventing that particular substrate from being efficiently recognized and degraded by the proteasome. Since in yeast there are more deubiquitinating enzymes (16) than ubiquitin conjugating enzymes (11), it is unlikely that all deubiquitinating enzymes participate only in recycling functions. If this model was to be true, the half-life of a substrate would be determined by the balance between the ubiquitinating and the deubiquitinating enzymes. In this case, a change of expression of either the ubiquitinating or deubiquitinating enzymes would lead to a change of the half-life of the substrate. If this substrate happens to be a regulator of the cell cycle, then the effects could be tumorigenic. One possible example of this is the Tre-2 proto-oncogene. The full length Tre-2 is a deubiquitinating enzyme for which the substrates remains unknown. A truncated isoform was found to be transforming. This isoform is lacking the His domain and therefore deubiquitinating activity (Nakamura et al., 1992). These observations suggest that at least one substrate of full length Tre-2 is a tumour suppressor, whose half-life is prolonged

by the deubiquitination action of Tre-2. The truncation of Tre-2, which renders it catalytically inactive, thus reduces the stability of the tumour suppressor which in turn promotes tumorigenicity. The truncated form of Tre-2 may even act as a dominant negative.

Another example of a deubiquitinating enzyme that acts to stabilize a particular substrate(s) is the *Drosophila* deubiquitinating enzyme Fat facets (faf). The faf gene is specifically required for eye development in *Drosophila* (Fischer-Vize et al., 1992). Mutant flies are viable but have abnormal eye morphology caused by the presence of extra photoreceptor cells in each eye facets. Faf is a Ubp, the largest in the family, and its deubiquitinating activity is required for its function (Huang et al., 1995). In addition, the faf phenotype is suppressed by a mutation in a proteasome subunit gene (Huang et al., 1995). One substrate has been proposed for faf, liquid facets (lqf), a newly described gene involved in the endocytosis pathway. A slight increase of this protein in the appropriate cells is sufficient to completely abolish the faf phenotype (Cadavid et al., 2000) suggesting that the function of this deubiquitinating enzyme is the regulation of the lqf protein levels. There are indications that the lqf protein is not the only substrate of faf. The mouse homologue, Fam seems to be involved with different kinds of pathways (Taya et al., 1998), observations that are also supported by evidence discovered with the *Drosophila* faf studies (Li et al., 1997).

1.2.3 Alternative pathways

The normal sequence of events in the ubiquitin pathway is the polyubiquitination of proteins followed by their complete degradation. However, there are some cases in which these two events are independent of each other. One example is the degradation of the ornithine decarboxylase (ODC) protein. This protein has been shown to be degraded by the

proteasome but this degradation is not dependent on prior ubiquitination. In this case the recognition by the proteasome is mediated by the interaction of ODC with antizyme, a negative regulator of ODC's enzymatic activity (Hayashi et al., 1996; Murakami et al., 1992). Another example demonstrating the uncoupling of ubiquitination and degradation is the limited processing of p105, a precursor protein of the transcription factor NF- κ B, (Oran et al., 1995; Palombella et al., 1994). This protein is ubiquitinated and targeted to the proteasome, but unlike most proteins, the degradation by the proteasome is incomplete. The partial degradation (from the C-terminal) results in the specific formation of p50, a subunit of the active transcription factor NF- κ B. This partial processing is regulated by phosphorylation (Heissmeyer et al., 1999), and is dependent on the appropriate positioning of a glycine rich stop signal (Lin and Ghosh, 1996).

1.2.4 Ubiquitin-like proteins

In the last few years, proteins with some degree of homology to ubiquitin have been discovered. At least eight have been identified in mammalian systems with SUMO-1 (SMT3 in yeast) and NEDD8 (Rub1 in yeast) being the most studied (Jentsch and Pyrowolakis, 2000). Like ubiquitin these molecules are also conjugated to proteins in a manner very analogous to the ubiquitin pathway. They have E1 enzymes (sometimes in the form of a dimer) that are specific for each ubiquitin-like molecule as well as specific E2s. Some substrates even require E3 enzymes. Members of the PIAS family are required for conjugation of SUMO on yeast septins (Johnson and Gupta, 2001), p53 (Kahyo et al., 2001) and LEF1 (Sachdev et al., 2001). Although conjugation of SUMO-1 to p53 *in vitro* requires only p53, SUMO-1, the SUMO-1 activating enzyme and ubc9 (E2) (Rodriguez et al., 1999).

Unlike the conjugation of ubiquitin, these proteins cannot form chains, with the possible exception of SUMO-2 and 3 (Tatham et al., 2001) and the modification of proteins does not target them to the proteasome for degradation.

The functions of these conjugates are not fully elucidated. Although conjugation does not lead to degradation, in some instances its effects parallel the ubiquitin pathway and can affect a protein half-life indirectly. Conjugation of SUMO-1 to I κ B α occurs on lysine residue K21 which is also utilized for ubiquitin modification (Desterro et al., 1998). Conjugation of ubiquitin which is induced by phosphorylation results in the degradation of I κ B α and the activation of NF- κ B. Modification by SUMO-1 which is inhibited by phosphorylation of I κ B α , prevents ubiquitination and the degradation of I κ B α and thus the activation of NF- κ B (Desterro et al., 1998). p53 is another protein that can be modified by both ubiquitin and SUMO-1. It is still unclear if the modification by SUMO-1 alters the stability of p53, but it has been shown to result in an increased transactivational ability of p53 (Gostissa et al., 1999; Rodriguez et al., 1999).

Another possible function of the modification by ubiquitin-like molecules is to affect localization of the proteins or the stabilization of complexes. Conjugation of PML and SP100 with SUMO-1 is required for their localization in the PML nuclear bodies. Modification of RanGAP1 by SUMO-1 is also required for stable interactions in the nuclear pore complex (Mahajan et al., 1997; Matunis et al., 1996). All members of the cullin protein family are modified by the NEDD8 protein (Hori et al., 1999). The cullins are part of a subtype of E3 complexes and these complexes must be formed prior to their modification by NEDD8 (Kamura et al., 1999; Liakopoulos et al., 1999). The role of these modifications is thought to be the stabilization or the activation of the ligase complex formed (Jentsch and

Pyrowolakis, 2000) and is required for efficient ubiquitination of p27 by the SCF^{Skp2} complex (Podust et al., 2000) and for the degradation of p53 by adenovirus E4orf6/E1B55K complex (Querido et al., 2001).

1.3 Thesis Work

A few years ago, in an analysis of genes linked to the Mpv 20 retroviral insertion site, the *Unp* gene was cloned (Gupta et al., 1993). It was named *Unp* for the preliminary evidence that suggested it was a ubiquitously expressed nuclear protein (Gupta et al., 1994). It was detected by northern analysis in every mouse tissues tested, and in fractionation experiments it was detected exclusively in the nuclear fraction. When overexpressed, *Unp* is an oncogene. This was first demonstrated in nude mice experiments where *Unp* transfected NIH3T3 cells consistently produced tumours (Gupta et al., 1994). In addition, its human homologue, USP4 (previously named *Unph*), was shown to be overexpressed in specific types of human lung tumours (small cells and adenocarcinomas) (Gray et al., 1995). The predicted amino acid sequence of *Unp* was found to contain the 2 domains, Cys and His that had been associated with deubiquitinating activity in yeast Ubp1, 2 and 3, suggesting that *Unp* is also a deubiquitinating enzyme. Interestingly, *Unp* also possesses two regions, (CR1 and CR2 regions) that in viral proteins (like adenovirus E1A) mediates binding to pRb leading to the hypothesis that *Unp*'s tumorigenic activity, when overexpressed, may be a result of stabilizing a protein in the pRb pathway by cleaving ubiquitin from its chain, thus preventing its degradation by the proteasome.

Chapter 2

Materials and Methods

Plasmid construction

Site-directed mutagenesis (Cys, CR2, CR1 and NLS mutants)

These mutations were created using the “Altered Sites *in vitro* Mutagenesis System” kit from Promega. This consisted of cloning the Unp cDNA into their vector pAlter and producing single stranded DNA using the M13 phage. Mutagenic oligos were then annealed to produce the desired mutations. Clones that seemed positive for the mutation by restriction digest were sequenced and sub-cloned into the pCDNA3 vector (Invitrogen), a mammalian cell expression vector in which the gene of interest is expressed under the strong constitutive pCMV promoter. A resistance gene, coding for G418 resistance is also included in the vector under the SV40 promoter. The oligos used were (mutation underlined): CR1- ATT CTA GAC GGA CTG CTC GAG GAC CTG AAC CGC NLS- ATG TCA CAG CCG CAG AAG AAT TCG AAG GCG GCA GTA GCC CTG CR2- GAC TTT GGT GGG CCC AGA ATG T. Genbank accession number for Unp cDNA is 6755942. The Cys mutant was given by Rohan Baker (Australian National University, Canberra, Australia), and the details of its construction are published (Gilchrist et al., 1997)

PCR deletion (acidic domain mutant)

The acidic domain mutant (Δ DE) was created by PCR reaction with oligos flanking the domain, facing away from it. (1- ATC ACT TAA GAT ACC TAC TCC TAG AGC and 2- TCA TCT TAA GAG CGC CCA AAA GGT GAA). The PCR reaction, which resulted in a linearized plasmid lacking the domain, was ligated together after digest with Afl II

enzyme to regenerate the sticky ends. This restriction site was engineered in order to keep the sequence in frame.

IRES-based vectors

The cDNA from WT and Unp mutant was cut from the pcDNA3 vector with SpeI and ligated into the pECE11 vector that was linearized with XbaI (compatible ends). In this vector the gene of interest is expressed under the SV40 promoter, as well as the resistance gene, hygromycin. This creates a bicistronic mRNA from which the second gene (Hyg) is translated with the help of an internal ribosome entry site (IRES).

Bacterial GST-fusion constructs

The wt or mutant Unp cDNA was cloned in frame with the GST tag in the bacterial expression vector pGEX4T3, using a specially designed adapter linking the BamHI site from the GST to the BspEI site situated just downstream of the initiating methionine of Unp. This adaptor encoded the first amino acids of Unp that would otherwise be missing after the restriction digest. (Oligos: 1- GAT CGA TGG CGG AAG GCC GCG GCA GCC GTG AGC GAC and 2- CGC TCA CGG CTG CCC CGG CCT TCC GCC ATG)

Cell lines and culture conditions

NIH 3T3 (mouse), COS1 (monkey), 293T (human), and HeLa, (human) cells were maintained in alpha medium (Gibco) supplemented with 10% donor bovine serum.

HFF, primary human fibroblast cells, and P19 (mouse embryonal cell line) were maintained in alpha medium supplemented with 7.5% donor bovine serum and 2.5% fetal bovine serum.

H1299 cells, a human lung carcinoma cell line of epithelial morphology, a gift from Dr Branton, (Department of Biochemistry, McGill University, Montreal Quebec) were cultured

in medium supplemented with 10% fetal bovine serum. H1299 stable cells lines were cultured with 0.4 mg/mL Hygromycin (Roche).

Transfections

Transfection methods and conditions were optimized for each cell line, and done according to the manufacturers' instructions. NIH3T3 cells were electroporated at 450 V and 250 μ F. HFF cells at 300V and 960 μ F and COS1 cells at 220 V and 960 μ F. P19, 293T and Hela cells were transfected with Fugene6 (Roche) following the recommended protocols. H1299 were transfected with Effectine (Quiagen) following these conditions: 300 000 cells were plated in 6 well dishes the day before transfection. They were transfected with 2 μ g DNA with 10 μ L Effectine and 3.2 μ L enhancer according to the provided protocol.

Immunofluorescence

Since the only antibody available against the endogenous Unp protein did not function in immunofluorescence, Unp constructs were made with the Myc tag, for use with the α Myc antibody. Cells (COS1) transfected with these constructs were grown on coverslips, fixed with -20°C methanol for 10 minutes, washed in PBS, and incubated with the primary antibody for 1h at 37°C in a humidity chamber or overnight at 4°C. The primary antibody (α Myc) was diluted 1/100 in PBS+ 0.3% Triton X-100. After 3 washes with PBS, the coverslips were incubated with the secondary, an FITC conjugated goat α mouse, (Amersham, diluted 1/20, same buffer) for 45 min. at 37°C in humidity chamber, protected from light. The coverslips were washed again 3 times with PBS before one wash in anti-Fade buffer (Slow Fade kit, Molecular Probes) and mounted on slide using the buffered

glycerol mounting media provided in the anti-fade kit. Immunofluorescence pictures were taken both on a confocal microscope (a Leica Confocal Laser Scanning Microscope equipped with an FITC filter combination, operated by Andrew Vaillant situated in the biology department, at the University of Ottawa) and on a fluorescent microscope (Zeiss Germany Axiphot)

SDS-PAGE and immunoblots

Cells were lysed with 500 μ L (for 100 mm dishes, 300 μ L for 60 mm dishes) of lysis buffer containing protease and phosphatase inhibitors (20mM Tris pH 7.5, 150mM NaCl, 2mM DTT, 1% Triton X-100, 5% Glycerol, 2mM NaF, 2mM NaPPi, 50 μ M β -glycerophosphate, 500 μ M Na-vanadate, 200 μ g/mL PMSF, 5 μ g/mL leupeptin, and 2 μ g/mL aprotinin). After 20 minutes on ice, soluble proteins were recovered in the supernatant following a 10 min. centrifugation at 4°C to pellet cell debris. Proteins were mixed with 3X SDS sample buffer (NEB), boiled and run on 7.5% SDS polyacrylamide gel, containing a 4% stacking gel (pH 6.8). Proteins separated of SDS-PAGE were transferred to a nitrocellulose membrane (Hybond C+, Amersham) using a wet transfer method, at 400 mA for 50 min in transfer buffer (25 mM Tris-Cl, 192 mM glycine and 20% (v/v) methanol). The membranes were blocked 1 hour at room temperature or over night at 4°C in 5% skim milk in TBST (10 mM Tris-Cl pH 7.6, 150 mM NaCl, 0.05% Tween) prior to probing.

Membranes were incubated with primary antibody (see Table 2.1 for dilutions) diluted in 5% skim milk in TBST for 1 hour at room temperature. Following 3 times 8 minutes washes in TBST, they were incubated for 45 min. with the appropriate secondary antibody conjugated to horseradish peroxidase, and wash in the same manner. The signal

was detected after a 1 min incubation with a chemiluminescent substrate (Kirkegaard and Perry Laboratories) by exposure to X-ray film. When a phospho-amino acid antibody was to be used on a membrane, TBST was replaced by PBST (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄ and 0.05% Tween) at every steps to reduce the high levels of background inherent to these antibodies.

Immunoprecipitations

100 mm dishes of cells were lysed in 500 µL of lysis buffer and were incubated overnight with the appropriate antibody (see Table 2.1 for dilution). Antibody-antigen complexes were recovered by binding to protein G sepharose beads (Gammabind G Sepharose, Amersham Pharmacia) that had been previously washed in lysis buffer. Following washing in the lysis buffer, the proteins were eluted in SDS loading buffer (NEB) by boiling. The samples were then manipulated as described in the previous section.

Table 2.1 Antibody dilutions

Antibodies	clone #	company	dilution for IP	dilution for WB
UNP	CSM-11 (rabbit)	Zymed	1/100	1/500
Myc	9E10 (mouse)	donated by Dr Bell	1/50	1/200
pRb	G3-245 (mouse)	Pharmingen	1/100	1/200
p107	C-17 (rabbit)	Santa Cruz	1/100	1/200
p130	C-20 (rabbit)	Santa Cruz	1/100	1/200
E2F-1	KH95 (mouse)	Santa Cruz		1/500
E2F-1	C-20 (rabbit)	Santa Cruz	1/125	

pSer	PSR-45 (mouse)	Sigma	1/100	1/500
pThr	PTR-8 (mouse)	Sigma	1/50	1/100
pTyr	PY20 (mouse)	Transduction Laboratories	1/100	1/500
E1A	M58 (mouse)	Pharmingen	1/250	
Ub	Z0458 (rabbit)	Dako		1/600
Sumo-1		Zymed	1/250	
mouse	2 HRP-conj.	KPL		1/5000
rabbit	2 HRP-conj.	Cederlane		1/5000

Production and purification of fusion protein

The method used is a modification of the protocol given with the pGEX vector and incorporation of suggestions made by Takeshima et al (Takeshima et al., 1994). Briefly, 500 μ L of an overnight culture of TG1 bacteria containing the fusion plasmid was added to 1.5 mL of fresh LB-Amp and left to grow to exponential phase for 30–45 min. The cultures were induced with 1.5mM IPTG and grown for 4h at room temperature. The pellets from 6 such cultures were combined, and lysed in PBS + 1% Triton plus inhibitors (same concentration as with cell lysis buffer) by a freeze/thaw cycle followed by 4 rounds of sonification. The GST fusion proteins were recovered from the supernatant by incubating with 200 μ L of a 50% slurry of previously washed (50mM Tris pH 7.4, 75mM NaCl) glutathione-agarose beads (Sigma) for 1 to 2 hours at 4°C. After extensive washing in lysis buffer, the beads were kept as a 50% slurry in lysis buffer (with inhibitors) a 4°C. The expression was verified by running ~30 μ L of 50% fusion beads on PAGE gel followed by detection of proteins by silverstaining of the gel.

Silverstaining

The silverstaining protocol was obtained from Maniatis laboratory manual (Sambrook et al., 1989) and all solutions were made according to the protocol. The protein gel was fixed overnight in fixing solution (30% ethanol, 10% acetic acid). The next day, it was washed 2 times 30 minutes in 30% ethanol and 3 times 10 minutes in double distilled water. It was incubated for 30 minutes in 0.1% silver nitrate. Following a brief wash in water, the proteins were visualized by incubation in 2.5% sodium carbonate and 0.02% formaldehyde. When the signal was sufficiently strong, the reaction was stopped by incubation in 1% acetic acid.

Metabolic labelling of cells

Approximately 80% confluent 100 dishes of cells were washed and incubated with pre-warmed minus-cysteine, minus methionine media + 5% fetal calf serum for 30 minutes. The cells were then labelled with 200 $\mu\text{Ci/mL}$ of ^{35}S methionine/cysteine (Promix, Amersham) for 5h at 37°C with 5% CO₂. The cells were washed and lysed in presence of inhibitors. (See IP section)

GST-pull down (radioactive)

Lysates from *in vivo* labelled cells were pre-cleared with GSH-agarose beads overnight at 4°C and added to GST-fusion beads. GST alone was used for negative control. The beads were incubated 1 to 2 hours at 4°C, washed 5X with the lysis buffer (+inh.), boiled in SDS protein loading buffer (NEB biolabs) and loaded on a 7.5% polyacrylamide gel. After separation, the gel was fixed for 20 min. (10% acetic acid, 20% methanol, 70% H₂O)

dried, and exposed on a phosphorscreen. Results were visualized using the STORM phosphorimager and software (Molecular Dynamics)

GST-pull down (antibody detection)

These were done in essentially the same way as the radioactive version except that normal growing cells were used instead of *in vivo* labelled cells, and the gels with the migrated proteins were transferred to a nitrocellulose membrane (Hybond C+, Amersham) to be probed with specific antibodies.

Functional assays (bacterial)

Plasmids containing Unp and mutants, in the pcDNA3 vector, were electroporated in competent bacteria containing either the pAC-R- β gal or pAC-M- β gal (control) plasmids. These plasmids, a gift from Dr Rohan Baker (Molecular Genetics Group, John Curtin School of Medical Research, Australian National University, Canberra), contain the Ub-X- β gal fusion proteins, a substrate for deubiquitinating enzymes. The transformed bacteria were spread on LB/Chloramphenicol(35 μ g/mL)/Amp(100 μ g/mL)/Xgal and grown overnight at 37°C

Cleavage assay (*in vitro*)

Preparation of ³⁵S labelled Ub-GST substrate

Bacteria containing the pRB307 plasmid were donated by Dr Rohan Baker, and the preparation of labelled substrate was done following his protocol. Briefly, 500 μ L of an overnight culture was added to 50 mL LB/Amp and grown until OD600 was about 0.9. The

pellet was washed in M9 broth (6g/L Na₂PO₄, 3g/L KH₂PO₄, 1g/L NH₄Cl, 0.5g/L NaCl, 0,2% glucose, 1mM MgSO₄, 5x10⁻⁵% thiamine, and 0.1 mM CaCl₂). The pellet was then resuspended in same broth plus aminoacids (1/33 volume of Gibco MEM Non-Essential Amino Acid solution +Trp, His and Leu at 40 µg/mL) +40 µg/mL Amp + 1mM IPTG and grown for 1h at 37°C. The bacteria were labelled with 70 µL of Translabel, ICN (a mixture of ³⁵S met. and cys) for 3h before addition of unlabelled methionine to 1mm. After washes in M9 broth, the cells were lysed with sequential addition of 25% sucrose/50mM Tris pH8, lysosyme, and Triton X-100. The supernatant was diluted with 50 mM Tris pH7,2 and incubated with pre-washed glutathione-agarose beads for 2 hours. The beads were washed and the Ub-GST fusion protein eluted in 50mM Tris/5mM GSH pH9.6. Fractions containing the fusion (detected by autoradiogram of gel-migrated samples) were pooled and dialysed with 50 mM Tris pH7.2/1 mM β-mercapto-ethanol, aliquoted and stored at -80°C.

Cleavage reaction

The GST-UNP fusion or immunoprecipitated Unp (wt or mut) still bound to beads (~20 µL of 50% beads in buffer containing 2 mM DTT except where specified) were incubated with 5 µL of substrate for 1h at 37°C. The whole reaction was then boiled with 10 µL SDS sample buffer, run on a 10% PAGE gel, fixed, dried and exposed on a phosphorscreen.

CIP treatment

In the cases where dephosphorylation of the immunoprecipitated Unp was necessary, the IP/beads complexes were split in 2 equal parts. One part was washed in buffer minus phosphatase inhibitors (see IP) and adjusted to contain MgCl₂ to 0.1 M. To this, 1 µL of Calf

Intestine Phosphatase (CIP, NEB Biolabs) was added to the beads incubated for 1h at 37°C.

Production of Unp knock-out ES cell lines

Construction of targeting vector

A 5.3 kb EcoR1 fragment from phage clone UG 7-3 containing exons 7, 8 and 9 was used to clone the 5' and 3' regions into a bicistronic vector (containing an IRES β geo sequence)(Mountford et al., 1994) using linkers. A 2.5kb EcoR1-BsaW1 fragment corresponding to genomic base pairs 16622-19083 was first cloned into BluescriptII SK+ vector (in EcoR1-XmaI sites), followed by digestion at the EcoRV site (just outside of EcoR1) and ligation of a NotI linker. From this construct, the fragment was released by a NotI digest(present from linker and downstream of the XmaI site) and cloned into the NotI site 5' of the IRES. This fragment contains at its 3' end exon 7 minus the last 15 bp and part of the intron preceding exon 7. For the 3' fragment, first a bigger, 2.2 kb XbaI- XhoI fragment was cloned in the same sites in BluescriptII SK+ vector, followed by digestion at the internal EcoRV site and ligation of a XhoI linker. From this construct the fragment,corresponding to genomic base pairs 20123-21813, was released by a XhoI digest and cloned into the XhoI site downstream of the polyadenylation sequence in the bicistronic vector containing the 5' fragment. The 3' fragment contains the totality of exon 9 and part of the flanking introns.

Culture of ES cells

J1 ES cells passage 13 were obtained from John Bell's lab (Cancer Research Group, Ottawa Regional Cancer Centre) and cultured in ES cell media (DMEM (high glucose, without sodium pyruvate),+ 15% fetal calf serum, Gibco non-essential amino acids, Gibco

Glutamax, pen-strep and 0.1 mM β -mercaptoethanol) plus leukemia inhibitory factor (LIF) at 500 units/mL on irradiated feeder cells (mouse embryo fibroblast cells from Tik KO mice irradiated with 3000 rad)

Transfection of targeting vector

Targeting vector was linearized with SspB1 enzyme, and 50 to 100 μ g resuspended in 400 μ L electroporation buffer (20mM Hepes, 137mM NaCl, 5mM KCl 0.7mM Na₂HPO₄, 6 mM Dextrose and 0.1mM β -mercaptoethanol) for the electroporation of approximately 60 million cells at 400V and 25 μ F. The cells were plated in 4 X 100mm dishes containing each 3 million feeder cells. 24h following transfection, G418 was added to a final concentration of 0.2 mg/mL

Picking and freezing colonies

Colonies were usually ready to pick 10 to 12 days post selection. Picked colonies were trypsinized in 60 μ L for a few minutes at 37°C. Half of the colony was plated in a 96-well dish containing feeder cells, while the other half was plated in 24 well dishes without feeder cells. After 2-3 days growing in 96 well dishes, the colonies were trypsinized in 80 μ L and 80 μ L of media containing 2X serum and 20% DMSO was added. The cells were overlaid with 2 drops of autoclaved liquid parafilm and frozen at -80°C.

DNA extraction

When cells growing in the 24 well dishes attained confluency, 500 μ L of lysis buffer (100 mM TRIS pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 100 μ g/mL proteinase K) was added per well and left at 37°C until all clones had been incubated with lysis buffer at least overnight. The buffer (very viscous at this point) was then transferred into eppendorf tubes and the DNA was precipitated with 500 μ L of isopropanol.

Southern analysis

15 µg of DNA was digested overnight with the appropriate enzyme and analysed by standard Southern Blot analysis. The DNA fragment used to probe the blots is a 1kb EcoR I digestion product (from pKJ119 plasmid, which contains a fragment of Unp's genomic DNA) containing exon 5 and 6. It is situated outside of the region cloned in the targeting vector.

Chapter 3

Results

3.1 Characterization of Unp

Following the cloning and sequencing of Unp (Gupta et al.,1993), its protein sequence was compared with the sequences in protein databases in the hope of finding domains that may give indications of its role. Three types of domains were found with homology to other proteins. The first group consists of two regions (termed Cys and His domains) that match consensus sequences that are highly conserved in a family of proteins, the ubiquitin specific proteases, (Ubps). The second group consists of exact match of two motifs (CR1 and CR2) common in three transforming viral proteins implicated in the interaction with the pRb tumour suppressor. The other motif has similarity to the nuclear localization signal (NLS) found in the p53 protein.

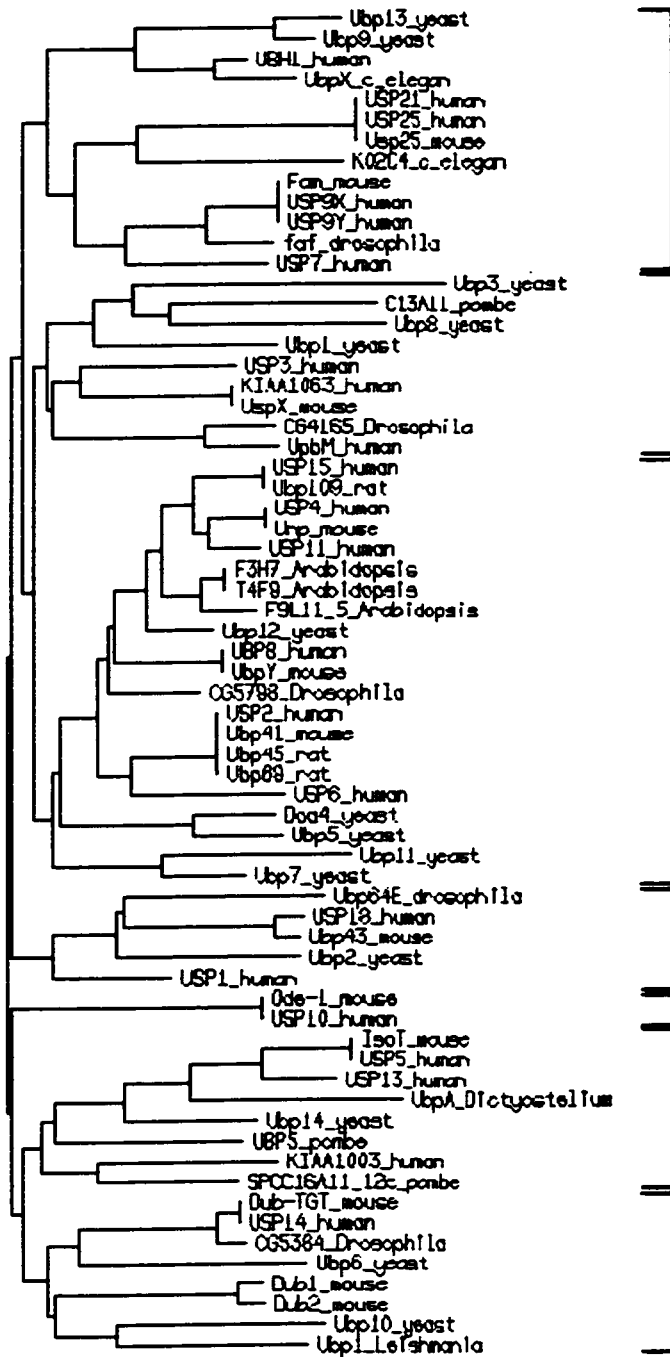
The Ubps were originally discovered in yeast and were best characterized in that system. Despite being very diverse in nature, they share a few regions of homology including the highly conserved cysteine (Cys) and histidine (His) domains. When including Unp with these proteins in a block analysis, it becomes evident that Unp also possesses these regions of homology. Figure 3.1.1 shows a recent result of a block analysis performed with Ubps of mouse, human, *Drosophila*, yeast, and rabbit origins. In addition to blocks containing the Cys domain (Block A) and the His domain (Block E), other blocks of homology are found. These blocks are very similar to the results obtained in the analysis done with the yeast Ubps (Wilkinson 1997), indicating that they are general domains

Figure 3.1.1 Block analysis of deubiquitinating enzymes

Deubiquitinating enzymes of different species were analysed for blocks of similarity with a Blocks analysis. Arrows points to the three residues forming the catalytic triad. The other conserved histidine residue is marked with an asterisk.

involved in the function of the enzymes. On the top of each block is indicated a consensus sequence that is more stringent than the one presented in Wilkinson's review (Wilkinson 1997). The presence of these domains in Unp suggest that it is a member of this family and possesses deubiquitinating activity. Assuming for the moment that Unp is a Ubp, we can analyse its structure in more details. Wilkinson refers to these domains as the core catalytic domain of Ubps. In addition to the Cys and His domains, the function of the other sequences identified in the blocks is not known with the exception of a conserved aspartic acid residue, present in Block B, which in combination with the cysteine and histidine residues (indicated with arrows) constitute the catalytic triad of the active site of the enzymes. Like many other Ubps, including yeast Ubp2 and Doa4, human Tre-2, and drosophila Faf, Unp possesses a N-terminal extension. It also shares with its closest relatives (see figure 3.1.2 and description below) an insertion of approximately 300 (400 for yeast Ubp12) amino acids between Blocks C and D (Unp, TRE-2, USP11, USP15 and Ubp12 in figure 3.1.2). These extensions and insertions are assumed to confer substrate specificity and localization to the enzymes (Lin et al., 2000; Lin et al., 2001). The putative NLS falls in the insertion, but interestingly, the CR1 and CR2 domains fall into Blocks B and C respectively. The relation of Unp with the other Ubps was determined by constructing a phylogenic tree of available Ubps. From this analysis, it was determined that the most closely related yeast Ubp is Ubp 12. A recent version of the tree analysis is shown in figure 3.1.2. This tree was constructed with the help of the Grow Tree software at the Seqweb website of the University of Ottawa. The Cys and His domains, the most conserved regions in Ubps, were extracted from more than 60 Ubps found in GenBank and entered into the Grow Tree software. The correction method used was the Jukes-Cantor with a gap weight of 8. The sequences in this tree can

Figure 3.1.2 Phylogenic tree of deubiquitinating enzymes
The tree was constructed with sequences of the Cys and His domains of available Ubps with the Grow Tree software at the University of Ottawa SeqWeb website.



10.00
substitution per 100 residues

VII

VI

V

IV

III

II

I

10.00

be divided in six early divergent groups. In this classification, Unp belongs in group 1 which is also the largest, at least to date. This group also includes USP4, Unp's human homologue, yeast Ubp12, and USP6, a human proto-oncogene previously named Tre-2. In addition to its human homologue USP4, the closest relatives to Unp are the recently cloned human USP11 and USP15, and rat Ubp109 which is the homologue of human USP15. A complete sequence alignment, shown in Figure 3.1.3, shows that the similarity between these proteins extends to more than the Cys and His domains. High sequence homology is detected throughout the sequences, except for the extreme C-terminal end and a region between the CR2 up to, and including, the NLS. The CR1 and the CR2 domains are also present in all these sequences. In the three human proteins (USP4, USP11 and USP15), the identity between them varies from around 50% (4 and 11) to around 60% (4 and 15), excluding approximately 200 N-terminal amino acids missing in USP11.

Since the Ubps have been mostly studied in the yeast organism, it was hoped that finding the closest yeast relative would give indications on Unp's role as a mammalian Ubp. Unfortunately, the function of yeast Ubp12 is also unknown (Hochstrasser, 1996). Our approach in discovering Unp's function was to perform a mutational analysis of the different domains identified in Unp and investigate their effects on possible enzymatic activity, sub-cellular localization, and protein interactions. Another, complementary approach taken, was to generate mice null for Unp to study the effects caused by the absence of a functional Unp protein.

Mutations in the identified domains were created by site-specific mutagenesis. The sequence of these domains as well as the mutations created are shown in figure 3.1.4. In addition to the domains identified by the homology search, a region of Unp was found to be

Figure 3.1.3 Sequence alignment of Unp and other closely related Ubps
The sequence alignment was produced with the Pretty software at the University of Ottawa SeqWeb website. The Cys and His domains are underlined, the CR1 and CR2 motifs are shaded, the NLS is shown in bold, while the acidic domain is italicized.

	1					70
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	mgssdvssre	cslvynedpd	ftdgttppcdr	lqvdlmavid	dkdei.kqesv	pvsdreiedt esdasavssf
Consensus	-----	-----	-----	-----	-----	-----
	71					140
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	asaneliaep	haasetnigt	ngqdgrrvle	qqrdrvvarli	eenketqkeg	dkvcivpkvw y...dkffdp
Consensus	-----	-----	-----	-----	-----	-----
	121					210
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	dvtdepedi..gpint	rmicrdfenf	vledy..nrc	pylsiaepvf	nfiseiygmt sqsyppvtnl
Consensus	DSWD-Y--G-	-N--PGPIDN	SGL--D---Q	-LKEHLIDEL	DY-L-P-E-W	NKL--WY---
	211					280
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	veqgmfvkhc	kvevylltelk	lcengnmannv	vtrrfskadt	idtiekeirk	ifsipdekert rlwnkysnt
Consensus	V--G-FVKHC	KVEVYL-ELK	LCEN-----NV	----FSKADT	I-TIEKE-RK	-F-IP-E-E-
	281					350
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	feinkpdpst	iqdagl.yqg	qvlvieqkne	dgtwpr....gps..	...tpnvkns nyclpsytay
Consensus	-E-L-K-D-T	-QDAGL-YQG	QVLVIE--NE	DGTWPR----	-----PS--	-----S
	351					420
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	kn.....ydys	epgrnne.qp	gicqlslqln	tcfmnsalgc	lsntpplitey
Consensus	-N-----G-	-----YN--	EP-----QP	GLCGL-NLGN	TCFMNSALQC	LSNTPPPLTEY
	421					490
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	flndkyqeel	nfdnplqmrq	eiakysaeli	kqmwsgkfsy	v.....tpr	afktqvgrrfa pqfsgyqqqd
Consensus	FL-D-Y--E-	N-DNPLGM-G	EIA-AYAELI	KQMWSG----	V-----P-	-FKTQVGRFA PQFSGYQQCD
	491					560
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	sqellaflld	glibedlnrri	kkpyiqlkda	dgrpd.....	..kvvaeaw	enhkkrnds i vdtfhgflk
Consensus	SQELLAFLLD	GLIBEDLNR--	KKPY-ELKDA	-GRPD-----	---VVA-EAM	ENH--RNDVS
	561					630
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	stlvpcacq	isvtfdfpcy	ltlplpakk.	.ertlevyiv	rmcpltkpmq	ykvvvpkign ildlctalsa
Consensus	STLVPCACQ	VSVTFDFPCY	LTLPPLP-KK-	--R--EVFLV	PMDP---P-Q	Y-V-VPK-G-
	631					700
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	lsgipadkmi	vtdiynhrfh	rifandenls	s.....im	erddiyvfe.i n..nrteat
Consensus	LSGI-A--M-	V-D-YNHRFH	-IF-MDE-LS	-----I-	-RDDI-VYE-	-----V
	701					770
USP15	-----	-----	-----	-----	-----	-----
Ubp109	-----	-----	-----	-----	-----	-----
Unp	-----	-----	-----	-----	-----	-----
Usp4	-----	-----	-----	-----	-----	-----
USP11	-----	-----	-----	-----	-----	-----
Ubp12	.ehvii...p	..vclre...kfrh	ssythh.tgs	sl.....	..fgqplm avprnn.ted
Consensus	-E-----P	--V-LRE---	-----S	-----G-	-L-----	---FGQP-L-

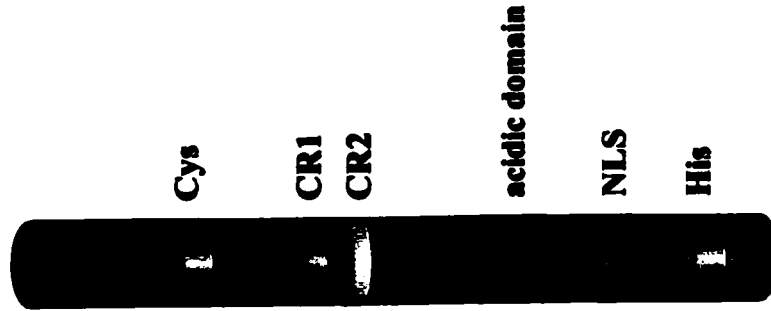
	771					840	
USP15	klynllllrm	cryvkistet	eetegslhcc	kdqningnqp	ngiheegsps	emetdepdde	ssdqqlpse
Ubp109	klynllllrm	cryvkistet	eetdqlrcc	edqningnqp	ngiheegsps	emetdepdde	ssdqqlpse
Unp	slyqavcdri	sryvkqplpd	eflsspie..p	gacngsrssy	eqdeeeemch	qeegkeqise
Usp4	slyqavcdri	sryvkqplpd	efgsspie..p	gacngsrnsc	eqdeeeemch	qeegkeqise
USP11	glynvlymrl	sryvtpnsd	deddgd....	ekeddeedkd	dvpgpstqgs
Ubp12	eqykdlqyt	sikvtdkds	ffsikilsve	keqqfasnrr	tqpnfwtpis	qlnlakatdi	ddkledvvd
Consensus	-LY-----R-	SRYVK-----	E-----L---	-----P		E-E-DE--D-	-----E--SE
	841					910	
USP15	nen.sqseds	vvg.....dn	dsenqlcted	tckg..qltg	hkkrr..lft.	fqf.nnlgnt	diny.ikddt
Ubp109	nen.sqseds	vvg.....dn	dsenqlctee	tckg..rltg	hkkrr..lft.	fqf.nnlgnt	diny.ikddt
Unp	veg.sgeddky	..g.....nd	hse.....aq.	kvkg..q..p	rhrk..lft.	fslvnscgta	din.....
Usp4	veg.sgedep	..g.....nd	pset....tqk	kikg..q..p	cpkr..lft.	fslvnsygt	din.....
USP11	lrd.pepeqa	gps.....sq	vtncrpfild	ncigtswpp	rrrrkqlft.	lqtvnsngts	drtt.spee.
Ubp12	iyssslvdc	aegvlmqvdd	eqdteqseak	nfskpfqsgd	deenketvtn	nenvntndr	deemeltddv
Consensus	-E--S---D-	--G-----D-	-SE-----	--KG--Q---	--KR--LFT-	F--VN--G--	DIN-----
	911					980	
USP15	rhirfddrql	r.....	ldersflal	dwdpdkkry	fdenaaedfe	khesv.....
Ubp109	rhirfddrql	r.....	ldersflal	dwdpdkkry	fdenaaedfe	khesv.....
Unp	.slatdqkll	k.....	lnsrstlai	dwdsetrsly	fdeqeseace	khism.....
Usp4	.slaadgkll	k.....	lnsrstlam	dwdretrrly	ydeqeseaye	khvsm.....
USP11	eedastepel	tdkpealdki	kdsitstpfa	lismndiivc	ewselgsnea	fsddkiynwe	npatlpnke!
Ubp12
Consensus	-----D--L	-----	-----	-L--RS-LA-	DWD-----Y	FDE---E--E	KH-S-----
	981					1050	
USP15eykpp	kkpfvklkdc	ielfttkekl	gaedpwycpn	ckehqqatkk	ldlwsllpvl	vvhlkrfsys
Ubp109eykpp	krpfvklkdc	ielfttkekl	gaedpwycpn	ckehqqatkk	ldlwsllpvl	vvhlkrfsys
Unp eykpp	kkaavalrec	ielfttmetl	gehdpwycpt	ckkhqqatkk	fdlwsllpvl	vvhlkrfsyn
Usp4 eykpp	kkttvalrdc	ielfttmetl	gehdpwycpn	ckkhqqatkk	fdlwsllpvl	vvhlkrfsyn
USP11gyvm..	kkapvrlqec	ielfttveti	ekenpwycps	ckqhqlatkk	ldlwsllpvl	vvhlkrfsyt
Ubp12	enaklersna	kertitlddc	lqlfskpeil	gltswycpt	ckehrqtakq	iqlwntpdil	lhlkrfsesq
Consensus	-----	KK--V-L-DC	IELFTT-E-L	G--DPWYCP-	CK-HQQATKK	-DLWSLP-IL	VVHLKRFSY-
	1051					1120	
USP15	rymrkldtl	vdfpindidm	seflinp..n	agp..cryn!	<u>lavsnhyggm</u>	gqghytafak	nkddqkwyff
Ubp109	rymrkldtl	vdfpindidm	seflinp..n	agp..cryn!	<u>lavsnhyggm</u>	gqghytafak	nkddqkwyff
Unp	rywrkldtv	vefpvralnm	sefvcd..s	arp..yvydl	<u>lavsnhygam</u>	gvghytayak	nrlngkwyff
Usp4	rywrkldtv	vefpvralnm	sefvcd..s	arp..yvydl	<u>lavsnhygam</u>	gvghytayak	nrlngkwyff
USP11	kfsrekldtl	vefpirdldf	sefvicpque	snpekyvdl	<u>lavsnhyggm</u>	rdghyttfac	nkdsqkwyff
Ubp12	rsfsdkidat	vnfpitdldl	sryvvykdd.	..prgliydl	<u>yavdnhygg!</u>	gqghytafak	nfadkwyff
Consensus	RY-RDKLDT-	V-FPI-DLDM	SEFV-----	A-F-----	<u>YDL IAVSNHYGGM</u>	G-GHYTA-AK	NK--GKWYFF
	1121					1190	
USP15	ddssvstase	dqivskaayv	lfyqrdq...	...tfs....	..gtgffpld	retkgasaat	g:plsdeds
Ubp109	ddssvssase	dqivskaayv	lfyqrdq...	...tfs....	..gtgffpld	retkgasaat	gvplsdeds
Unp	ddssvslase	dqivtkaayv	lfyqrrd.de	castss....	..lgsfpqsd	gqvklssshq	gm...gdeea
Usp4	ddsnvslase	dqivtkaayv	lfyqrrd.de	fyktps....	..lssqssd	gqtrpsssqg	gf...gdeea
USP11	ddsnvsvpne	nqieskaayv	lfyqrdqvar	rllspa....	..gssqapas	pacssppsse	fmdvn----
Ubp12	ddsrvtetap	ensiaqsayl	lfyirrhkdg	nqlgssklqe	tiqsrhgyd	erikklydeq	mkiyefnkt
Consensus	DDSSVS-ASE	DQIV-KAAYV	LFYQR-D---	---T-S---	---S---D	---K--S---	G-----D---
	1141					1200	
USP15	ndndndiene	ncmhtn----	-----	-----	-----	-----	-----
Ubp109	ndndndiene	ncmhtn----	-----	-----	-----	-----	-----
Unp	ymdntn----	-----	-----	-----	-----	-----	-----
Usp4	csmdtn----	-----	-----	-----	-----	-----	-----
USP11	eeedvsddm	ecnedvqape	ysnrslvgh	ietqdcnded	dnddgernt	grrklrlkk	vyknsqqlgs
Ubp12
Consensus	---D-----	-----	-----	-----	-----	-----	-----

Figure 3.1.4 Domains present in Unp protein

A. Schematic representation of the Unp protein showing the relative position of the putative domains.

B. Sequences of the domains shown in A and the mutations that were generated.

A



B

Cys	³⁰³ GLGNLGNTCFMNSALQ ³¹⁸	(C311A)
CR1	⁴⁰⁵ LHE ⁴⁰⁷	(H406L)
CR2	⁴⁵⁹ LXCXE ⁴⁶³	(C461G)
Acidic	⁶⁵⁷ EGDEEEGKEQLSEVEGSGEDDQGDDHSE ⁶⁹¹	(Δ657-691)
NLS	⁷⁶⁵ SQPQKKK ⁷⁷²	(K770N/K771S)
His	⁸⁶⁴ YDLIAVSNHYGAMGVGHY ⁸⁸¹	
		Δ5' (Δ1-168)

highly acidic, composed of more than 50% of aspartic and glutamic acid residues. This region was also mutated utilising a PCR-based deletion strategy. Following mutagenesis, a small region enclosing the mutation was subcloned in the wild type Unp expression vector and sequenced to confirm the presence of the desired mutation as well as for the absence of undesired mutations.

To determine whether Unp is a deubiquitinating enzyme as suggested by its sequence, it was tested with a bacterial ubiquitin cleavage assay, a standard method that has been used to confirm deubiquitinating activity of many other enzymes. This qualitative assay is based on the N-end rule pathway, in which a protein's half-life depends on the nature of its N-terminal amino acid. One group of amino acids, including the usual methionine (M) which is coded by the translation initiating AUG codon, confers a long half-life while others including arginine (R), confer a short half-life (Tobias and Varshavsky, 1991). In this assay, a plasmid encoding the enzyme to be tested is transformed in bacteria which are also expressing a linear fusion protein composed of ubiquitin and β -galactosidase. This type of engineered fusion has been demonstrated to be a general substrate of deubiquitinating enzymes. The transformed bacteria are spread on LB-plates containing X-gal and IPTG. If the fusion protein is in the form of Ub-M- β gal, the arising colonies will be blue regardless of whether or not the fusion is cleaved by the putative enzyme since both forms would contain a methionine as their N-terminal amino acid. It thus serves as a control. However, if the fusion is in the form of Ub-R- β gal, the color of the colonies will be dependent on the activity of the enzyme. An active enzyme will cleave the fusion resulting in the production of a R- β gal polypeptide containing a destabilizing N-terminal amino acid. As a consequence,

blue colonies. To control for the quality of the X-gal on the plates, transfections of a particular construct in bacteria containing each fusion type were plated on one-half of the same plate. As seen in Table 3.1.1, wild-type Unp exhibits deubiquitinating activities seen from the formation of white colonies. As a control, the cysteine residue in the Cys domain, C311, has been mutated to an alanine, a type of mutation that results in inactive enzyme with other Ubps. As expected, this mutant loses all activity, resulting in the formation of blue colonies regardless of the identity of the substrates. In addition to the Cys mutant, only one other mutation was found to have an effect, although unexpected, on activity, the CR2 mutant. This mutant displayed only partial activity, as seen with the formation of colonies of a much lighter blue color than the negative control, but clearly not white. Since the pCMV promoter was found to be sufficiently leaky in bacteria to produce the amount of protein required for the assay to function (Baker, personal communication), the Unp cDNAs previously cloned in the mammalian expression plasmid (pcDNA3) were used for the assay instead of subcloning in a bacterial expression plasmid. However the assay did not work with cDNAs later cloned in the bicistronic pECE II vector, which utilises the SV40 promoter, probably due to insufficient levels of proteins in bacteria expressed from this promoter.

In cell fractionation experiments Unp is detected exclusively in the nuclear fraction (Gupta et al., 1994). Since the available Unp antibody was not useful in immunofluorescence experiments (and fairly weak in western blots), all Unp constructs were tagged on the C-terminal with the 6xMyc epitope, which can be recognized with the 9E10 monoclonal antibody. The C-terminal end was chosen to prevent the detection of partially translated proteins that may happen with a N-terminal tag. Although there is always the possibility of translation using internal start sites, the majority of the signal should be full

Table 3.1.1 Results of the bacterial assay

Constructs coding for wt Unp and mutants (in pcDNA3 vector) were transformed in bacteria containing the substrate Ub-X- β gal and plated on LB-Amp containing IPTG and X-gal. White colonies indicate an active deubiquitinating enzyme.

<u>Constructs</u>	<u>Colonies</u>	<u>Activity</u>
Wild-type	white	yes
Δ5'	white	yes
Cys	blue	no
CR1	white	yes
CR2	light blue	partial
NLS	white	yes
Δ acidic domain	white	yes

length products. Transfection of a Myc tagged Unp in COS-1 cells was also shown to fractionate with the nuclear fraction, both with soluble nuclear proteins and in the insoluble nuclear fraction (Tibbo, 1997). Localization of Myc tagged Unp, wild type and mutants was verified by indirect immunofluorescence. These constructs were transfected by electroporation in COS-1 cells, and following incubation with the proper antibodies, the immunofluorescent signal was observed with a confocal microscope. A representative sample of the pictures obtained are shown in figure 3.1.5. In most cases, the expression of Unp was seen throughout the cell including the nucleus and the cytoplasm. However, the highest level of expression was peri-nuclear, as seen in a bright ring around the nucleus, which was even more pronounced in the Cys mutant (panel D). In the case of the putative NLS mutant, two kind of results were obtained. Some cells had an expression that was similar to wild type cells (panel F), while others had expression in the cytoplasm only, showing a dark nucleus which was also lacking the ring (panel E). The constructs used in this experiment were all missing the first 168 amino acids, and the immunofluorescence experiment was subsequently repeated with full length corrected constructs as well as with additional mutants. With a fluorescent microscope, no differences were observed between the 5' end deleted and the full length Unp. Figure 3.1.6 shows a representative sample of the pictures obtained. In many cells, the pattern of expression appeared very filamentous (in particular the cell shown in panel F) as if the protein was bound to a filamentous structure in the cell. The specificity of the antibodies is demonstrated by the lack of signal detected in vector-transfected cells (panel A in both Figure 3.1.5 and 3.1.6)

Since these experiments were performed with transiently transfected cells (thus with highly expressed proteins levels), it is possible that the localization of Unp is affected by

Figure 3.1.5 Confocal microscopy of wt Unp and mutants

COS1 cells were transfected by electroporation with **A** pcDNA3 vector; **B** and **C** WT (pDG 50); **D** Cys mutant (pDG67); **E** and **F** NLS mutant (pDG68); and plated on coverslips. Transfected cells were fixed with methanol, stained with anti-myc (9E10) and visualized with an anti-mouse FITC-conjugated secondary antibody. Images were taken with the confocal microscope at the department of Biology of the University of Ottawa. These constructs all have the first 168 amino acid deleted.

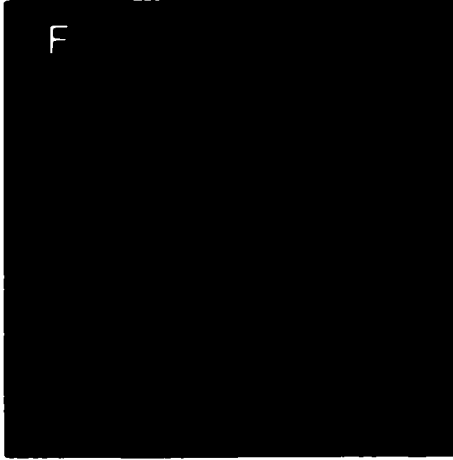
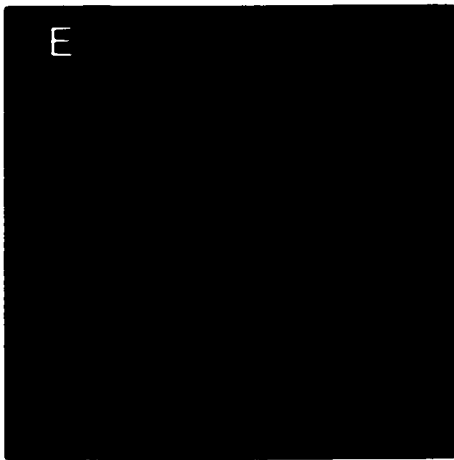
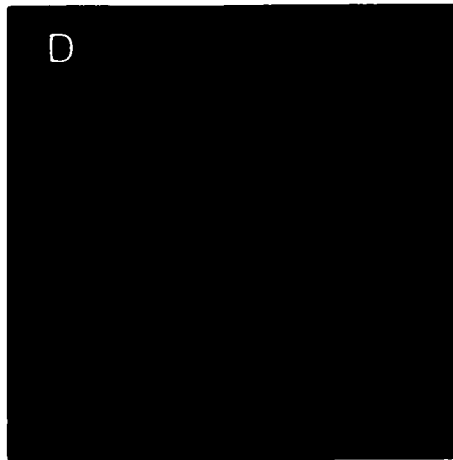
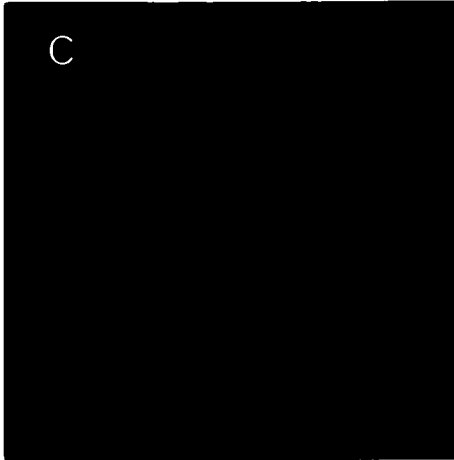
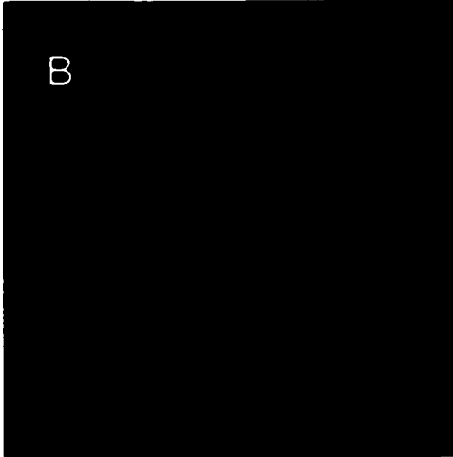
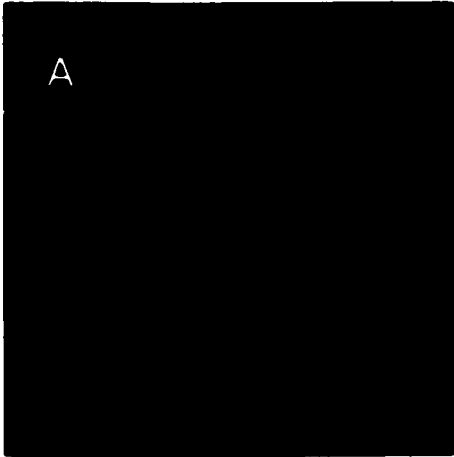
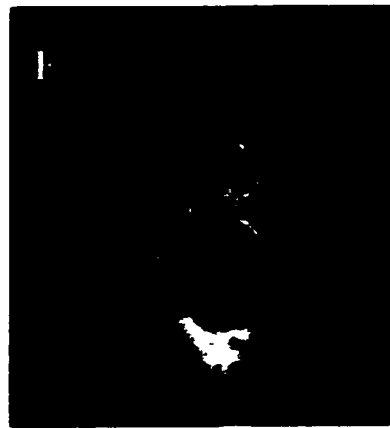
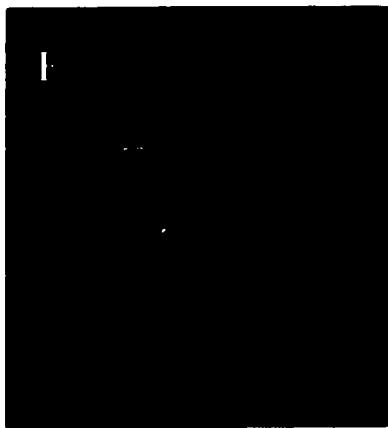
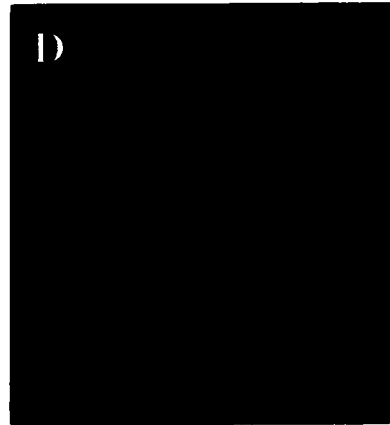
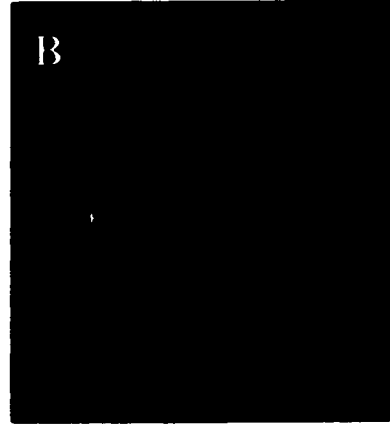
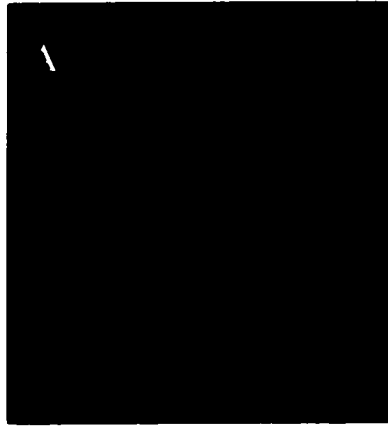


Figure 3.1.6 Immunofluorescence of full length wt Unp and mutants
Full length constructs of Unp and specific mutants were transfected in COS1 cells.
A pcDNA3 vector; **B** WT Unp (pDG104); **C** $\Delta 5'$ (pDG50); **D** CR1 mutant (pDG106);
E CR2 mutant (pDG107); **F** NLS mutant (pDG108). Cell were treated as in Figure 3.1.5.



such overexpression. In order to counteract that possibility, stable expression of Unp, wild type or mutants, was attempted in different cell lines, with a variety of expression vectors and transfection methods, all without success, Table 3.1.2A. When pcDNA3 was used as a vector, colonies were obtained, but no expression was detected from them, either by western or by immunofluorescence. With the pECEII vector (created by Ninan Abraham), Unp and the selection gene, hygromycin, are both translated from a bi-cistronic message, with the hygromycin gene being translated with the help of an internal ribosome entry site (IRES) sequence. With this type of vector, colonies surviving in the selection media should be expressing both genes. In transfection utilizing this vector, no colonies were obtained, with the exception of one experiment. In one NIH 3T3 transfection, colonies with low levels of expression were originally detected, but expression was rapidly lost, in about 3-4 passages. Only later, when transfections were carried out in p19 and H1299 cells were stable transfections possible to obtain, Table 3.1.2B. Expression in these colonies was clearly detected by western as shown in Figure 3.1.7, but the signal was just barely above background in immunofluorescence detection, with the anti-myc antibody, therefore not sufficiently strong to determine its localization. Deletion of the acidic domain had a greater effect than expected on the migration of this mutated protein, as seen on the western blot in Figure 3.1.7, lanes labelled Δ DE. It was not expected that deletion of 29 amino acid would have a detectable effect on the migration of a protein with a molecular weight over 100 KDa. This particular mutation was also observed to be generally expressed at higher levels than the other constructs.

The traditional (bacterial) assay for deubiquitinating activity requires that the protein

Table 3.1.2 Attempts at generating stable cell lines expressing wt Unp or mutants
A Attempts that failed to result in positive colonies
B Attempts that succeeded in producing stable colonies.

A

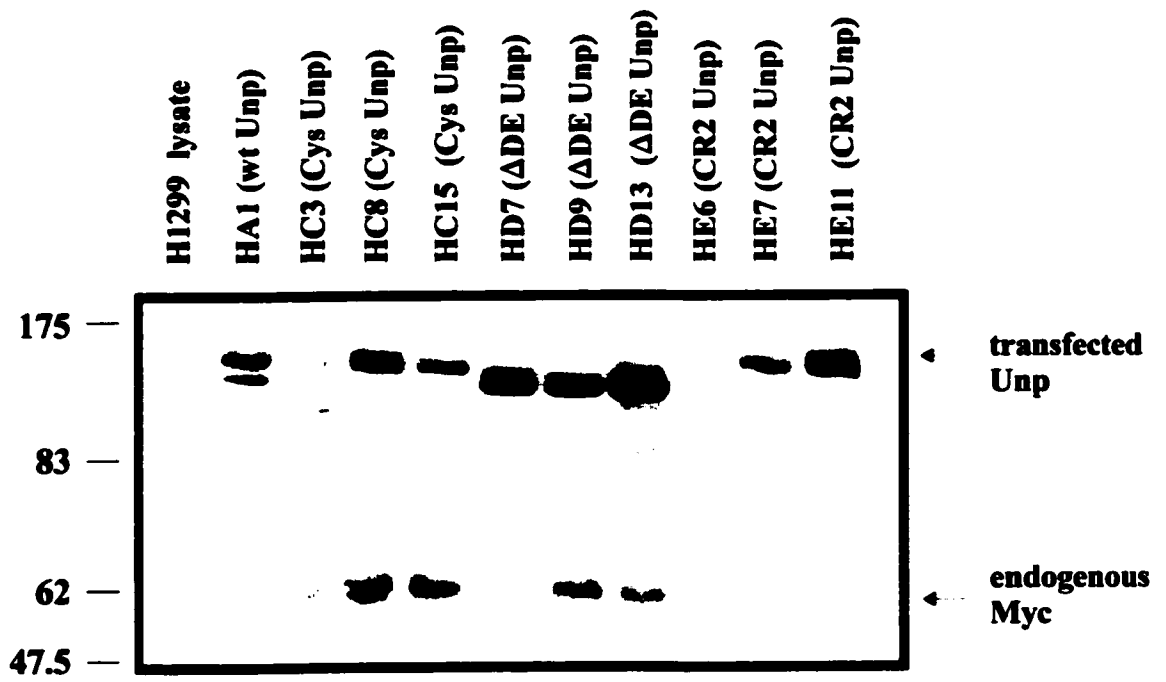
Date	Construct	Vector	Cell Type	Method
Sept 94	Wt, Cys, $\Delta 5'$	pcDNA3	NIH3T3	electroporation
Apr 95	CR2, CR1, NLS ($\Delta 5'$)	pcDNA3	NIH3T3	electroporation
May 95	CR2, CR1, NLS ($\Delta 5'$)	pcDNA3	HFF	electroporation
Sep 95	Wt, Cys, NLS	pcDNA3	HFF	electroporation
Feb 96	Wt, Cys (myc)($\Delta 5'$)	IRES-based	NIH3T3	electroporation
June 96	all (myc)($\Delta 5'$)	pcDNA3	NIH3T3	electroporation
Nov 96	Wt, Cys, ΔDE (myc)	pcDNA3	NIH3T3	electroporation
Apr 97	Wt (myc)	IRES-based	NIH3T3	electroporation
May 97	Wt (myc)	IRES-based	293T	Lipofection
June 97	Wt (myc)	IRES-based	Hela	CaPO4
Sept 97	Wt (myc)	IRES-based	NIH3T3	electroporation

B

Oct 97	Wt (myc)	IRES-based	P19	CaPO4
Nov 97	Wt, Cys, CR2, ΔDE	IRES-based	P19	CaPO4
Oct 99	Wt (myc)	IRES-based	H1299	Effectine
Jan 00	Cys, CR2, ΔDE (myc)	IRES-based	H1299	Effectine

Figure 3.1.7 Western blot analysis of H1299 stable colonies

H1299 cells were transfected (Effectine, Quiagen) with IRES based constructs of wt Unp and mutants. 40 µg of proteins were migrated on a SDS-PAGE (7.5%), transferred to a nitrocellulose membrane and probed with the anti-myc 9E10 antibody. The antibody also recognized the endogenous human myc protein serving as a loading control. The migration of molecular weight size markers in Kilodalton (kD) is indicated on the left of the figure.



α Myc

be expressed in bacteria. In addition to the concerns raised by this (correct folding and post-translational modifications), it is not possible to manipulate the conditions in this assay. It is also only qualitative, and it is not possible to quantitate differences of activity. An *in vitro* cleavage assay became available where the source of deubiquitinating enzymes can be from mammalian cells as well as from bacteria. In this assay, a radioactive substrate in the form of a linear fusion of ubiquitin to GST is produced in bacteria and purified using glutathione beads. This substrate is incubated in the presence of a deubiquitinating enzyme, either immunoprecipitated from mammalian cells or purified from expressing bacteria. The products of the reactions are separated on an SDS-PAGE and visualized by autoradiography. With this assay the activity of Unp and of the mutants was confirmed, whether the source of Unp was immunoprecipitates (with the myc antibody) of transient transfections or stable cell lines, or immunoprecipitates of the endogenous Unp protein. Figure 3.1.8A shows the results of a cleavage assay performed with the Unp protein immunoprecipitated from transient transfections in 293T cells. As expected from the previous assay, the Cys mutation completely abolished activity of wild type Unp. In this particular experiment, no decrease of activity was observed with the CR2 mutant although such a decrease was observed when this mutant was immunoprecipitated from the stable cell line (panel B). With the CR1 mutation alone, no effect on activity was observed, although its combination with the CR2 mutation always resulted in the elimination of the activity. No effect on activity was observed for the acidic domain deletion as well as the 5' deletion and the nuclear localization signal mutant.

In this assay, the conditions of Unp's activity can be better manipulated and they were shown to be dependent on the presence of a reducing agent. Normally DTT was added

Figure 3.1.8 *In vitro* activity test of wt Unp and mutants

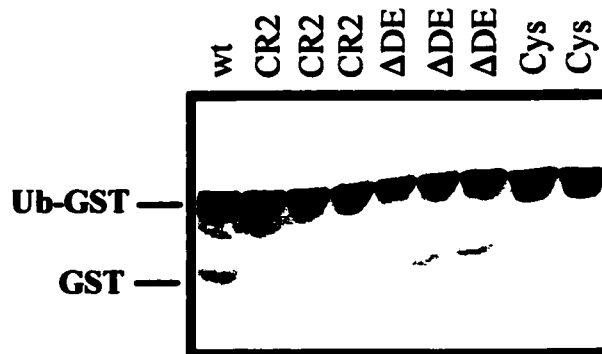
Myc tagged Unp from 2 mg of proteins was immunoprecipitated with the anti-myc 9E10 antibody from lysates of **A transient transfections; **B** stable transfections and incubated with 5 μ L of the 35 S substrate. The bands were separated on a 10% SDS-PAGE, the gel was dried, exposed on a phosphoscreen and visualized on the STORM (Molecular Dynamics).**

A



transient transfections

B



stable colonies

in the immunoprecipitation buffer. When it was omitted, a drastic decrease of activity was observed, as seen in Figure 3.1.9.

One possibility for the reduced activity of the CR2 mutant is the elimination of a zinc finger motif. The sequence of the CR2 motif consists of LXCXE. The subsequent amino acid in Unp is a cysteine giving an alternative motif of CXXC, which could be part of a zinc finger. In this case, the mutation of the cysteine to glycine would disrupt binding to zinc possibly explaining the reduced activity observed. If this model were true, it would imply that binding to zinc is important for Unp activity. This possibility was tested by adding increasing amount of EDTA to the immunoprecipitation buffer. EDTA is a chelating agent that has affinity to divalent ions, including zinc. Thus, increasing amounts of EDTA should lead to reduced amounts of zinc available for binding to Unp. If zinc binding is important for Unp's activity this would result in a decrease of activity that is reversible by addition of zinc to the reaction. As seen in figure 3.1.9, this is not the case since an addition of EDTA up to 30 mM (15 times the concentration in normal lysis buffer, panel B) does not result in any change in Unp's activity. Adding more zinc to the reaction also does not increase the activity of Unp (panel A).

3.2 Protein Binding Analysis

In order to identify proteins binding to Unp, whether these are substrates or just interacting proteins (and possibly regulators), a GST pull-down approach was chosen. For this strategy, a GST-Unp fusion construct was created by subcloning the myc-tagged Unp cDNA in frame with the GST coding sequence in a bacterial expression plasmid pGEX4T3

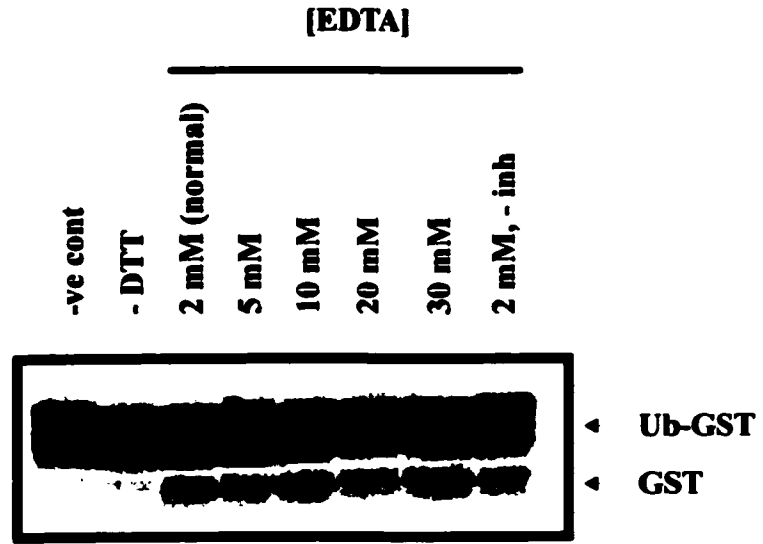
Figure 3.1.9 Effect of DTT and zinc on Unp's activity

Unp from 1 mg of proteins was immunoprecipitated from untransfected H1299 cells and washed with the conditions indicated in each lane. The immunoprecipitated Unp was incubated with the ³⁵S labelled substrate under the same conditions. The bands were separated on a 10% SDS-PAGE, the gel was dried, exposed on a phosphoscreen and visualized on the STORM, Molecular Dynamics.

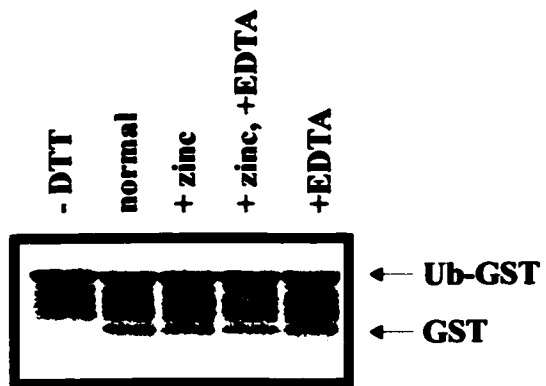
A Variation of the concentration of EDTA from the usual 2 mM in lysis buffer to 30 mM.

B Addition of 10 mM Zinc, or 20 mM EDTA, or both.

A



B

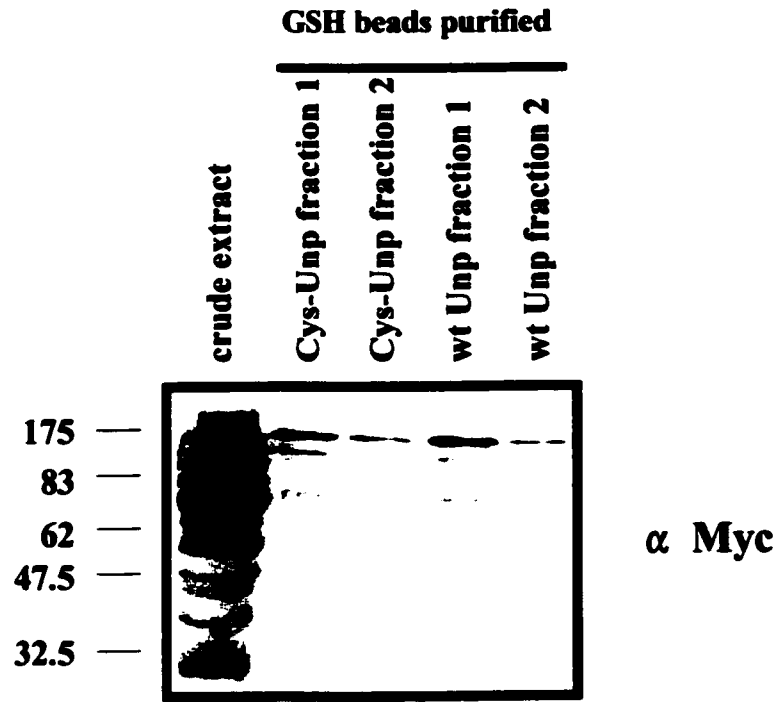


(Promega). A fusion construct was also made with the Cys mutant because of the possibility that a catalytically inactive mutant may bind more strongly to its substrate(s). The Unp fusion proteins expressed from these constructs contain the GST tag at their N-terminal end in addition to the Myc tag at their C-terminal end, resulting in proteins with a molecular weight of approximately 170 KDa. The bacterially expressed fusion proteins were purified with glutathione beads and expression levels were verified by western blot analysis. Figure 3.2.1A shows a western blot probed with the myc antibody. With both constructs, the binding to the beads resulted in the purification of full length proteins. The proteins detected in this manner should be full length since they were purified by affinity for the tag (GST) at one end and detected by the tag (myc) at the other end. To confirm, a blot was also probed with an antibody that recognizes the GST tag, panel B. Since the intensities of the bands were weaker than expected, a bacterial fractionation was done to determine in which bacterial compartment the protein was expressed. The localization and form of expression of the protein can determine which method of preparation is the most efficient, especially in the case of periplasmic expression. As seen in Figure 3.2.2, the majority of the expression was detected in the insoluble pellet, most likely in the form of inclusion bodies. As expected from the previous preparation, expression was also detected in the soluble fraction, although to a lesser degree, but practically no expression was detected in the periplasmic fraction. To avoid the problem of solubilizing the inclusion bodies, which requires the denaturation of the proteins, followed by a renaturation step, the cultures were grown and induced at 25°C. It had been suggested by Takeshima et al. (Takeshima et al., 1994) that induction at room temperature greatly reduces the production of inclusion bodies for some proteins, thus increasing the yield of soluble proteins. Using that condition, in combination with a slightly

Figure 3.2.1 Purification of bacterially expressed GST-Unp fusion proteins

Bacteria expressing GST-Unp fusions were lysed and the fusions were purified with glutathione beads. The proteins were eluted by competition with free glutathione and alkaline pH in successive fractions. The elution fractions were migrated on SDS PAGE and probed **A with anti-myc and **B** with anti-GST antibodies**

A



B

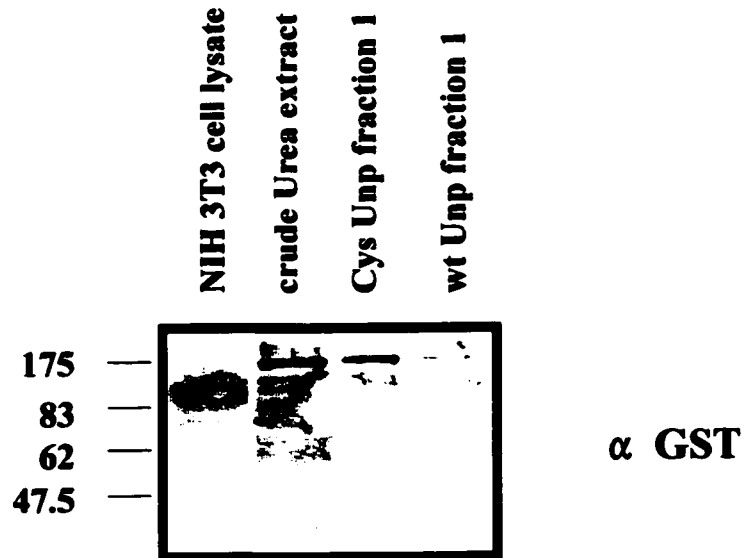
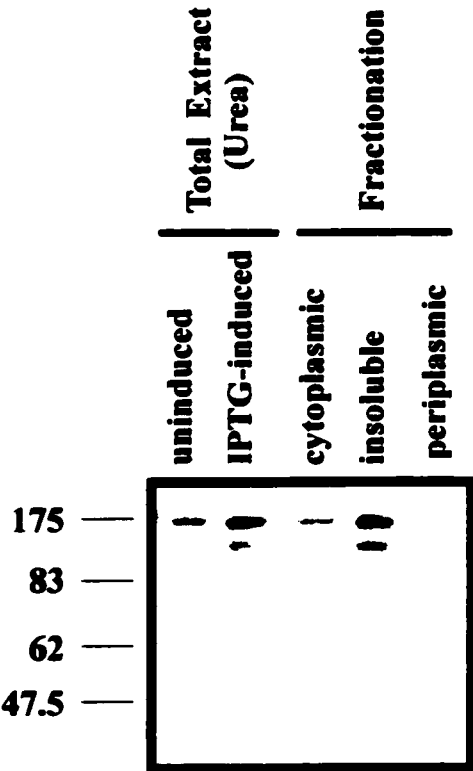


Figure 3.2.2 Bacterial fractionation and expression of fusion proteins

The bacteria expressing wt GST-Unp fusion were fractionated in cytoplasmic, insoluble and periplasmic fractions. Aliquots from each fractions as well as total lysates were migrated on SDS-PAGE and the expression was verified by probing with the anti-myc antibody.



reduced concentration of IPTG (30 mM instead of 40 mM), the yields were indeed increased and the purified proteins were visible on a silverstained polyacrylamide gel, figure 3.2.3. In addition to the full length product, which can be detected by western blot analysis with the myc antibody, many other smaller bands were present that were absent in the control GST alone lane. Since these were also purified with the GSH beads, they are most likely products of an incomplete translation or partial degradation, and should not interfere with the binding assay. With the induction at room temperature, the expression of the fusion proteins improved, but was still not as efficient as the GST alone since a dilution of 1/10 was required to see comparable amounts of proteins, suggesting that the production of inclusion bodies had not been completely eliminated.

With these conditions, enough proteins were produced and purified on beads to attempt the pull down assays. This assay was performed with the purified proteins still attached to the beads, providing means of separating the fusion and bound proteins from the unbound ones. For these, NIH 3T3 cells were metabolically labelled with ³⁵S methionine and cysteine. Following the 5 hours labelling reaction the cells were lysed, and the lysates were pre-cleared with GST bound beads which were produced in the same manner as the Unp fusion proteins, from the pGEX vector. The pre-cleared lysates were then incubated with GSH-beads bound with either GST alone or in fusion with the wild type or Cys mutant Unp. After extensive washing, the bound proteins were eluted in SDS sample buffer, migrated on an SDS PAGE and visualised by autoradiography. A typical result, migrated on a 7.5% gel is shown in figure 3.2.4. Arrows are pointing to the bands which are present in the Unp lanes but not in the GST alone control lane (with the exception to the smallest band which is also present in the GST lane, but is much more intense in the wild type Unp

Figure 3.2.3 Silverstain of GST-fusions

Bacteria expressing GST-Unp fusions were induced at room temperature to reduce the formation of inclusion bodies. Fusions were purified by incubation with glutathione beads and following extensive washing, aliquots (1/5 of the beads from 12ml of bacteria culture) were eluted directly in SDS sample buffer and migrated on SDS-PAGE. Proteins were visualized by silverstaining the gel.

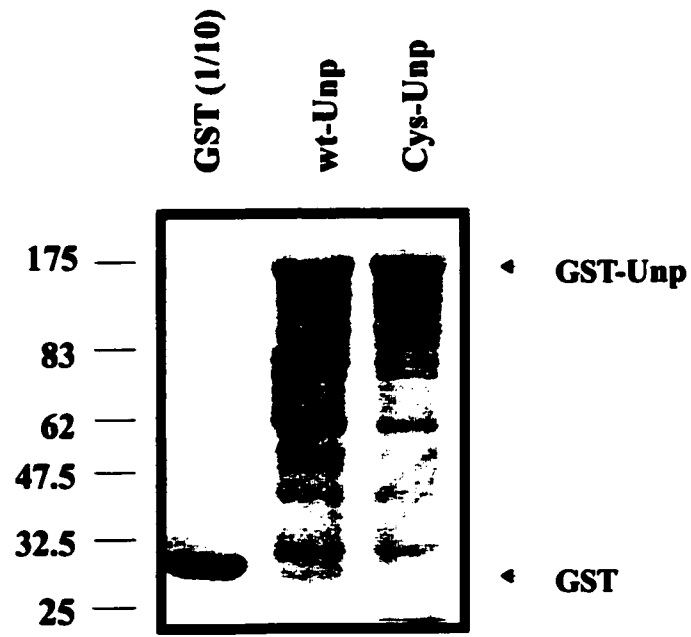
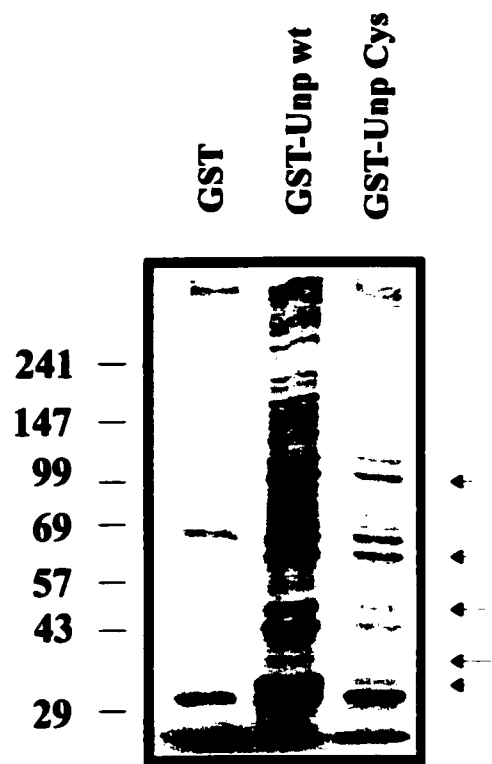


Figure 3.2.4 ³⁵S-labelled pull down

Two 100mm dishes of NIH3T3 cells were metabolically labelled with ³⁵S Met and Cys (Promix, Amersham) and 1/3 of the combined lysate was incubated with each of GST or GST-Unp fusion containing beads. The amount of beads used was 2/5 of the prep (from 12 mL of bacterial culture, which is double the amount used for figure 3.2.3). Interacting proteins were eluted in SDS sample buffer and migrated on SDS-PAGE. The gel was dried, exposed on a phosphoscreen and visualized on the STORM, Molecular Dynamics. Bands present in lanes with fusion proteins and absent in control GST alone lane are indicated with arrows at the right of the figure.



lane). The samples were also migrated on a 12.5% gel to obtain a better resolution of the smaller molecular weight proteins (not shown). The bands that were seen consistently in most experiments are indicated in Table 3.2.1. One of these, with a molecular weight around 100 KDa, may be pRb. This is a definite possibility due to the presence of the CR1 and CR2 domains in Unp. Figure 3.2.5 shows a co-immunoprecipitation experiment that was performed in transfected NIH 3T3 cells. The cells were transfected with Unp, either alone or with wild-type or mutant pRb. Unp was immunoprecipitated from the lysates with the myc antibody and migrated on SDS PAGE. Binding to pRb was seen by probing with an antibody recognizing the pRb protein. Clear binding was observed when both Unp and wild-type pRb were transfected. The small amounts of binding seen in the other lanes may be due to the endogenous pRb present in the cells.

In other pRb interacting proteins containing the CR1 and CR2 domains, mutation of the cysteine residue in the CR2 domain has been shown to be sufficient to abolish pRb binding. A GST-CR2 Unp fusion construct was generated and its expression in bacteria verified. Since the yields were usually slightly lower than for the other fusions, the amounts added to the assays were compensated in consequence. A GST pull down experiment was performed with the three Unp fusions. Since it was binding to pRb that was investigated at this point, the assay was performed with unlabelled cell lysates (again NIH3T3) and the interaction was detected by western blot analysis with an anti-pRb antibody. Figure 3.2.6A shows a silverstained gel containing approximately the same amount of proteins for the three GST fusions. In panel B, binding to pRb was detected with both wild type and Cys mutant Unp, but not in GST alone or with the CR2-mutated Unp, suggesting that the interaction is not an artefact. Similar results were obtained using lysates from both p19

Table 3.2.1 Results of ^{35}S GST pull-down

Summary of the consistent Unp interacting bands detected in the ^{35}S pull-down experiments. These results are a combination of results from 7.5% and 12.5% gels of three independent experiments.

<u>Size</u>	<u>Frequency</u>
~100 KDa	3/3
~70 KDa	2/3
45 to 60 KDa	3/3
~35 KDa	2/3
20 to 25 KDa	3/3

Figure 3.2.5 Co-immunoprecipitation in transfected NIH3T3 cells
NIH3T3 cells were transfected with myc-tagged Unp, alone or with pRb. Lysates (approximately 2 mg in 500 μ L) were immunoprecipitated with anti-Myc migrated on SDS PAGE and probed with anti-pRb. 10 μ L of the lysate was run in lane 1.

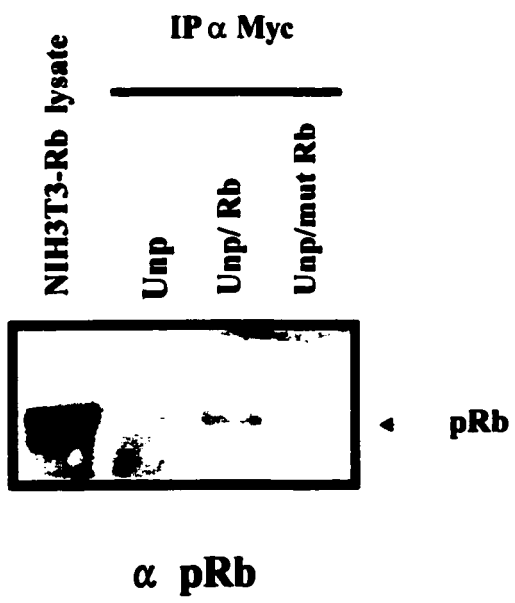
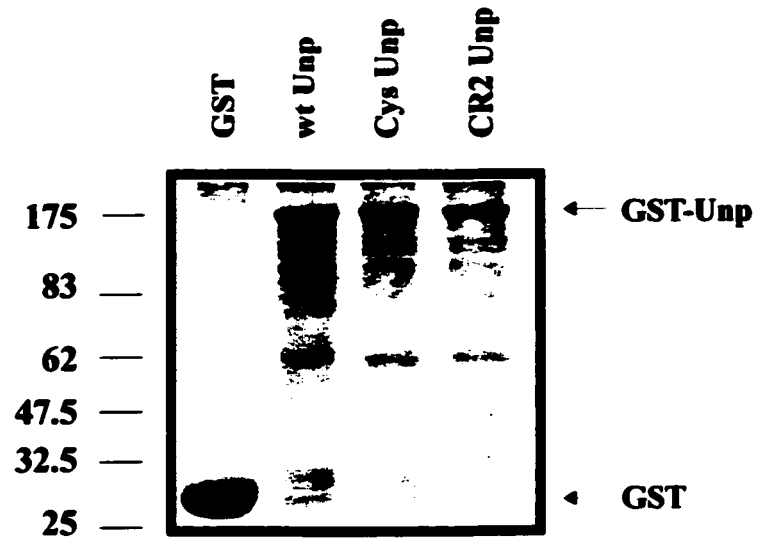


Figure 3.2.6 Cold GST pull-down - pRb

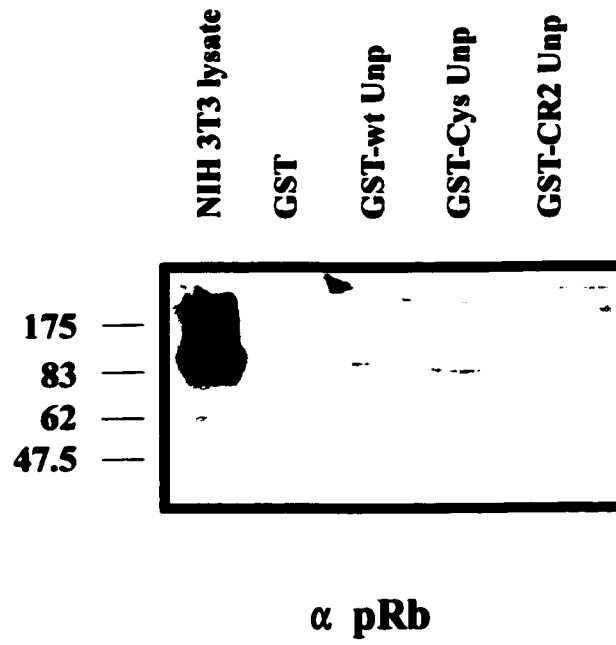
A Silverstained gel with GST and the three GST-Unp fusion proteins (as described in Figure 3.2.3).

B GST pull-down with unlabelled, untransfected NIH3T3 cell lysate, with 2/5 of the GST protein preps (double the amount used in panel A). The interacting proteins were migrated on an SDS-PAGE and transferred on a nitrocellulose membrane. The blot was probed for the presence of pRb. 1% of input lysate for GST pull down was loaded on lane 1.

A



B



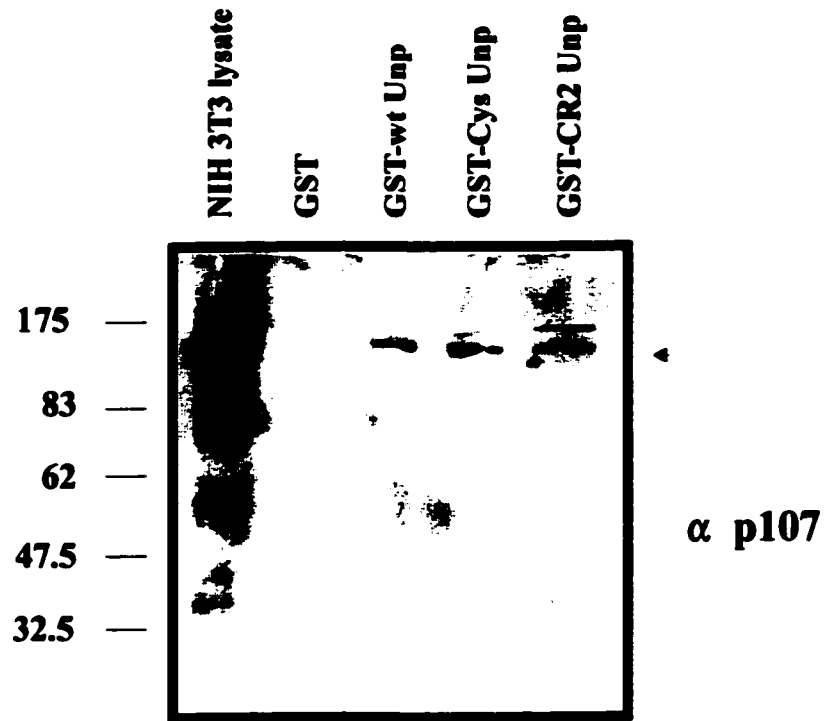
(mouse) and A431 (human) cells (not shown).

pRb is part of a family which also includes p107 and p130. Since Unp is binding to pRb, the possibility existed that it would also bind to p107 and p130. To test this hypothesis, the pull down blots were also probed with antibodies against these proteins. As seen in figure 3.2.7, Unp was able to bind to both although with obvious differences from the pRb interaction. In contrast to the results obtained with pRb, binding of Unp to p107 occurred with the CR2 mutant in addition to the other Unp fusion, panel A., while no interaction was detected in the GST alone lane. Two type of result were seen for p130. In some cases, as seen in panel B, binding of Unp to p130 was only observed in the lane where Unp's CR2 domain was mutated, but in other cases, binding was observed in all three Unp-fusion lanes. The first type of result was mostly observed when using subconfluent NIH3T3 cells while the second type of result was mostly observed in lysates from confluent NIH3T3, or with p19 and A431 cells. The fact that both p107 and p130 were able to bind the CR2 mutant indicated that unlike pRb, these proteins bind some other motif(s) in Unp. The most likely candidate is the CR1 domain.

GST-fusion constructs were made for two more mutants, the CR1 and a CR1/CR2 double mutant. Since our collaborator had also made a GST-CR2 mutant of Unp that was different from ours (theirs is a cysteine to serine mutation, whereas ours is a cysteine to glycine mutation), it was also used in the experiments. Figure 3.2.8 shows a silverstained gel of all the fusions, including the CR2(S), which migrates slightly faster than the others. The results of the pull down are shown in figure 3.2.9. In panel A we see that pRb bound Unp through Unp's CR2 domain as all the mutants that contained an intact CR2 domain were able to bind pRb, but none of the three constructs with mutated CR2 domain would. On the

Figure 3.2.7 Cold GST pull-down - p107 and p130
GST pull-down with unlabelled NIH3T3 cell lysate. The interacting proteins were migrated on an SDS-PAGE and transferred on a nitrocellulose membrane. The blot was probed for the presence of **A** p107 and **B** p130. Same conditions were used as for Figure 3.2.6.

A



B

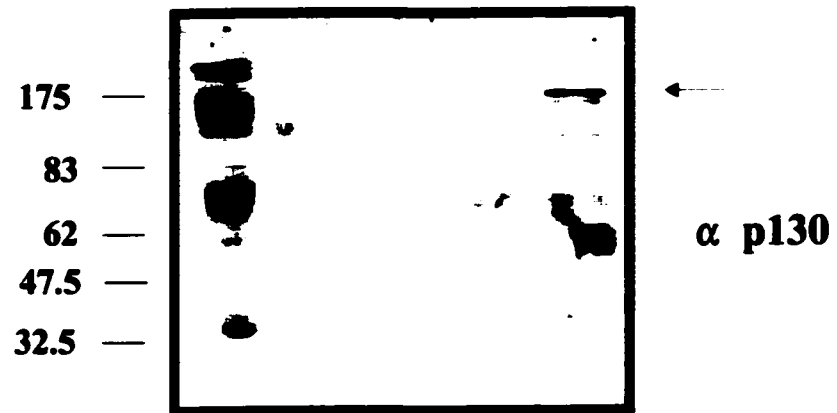


Figure 3.2.8 Silverstained gel of all GST-fusions
GST-fusions were generated for CR1 mutant as well as a double CR1/CR2 mutant. Proteins were prepared as previously described. 1/5 of the preps (from 12 mL bacterial cultures) were migrated on SDS-PAGE and visualized by silverstaining.

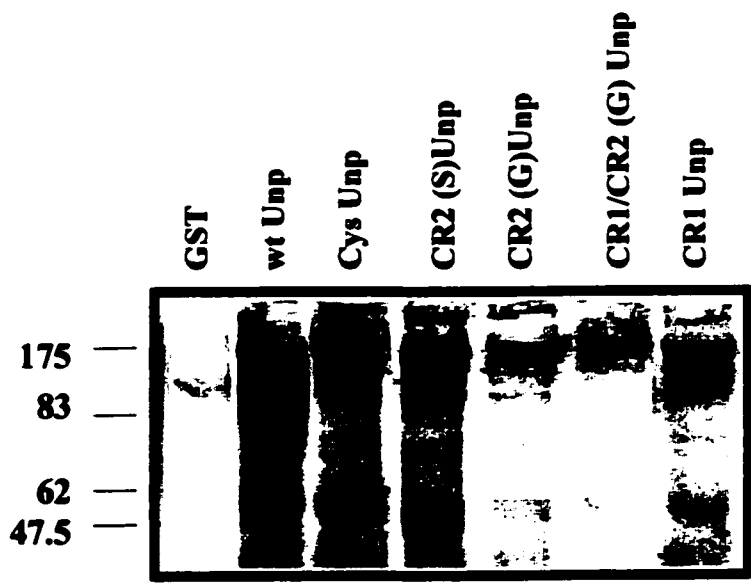
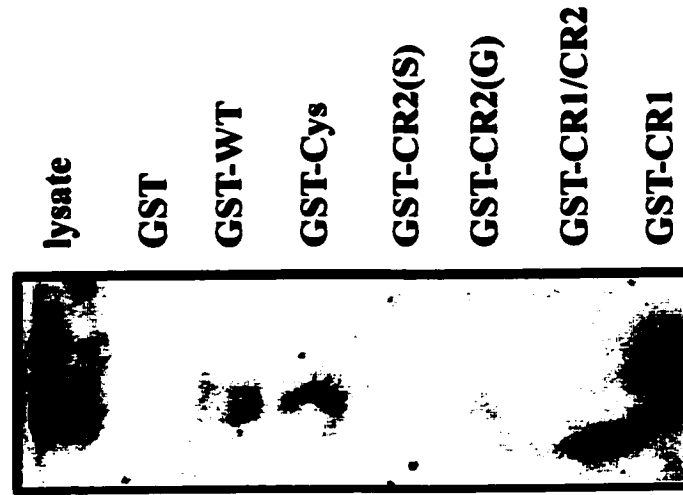


Figure 3.2.9 Cold GST pull-down with all mutants

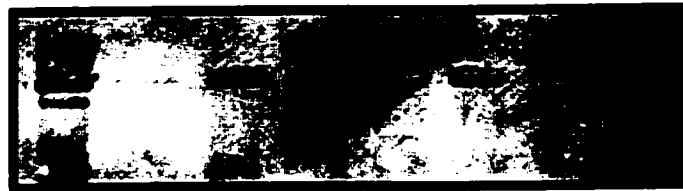
GST pull-down with unlabelled NIH3T3 cell lysate. The interacting proteins were migrated on an SDS-PAGE and transferred on a nitrocellulose membrane. The blot was probed for the presence of A pRb; B p107; C p130; and D E2F-1. Lane 1 (lysate) represent 1% of input.

A



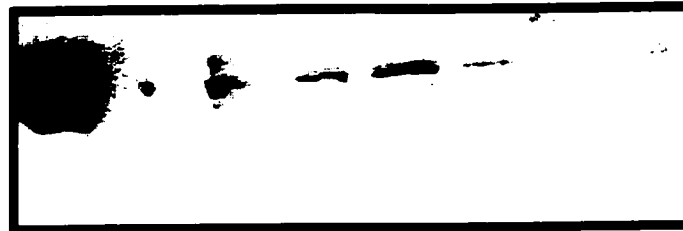
α Rb

B



α p107

C



α p130

D



α E2F-1

other hand, p107 bound neither through Unp's CR1 nor CR2 domains, as all the mutants, including the CR1/CR2 double mutant, were able to bind it (panel B). Interestingly, p130 seemed to be able to bind Unp through both the CR1 and/or the CR2 domains since only the double mutant was unable to bind p130 (panel C). As a control, binding to E2F-1 was tested, and no interaction was detected (panel D). Same negative result was obtained in probing for cyclin D1, (not shown). The results obtained from the GST-pull down experiments are summarized in Table 3.2.2.

The activity of these GST fusion proteins was tested, Figure 3.2.10, to assure the proper conformation of the purified proteins. As expected, activity was detected from wild-type Unp but not with the Cys mutant. As observed in most of the previous cases, a reduced activity was associated with the CR2 mutant, which was completely abolished in combination with the CR1 mutant. The CR1 mutation alone had no effect on activity as previously seen.

With the availability of a commercial Unp antibody (useful for western and immunopurification), it became possible to verify the interaction in a more physiological setting. Co-immunoprecipitation of both endogenous Unp and pRb could now be attempted in untransfected cells. Panel A in Figure 3.2.11 shows a co-immunoprecipitation experiment with the Unp antibody. As seen in the longer exposure, Unp is capable of binding both the hypophosphorylated and hyperphosphorylated forms of pRb. In contrast, pRb that was co-immunoprecipitated with the E2F-1 antibody was only of the hypophosphorylated form as expected from the functional aspect of pRb in the cell cycle. Co-immunoprecipitation was also observed with the pRb antibody, panel B, although this binding was antibody specific and only observed with the goat antibody.

Table 3.2.2 Summary of the GST pull-down results

All the results obtained in the GST pull-down experiments are summarized in the form of a table indicating all the possible Unp mutant / pocket protein interactions. A “+” sign indicates a positive interaction.

	pRb	p107	p130
WT	+	+	+
Cys	+	+	+
CR1	+	+	+
CR2	-	+	+
CR1/CR2	-	+	-

Figure 3.2.10 *In vitro* activity test with GST-fusions

The beads containing the GST fusions (1/5 of preps, from 12 mL bacterial cultures) were incubated with 5 μ L of the 35 S substrate. Bands were separated on a 10% SDS-PAGE, the gel was dried, exposed on a phosphoscreen and visualized on the STORM (Molecular Dynamics).

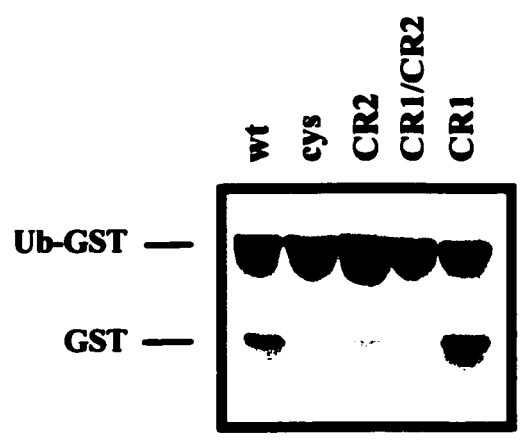
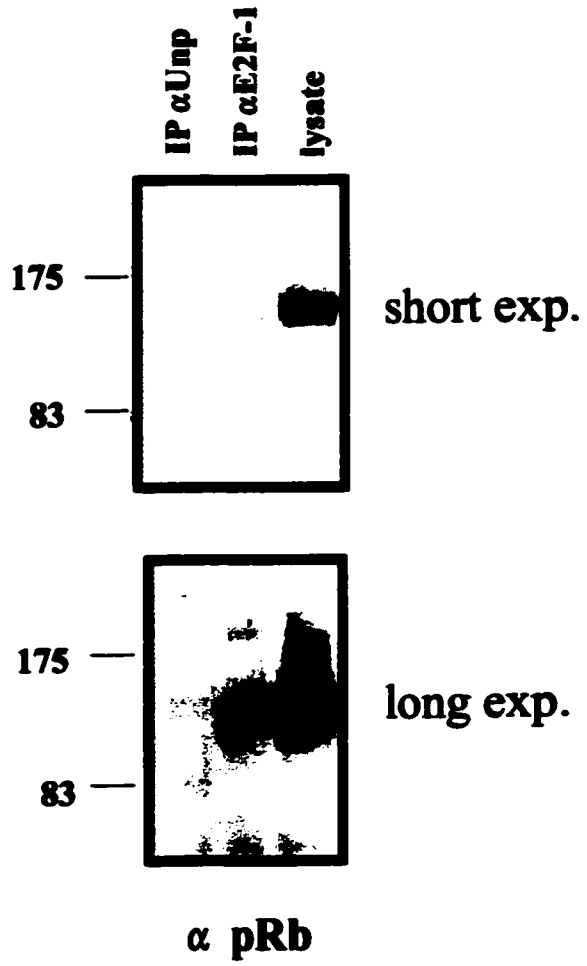


Figure 3.2.11 Co-immunoprecipitation with endogenous proteins.

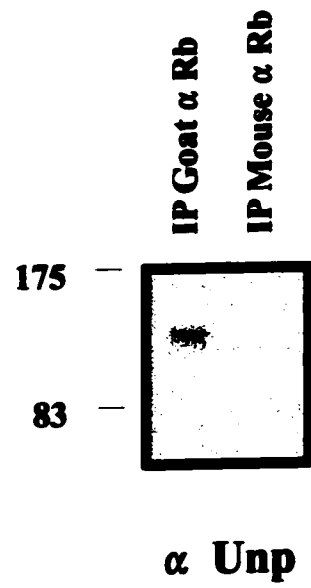
A 2 mg of untransfected H1299 cell lysate was immunoprecipitated with anti-Unp or anti-E2F-1 antibodies. The complexes were migrated on a 7.5% SDS-PAGE, transferred on a nitrocellulose membrane and probed for the presence of pRb. The lane labelled lysate represents 1% of input.

B 2 mg of untransfected H1299 cell lysate was immunoprecipitated with different anti pRb antibodies. The complexes were migrated on an 7.5% SDS-PAGE, transferred on a nitrocellulose membrane and probed for the presence of Unp.

A



B



3.3 Phosphorylation studies

Depending on gel conditions used, Unp can be seen as a tight doublet. There are several explanations possible for the presence of a doublet, one being differential phosphorylation. To address this question, Unp was immunoprecipitated from ³²P-orthophosphate labelled cells and resolved by SDS PAGE. Autoradiography revealed that both bands are phosphorylated (see figure 1A in Appendix 2). Furthermore, these bands were cleaved, digested and resolved by two-dimensional thin-layer chromatography and the predominant phosphoaminoacid was shown to be phosphoserine (Figure 1B, Appendix 2), although we cannot exclude phosphorylation of threonine or tyrosine at levels below detection in the system.

To confirm this data using a second approach, studies of Unp phosphorylation state was made using phospho-amino acid antibodies. A possible link to Unp's regulation by phosphorylation was also investigated.

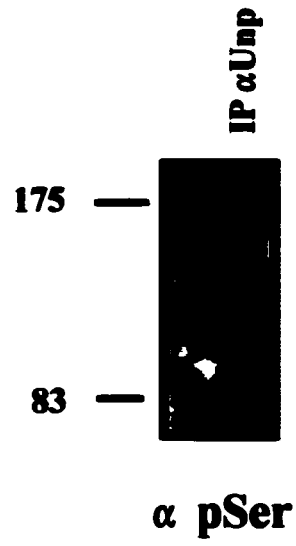
A cell lysate from untransfected H1299 cells was immunoprecipitated with an antibody against Unp. The precipitated proteins were migrated on an SDS-PAGE and were probed with antibodies against the phospho-amino acid epitopes. Only the phosphoserine-specific antibody detected a signal above background (Figure 3.3.1. panel A). Panel B shows the results when the experiment was performed in the reverse order. The H1299 cell lysates were immunoprecipitated with antibodies against the phospho-amino acid epitopes and the blot was probed for the presence of Unp. A clear signal was obtained in the lane immunoprecipitated with the pSer antibody, while the high background in the other lanes

Figure 3.3.1 Phospho-amino acid analysis

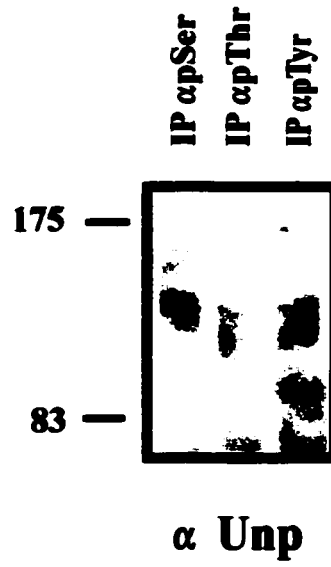
A 2 mg of untransfected H1299 cell lysate was immunoprecipitated with anti-Unp antibody. The complexes were migrated on a 7.5% SDS-PAGE, transferred on a nitrocellulose membrane and probed with an anti-phospho-serine antibody.

B 2 mg of untransfected H1299 cell lysates were immunoprecipitated with different anti-phospho amino acid antibodies. The complexes were migrated on a 7.5% SDS-PAGE, transferred on a nitrocellulose membrane and probed for the presence of Unp.

A



B



makes it impossible to be certain. The apparent bands in the pThr and pTyr lanes however, could be the result of Unp co-immunoprecipitated with proteins phosphorylated on threonine and tyrosine.

Immunoprecipitated samples were treated with the general phosphatase CIP in order to eliminate phosphorylation. Panel A in Figure 3.3.2 shows that the treatment was effective in eliminating the hypophosphorylated band in immunoprecipitates of pRb, while the doublet is still present in Unp immunoprecipitates. Although the doublet is still present, the phosphorylation on pSer was completely eliminated as seen in panel B, where in untreated immunoprecipitates, both bands were phosphorylated on serine residue(s) while that signal completely disappeared after treatment. The disappearance of the signal was not due to the loss of the protein since it was present when probed with an anti-Unp antibody. The CIP treated and untreated immunoprecipitates were tested for deubiquitinating activity with the *in vitro* cleavage assay. The result, shown in Figure 3.3.3 demonstrates that the phosphorylation was necessary for activity as the sample treated with CIP, in the last lane, had an activity comparable to the negative control.

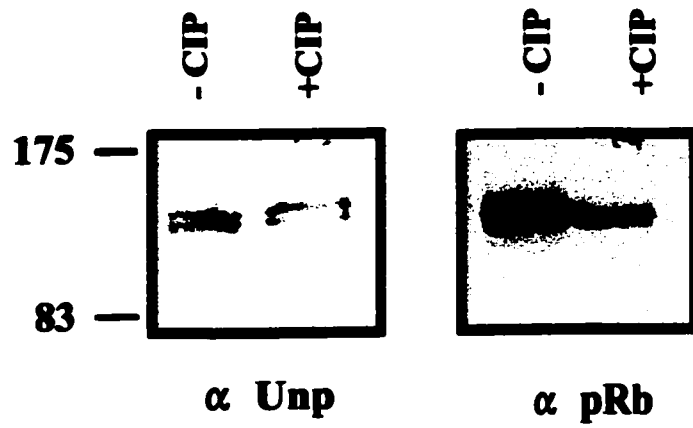
3.4 Knock-out of Unp

The biological roles of many genes have been elucidated by analysing the phenotype of mice in which the expression of these genes had been disrupted. These mice can be generated by random inactivation of genes (by insertion of retroviral provirus for example) or by the targeting of specific genes. Gene targeting in embryonic stem (ES) cells has become a very effective method to introduce specific mutations into the mouse genome.

Figure 3.3.2 Western of phosphatase treated Unp

2 mg of non-transfected H1299 cell lysate was immunoprecipitated with anti-Unp (anti-pRb for panel A, right). Half of each sample was treated with the phosphatase CIP. The proteins were eluted in SDS sample buffer, migrated on a 7.5% SDS-PAGE, and transferred on nitrocellulose membranes. The blots were probed with the indicated antibodies (Unp and pRb in panel A, pSer and Unp in panel B).

A



B

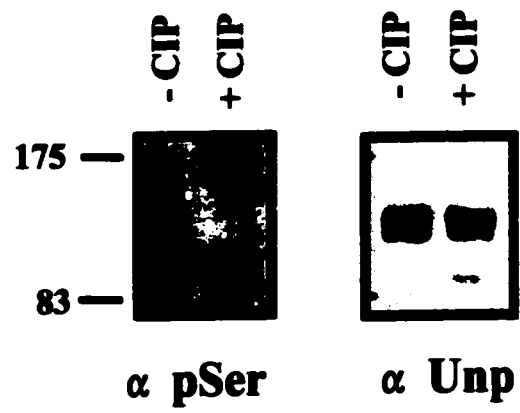
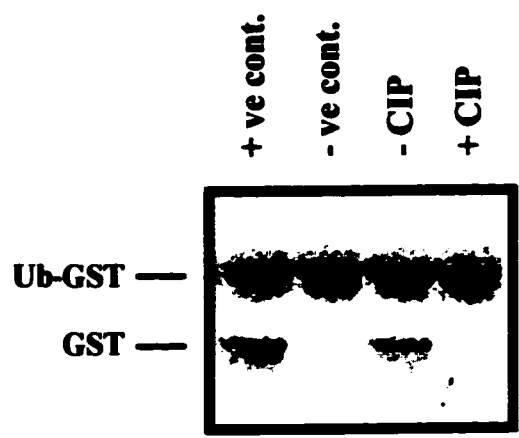


Figure 3.3.3 Effect of phosphorylation on Unp's activity

2 mg of untransfected H1299 cell lysate was immunoprecipitated with anti-Unp. Half of the sample was treated with the phosphatase CIP. Following incubation of the beads with 5 μ L of the 35 S substrate, the bands were separated on a 10% SDS-PAGE, the gel was dried, exposed on a phosphoscreen and visualized on the STORM (Molecular Dynamics). Positive control is bacterially purified Unp while negative control is beads alone.



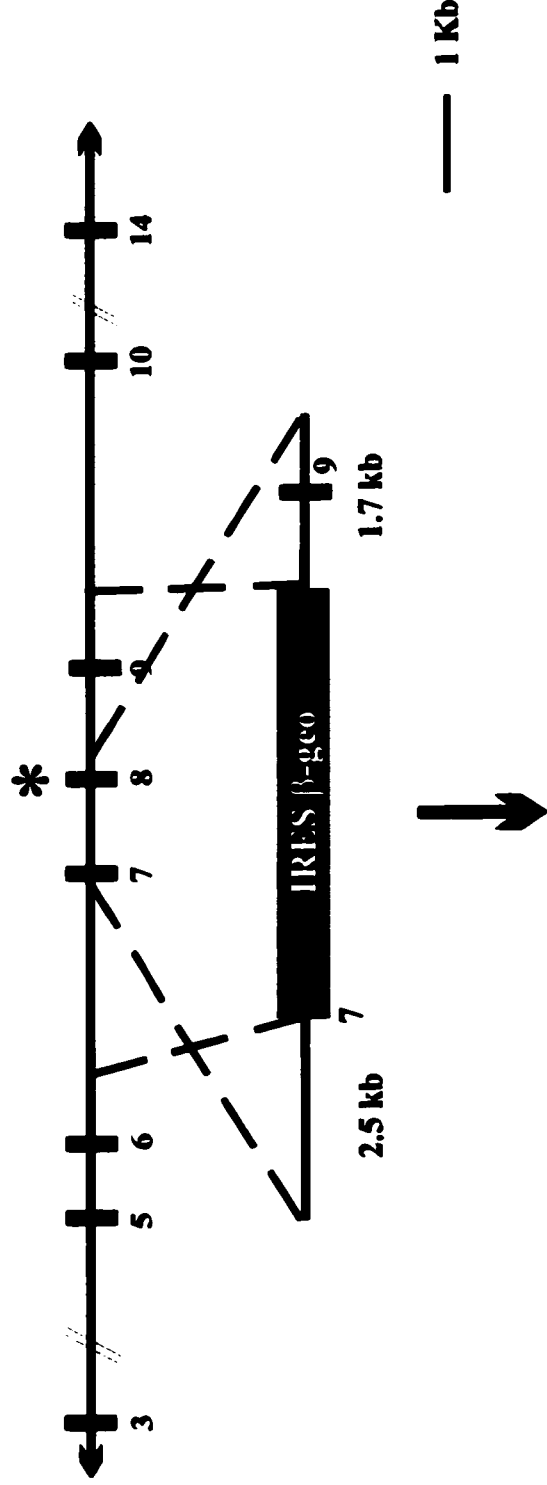
These cells, originating from the epiblast (primitive ectoderm)(Brook and Gardner, 1997), retain totipotency and as such are able to contribute to the formation of all tissues, including germ cells. Targeting in ES cells is achieved by homologous recombination of exogenous DNA containing the desired mutation, in a targeting vector, with the chromosomal DNA. Targeted ES cells are injected in blastocysts and implanted in pseudo-pregnant females. If the procedure is successful, chimeric mice will be born in which the contribution of the targeted ES cells can be estimated by the coat color. The injected ES cells originate from agouti colored mice while the recipient blastocysts originate from white colored mice. The chimeric mice are then bred with wild-type mice to test for germ-line contribution of targeted ES cells. These mice, heterozygous for the null mutation can be further bred to obtain homozygotes, if viable.

To further study the biological function of Unp, it was decided to generate mice null for Unp. Unp's genomic structure consist of 22 exons (Di Fruscio et al., 1998) with exon 8 containing all of the Cys domain. The strategy for creating a null mutant of Unp was to replace this exon with an IRES β geo selection cassette (Figure 3.4.1). Mutation of the cysteine residue in the Cys domain was shown to be sufficient to completely inactivate Unp's enzymatic activity (section 3.1.). In combination with the stop codons present in the selection cassette, deletion of exon 8 should result in a severely truncated, catalytically inactive protein that does not possess the CR1 and CR2 domains. To reduce the background of surviving ES colonies that arise from random integration in the genome a promoter-less strategy was chosen. In this approach, the selection marker (neo) is not expressed from a exogenous promoter included in the targeting vector. Instead, its expression is dependent on recombination of the vector in an actively transcribed gene since it is translated from a

Figure 3.4.1 Targeting strategy for Unp knock-out

The targeting strategy was to delete exon 8, marked with an asterisk, with a β -geo selection cassette. The targeting vector was constructed with a total of 4.3 kb of homology, the shorter arm (3') being 1.7 kb. Panel **A** shows the wild type genomic structure and the targeting vector while the panel **B** shows the structure of a targeted allele.

A



B



bicistronic mRNA with the help of an internal ribosome entry site (IRES) sequence (Mountford et al., 1994). Thus colonies will only be produced when there is homologous recombination and the resistance gene is expressed from Unp's promoter or when it randomly integrates in the coding sequence of another gene. In addition to reducing the background levels, this approach increases the fidelity of the homologous recombination event by eliminating the need for any exogenous transcriptional control elements (Mountford et al., 1994).

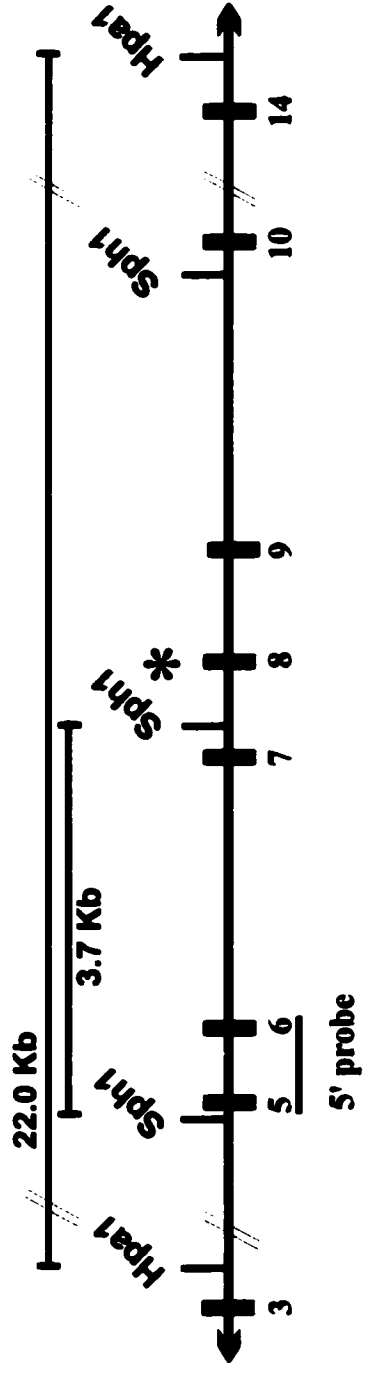
The efficiency of homologous recombination is dependent to some degree on the length of homology present in the targeting vector. A minimum length of homology of 1.7 kb is necessary for the homologous recombination to happen and an increase to 4.2 kb results in a 16-fold increase (Hasty et al., 1991). Further increases in length do not result in as drastic an increase in efficiency (Hasty et al., 1991). Also a minimum of 1 kb of homology on each arm is required for the recombination to occur with fidelity (Thomas et al., 1992). With these considerations in mind, a strategy was designed to generate a vector with a total homology of 4.3 kb, including a short arm of 1.7 kb from a 5.3 kb genomic fragment (see materials and methods). The targeting vector was generated by subcloning of fragments, without the use of PCR to avoid the problem of introducing mutations. Once the cloning was finished, all junctions were sequenced to confirmed their integrity.

After trying different combinations of restriction digests and probe fragments, a final working screening strategy was established, shown in figure 3.4.2. The 1.0 kb probe fragment containing exon 5 and 6, is situated outside the region that was cloned in the targeting vector, eliminating the possibility of false positives that can be created with internal probes combined with a restriction fragment size that coincides with the expected size.

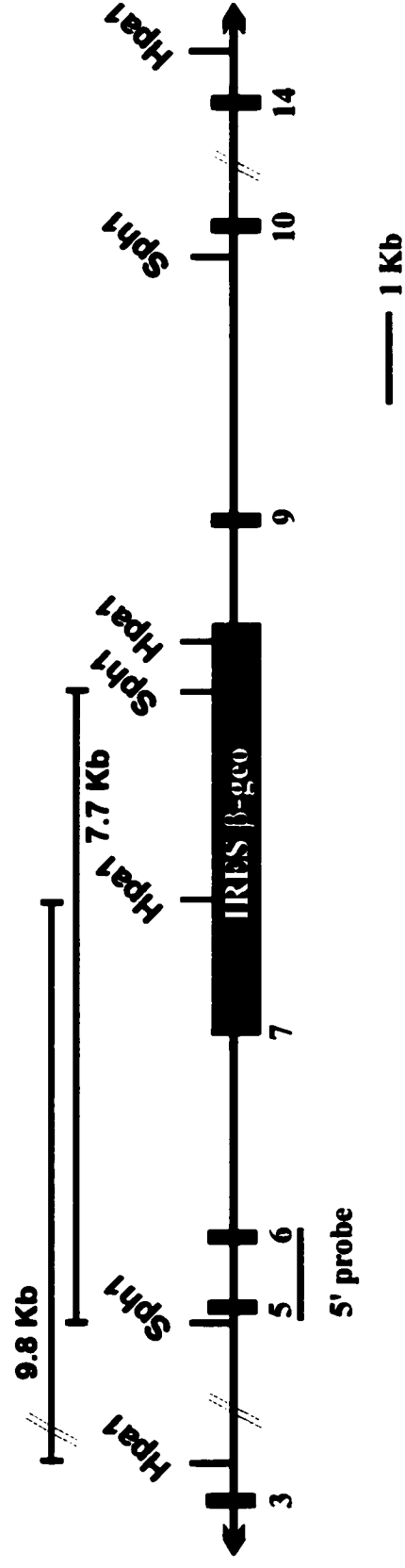
Figure 3.4.2 Screening strategy

Genomic structure of A a wild type allele and B a targeted allele, demonstrating the presence of the restriction sites and the size of expected fragments following a Southern blot analysis with the external exon5-6 fragment as a probe.

A



B

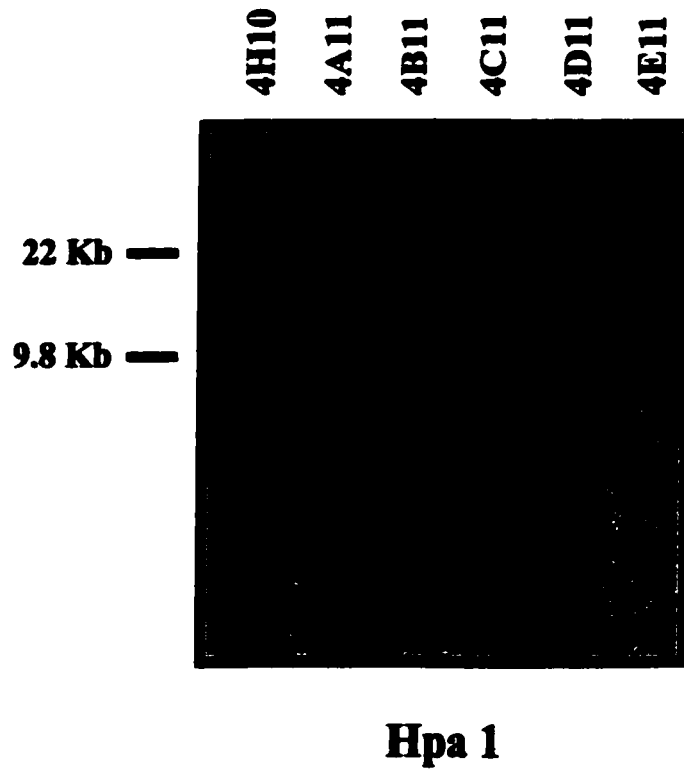


Depending on which of the 2 enzymes is utilised for a particular digest, the targeted allele will be shorter (Hpa1) or longer (Sph1) than the wild-type allele (see figure 3.4.2).

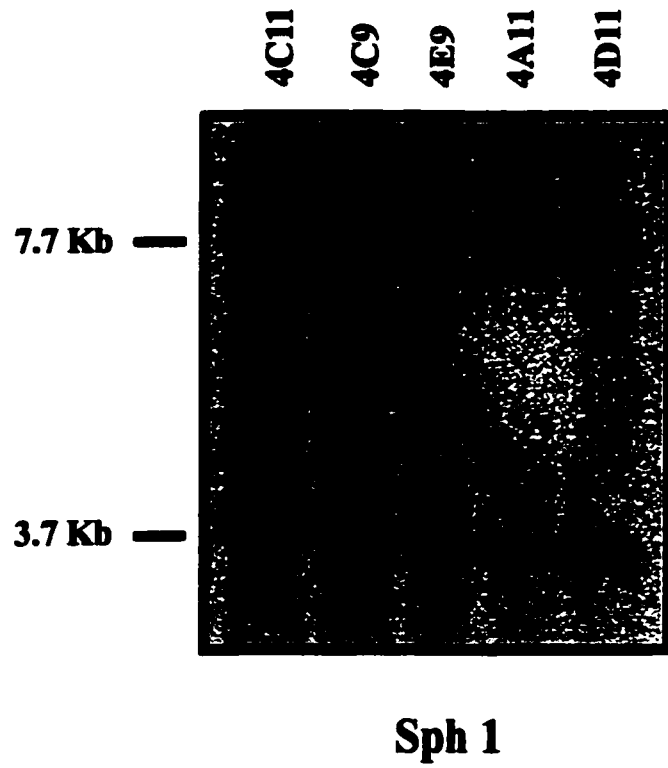
In one set of experiments 128 colonies were picked. Of those 86 grew that gave a signal either positive or negative, from these colonies 6 were determined positive with both screening strategies (two such blots are shown in figure 3.4.3). The efficiency of homologous recombination of 7% would be considered high with the original types of vectors, but is within expected range for the promoter-less vector used. These colonies were thawed, expanded and re-screened to confirm their state before being sent to Rudniki's lab (McMaster University, Hamilton, Ontario) for injection in blastocysts. Unfortunately no chimera were produced from these injections although one mouse had black eyes, a early sign of a possible chimera. Possible explanations were that the ES cells used were too old, or that the media/serum was not appropriate. To eliminate these possibilities, the transfection, selection and colony picking were redone in Rudniki's lab with his cells and media conditions that has worked for other KO studies. In this experiment, two more colonies were found to be positive and were used for injection. Again no chimeras were obtained.

Figure 3.4.3 Results of Southern blot analysis
Southern blot analysis of ES colonies digested with A HpaI and B SphI demonstrating positive colonies.

A



B



Chapter 4

Discussion

Characterization of Unp as a Ubp

In a homology search with protein databases, Unp was found to have domains of homology with the deubiquitinating family of enzymes. More than 60 Ubps have now been discovered from many species. Using the highly conserved Cys and His domains, a phylogenetic tree was constructed with the Ubps entered in the database. This tree is composed of six major groups with Unp fitting in the biggest, group 1. Sixteen Ubps were identified in the completely sequenced yeast genome and are distributed throughout the six groups. Of these, Ubp12 in group 1 is the closest yeast relative of Unp. Groups 1 and 5 contain clusters of yeast Ubps with no related mammalian homologues. This suggests that either these Ubps assume functions in yeast that have no equivalent in mammalian cells, or that mammalian Ubps related to these yeast Ubps have yet to be discovered. Since one of these Ubps, Ubp3, is implicated in gene silencing (Moazed and Johnson, 1996) which is not a yeast specific event, the latter possibility is more likely. In addition to Unp's human homologue, USP4, two other human proteins are highly related, USP11 and USP15. These three human proteins also share high sequence homology in regions outside the Cys and His domains, especially in a region encompassing the Cys domain and the CR1 and the CR2 domains. This suggests that they have evolved from a single gene and may have related functions in the cell. Ubp109, the rat homologue of USP15, was shown to be localized to both the nucleus and the cytoplasm (Park et al., 2000). The nuclear localization was dependent on only one of three similar nuclear localization signals, LKKR which is similar

to Unp's other stretch of basic amino acids, VKKK.

USP21, in group 6, has been shown to possess activity in cleaving NEDD8 conjugates in addition to the ubiquitin conjugates (Gong et al., 2000). This suggests that only a small variation was necessary to convert a ubiquitin cleaving enzyme to a NEDD8 cleaving enzyme. In contrast, enzymes involved in cleaving the SUMO-1 ubiquitin-like protein, SENP1, SUSP1, Ulp1 and Ulp2 do not have homology to the Cys and His domains of Ubps, indicating a greater divergence of the SUMO-1 pathway from the ubiquitin pathway than observed with the NEDD8 pathway. Not surprisingly, the NEDD8 protein is more closely related to ubiquitin (50%) than SUMO-1 (18%) (Jentsch and Pyrowolakis, 2000). With the exception of USP25 which has identical Cys and His domains, USP21 has no close relative. The high degree of conservation between the active site of USP21 and USP25 suggest that USP25 may also be a dual specificity protease.

As suggested by its homology with the Ubp family of enzyme, Unp is a deubiquitinating enzyme. This has been demonstrated with two different types of assays and in parallel in our collaborator's lab (Gilchrist et al., 1997). The first was the traditional assay, developed by Tobias and Varshavsky (1991) and used as a screening strategy that resulted in the cloning of yeast Ubp2 and Ubp3 (Baker et al., 1992). Since then, it has been used for confirming deubiquitinating activity of many proteins including the yeast DOA4 (Papa and Hochstrasser, 1993) and the *Drosophila fat facets* (Huang et al., 1995). From this assay the expected output is a yes or no (white or blue) answer to whether the protein is a deubiquitinating enzyme. The result of light blue colonies for the CR2 mutant was a surprise, as the assay was not expected to discriminate between the levels of activity. Unp

was also shown to be a functional deubiquitinating enzyme with a second assay, the *in vitro* cleavage assay. In this assay, the effect of the CR2 mutation was observed from the H1299 stable cells lines but not from the 293T transient transfections. These observations could be explained by a low efficiency of Unp toward linear fusion substrates, which are probably not their endogenous substrates. When comparing the levels of substrate cleavage of Unp with that of USP3, Ubp1 or Ubp2 (Sloper-Mould et al., 1999; Baker et al., 1999) performed by the same assay, it is clear that Unp does not cleave with the same efficiency. Combined with the low levels of expression expected from a mammalian vector in bacteria, this could have brought down the cleavage activity low enough to detect partial cleavage of the β gal substrate by the CR2 mutant. The high levels of expression in the transiently transfected 293T cells (cells known for their highly efficient transfections (>90%) and strong expression of transfected genes (Pear et al., 1993)) could mask the small effect of the CR2 mutation, which is visible in the lower levels of expression attained in the H1299 stable cell lines.

Unp possesses an N-terminal extension as well as an insertion in the middle of the core catalytic domain. These are assumed to confer specificity and localization to the enzymes. This concept was recently demonstrated by Lin et al. (Lin et al., 2001; Lin et al., 2000). Deletion of these domains in Unp may improve its catalytic activity toward linear fusion proteins, as well as toward polyubiquitinated proteins in general. Unp can cleave the isopeptide bond, at least when ubiquitin is conjugated to an ϵ -N of lysine (Layfield et al., 1999), although it was incapable of cleaving the more complex polyubiquitin chain on proline- β -gal (Gilchrist et al., 1997). This may be due to the extension and insertion which would direct the enzymatic specificity toward its cellular substrates.

There are two possible explanations for the effect of the CR2 mutation on activity. This mutation may cause a change in the protein folding, or it may destroy a zinc finger motif. The latter was considered a possibility since the CR2 domain consists of LXCXE and is followed by another cysteine residue. This CXXC motif in combination with a HXXH motif downstream could form a zinc finger domain. Similar type of zinc finger domain have been found to bind zinc in the human YVH-1 protein-tyrosine phosphatase (Muda et al., 1999) in which it has been shown to regulate its activity. Zinc binding has also been shown to regulate the function of bovine intestinal alkaline phosphatase (Bortolato et al., 1999), and to stabilise both the human Sonic Hedgehog (Day et al., 1999) and the myelin-associated glycoprotein (Kursula et al., 1999). If the reduced activity of this mutant is due to the elimination of a zinc finger motif, it would be expected that the activity of the wild type protein is dependent on the presence of zinc. However, no such dependence on zinc was observed, unless the zinc/protein complexes formed in the cell were bound tightly enough not to be displaced by the presence of high concentrations of EDTA, a formal possibility. In addition, there were some observations that support the misfolding model. The CR1 mutation, which had no effect on activity on its own, aggravates the loss of activity observed with the CR2 mutation alone. In the case of the transient transfection experiments for which no effect was observed with the CR2 mutant, a greatly decreased activity was observed with the CR1/CR2 double mutant. This additional effect of the CR1 mutation may be interpreted as an enhancement of the misfolding induced by the CR2 mutation. If misfolding is the reason for the decreased activity observed with CR2 mutant, it would have to be minor. This mutant still retains part of its activity and it is capable of binding both p107 and p130 proteins, suggesting that the overall folding is correct.

The possibility of assaying Unp's activity in an *in vitro* system as opposed to the bacterial assay has allowed us to better analyse the conditions necessary for Unp's activity. From these studies, it was clear that Unp requires a reduced environment to be active. This observation is not surprising when considering the identity of the amino acids that constitute the active site. It is now established that three amino acids, a cysteine, a histidine and an aspartic acid are essential in the formation of this site, and as such are sometimes referred as the catalytic triad (D'Andrea and Pellman, 1998). The reduced environment may be necessary for the cysteine residue to be in its proper conformation. Otherwise it may form disulfide bonds with other cysteine residues which would most likely affect the conformation of the active site.

A region of Unp was found to be highly acidic, 17 residues out of 32 (53%) are aspartic or glutamic acids. A deletion mutant of this region did not have any obvious effect on the activity or localization of Unp. This region may be involved in protein-protein interactions, possibly with Unp substrates, either as a direct binding site or through the stabilization of an interaction via a basic patch of the interacting protein.

Unp's sub-cellular localisation

Nuclear proteins bigger than approximately 45 KDa can no longer diffuse freely in and out of the nucleus (Miller et al., 1991). They must therefore be actively transported through the nuclear pores. Recognition of these proteins by the nuclear pore complex is mediated by short positively charged sequences termed nuclear localization sequences (NLS). The Unp protein contains a sequence (SQPQKKKK) which has similarity to one of the p53 NLS (NLSI, PQPKKKP) (Shaalsky et al., 1990) and to the SV40 large T antigen

NLS (PKKKRK) (Kalderon et al., 1984). The question was whether this sequence is functional. In the original fractionation experiment, endogenous Unp expression was detected exclusively in the nuclear fraction (Gupta et al., 1994). In addition, transiently transfected myc-Unp in COS1 cells was shown to fractionate to the soluble and insoluble nuclear fractions (Tibbo, 1997). However, the immunofluorescence experiments showed a different picture. Although there was some nuclear expression, seen by confocal microscopy, it is clear that Unp was not located exclusively in the nucleus. This discrepancy can be resolved with the hypothesis that Unp is binding to a structure outside the nucleus which under the conditions utilized would fractionate with the nucleus. Some observations suggested that this could be a possibility. In the images obtained with the confocal microscope, one of the most prominent features was the presence of an intense ring of fluorescence around the nucleus, which was more intense in cells transfected with the catalytically inactive protein. This may result from overwhelming the nuclear import machinery or by a preferred binding site on the nuclear membrane. In addition, pictures of cells transfected with full length Unp, taken from a fluorescence microscope, showed that the fluorescent signal in the cytoplasm appears very filamentous. Recent results obtained with a deconvolution microscope (Gray, personal communication) suggest that myc and GFP tagged Unp in transient transfection colocalizes with the ER protein BiP, in addition to some nuclear expression.

In a paper published in 1998 from a group that was interested in the human homologue of Unp (Frederick et al., 1998), the authors argued for an exclusively cytoplasmic localization of Unp. Their conclusion was based mostly on a fractionation experiment in which they detected Unp only in the cytoplasmic fraction, but was not supported by their

immunofluorescence experiments. In these the localization of Unp was determined by transfection of myc-tag transfected Unp visualized by fluorescent microscopy. In these experiments, they observed whole cell expression with some cells demonstrating nuclear exclusion. Their explanation for these observations was that the whole cell expression was due to a strong cytoplasmic signal masking the nucleus and that unambiguous nuclear-exclusion was evident only in fortuitously well-positioned nuclei. Since no confocal microscopy was performed to confirm nuclear exclusion, and our confocal data show clearly that there is some degree of nuclear expression, an alternative explanation is more likely. Apparent nuclear exclusion was also observed in our immunofluorescence experiment when visualised with a fluorescent microscope. In these the expression levels of transfected Unp was very high thus requiring a shorter exposure time when taking pictures. A weak signal in the nucleus would appear weaker in comparison with a strong signal in the cytoplasm. Another possible explanation would be cell cycle regulated exclusion from the nucleus. Such a relationship of localization with cell cycle progression has not been comprehensively studied, but is not apparent in preliminary experiments (Raynal, unpublished). The differences they observed in the fractionation experiments may be due to more stringent conditions in the fractionation buffer, which may not preserve endogenous interactions.

It still remains unclear to what degree the localization of Unp is due to its putative NLS. Mutation of this domain does not have the expected effect of eliminating all expression in the nucleus. However, by confocal microscopy the proportion of cells which presented dark nuclei and the absence of a ring was much higher in the NLS mutant than with wild-type Unp or other mutants. It would seem that this sequence participates in Unp's localization although it does not have the sole responsibility for Unp's localization. It is

possible that it is part of a bipartite NLS motif. The p53 NLS has recently been identified as a bipartite motif (Liang and Clarke, 1999), although there are no basic residues in the equivalent positions in Unp. There is however another stretch of basic amino acids in Unp that could serve as an NLS (411-RVKKK)(Rao-Naik et al., 2000).

Protein interactions

The CR1 and CR2 domains present in Unp (LHE spacer LXCXE) has been demonstrated to be a pRb binding domain in many other mammalian (RB P-1, RBP-2) and viral (SV40 largeT, ad5 E1A, HPV16 E7) proteins (Defeo-Jones et al., 1991). This suggested that these domains in Unp were also involved in binding pRb. The possible Unp-pRb interactions were first tested by a GST pull down assay. GST-Unp fusions were constructed for the wild-type and different mutants of Unp. Problems with the solubility of the fusion proteins, resulting from the formation of inclusion bodies by the bacteria were greatly reduced by inducing expression at room temperature instead of 37°C as suggested by Takeshima et al, 1994. This problem of purifying soluble protein is not inherent to Unp, but seems to be common with many Ubps (Wilkinson, 1997). With the new conditions it was possible to purify enough proteins to visualise on gel and to perform the binding assay. Full length proteins were obtained which was verified by the presence of both the GST and the myc tags. In addition to full length proteins, other smaller bands were seen. Since these were observed following GSH beads purification, and the GST tag being present at the N-terminus, they most likely result from premature termination of translation. It is not expected that these would interfere with the binding assay. With this assay it was demonstrated that Unp is indeed capable of binding pRb. The interaction is dependent on

an intact CR2 domain, but was not affected by the CR1 mutation. Mutation of the cysteine residue in the CR2 domain is sufficient to abolish binding, as had been observed with other pRb binding proteins (Corbeil and Branton, 1994). The CR1 domain may also contribute to stable binding but is not required in this assay.

Two proteins share homology with pRb and have a similar function in the cell. These proteins, p107 and p130 were also investigated as possible Unp interacting proteins. Both of them were found capable of interacting with Unp, although their interactions were mediated in a different manner from that of pRb. In contrast to pRb where only the CR2 domain was critical for interaction, binding of Unp with p130 can occur through either the CR1 or the CR2 domains. The only mutant that was unable to interact with p130 was the double CR1 and CR2 mutant. An interesting observation was made in some pull-down blots probed with a p130 recognizing antibody. In some experiments the interaction of Unp with p130 was not detected unless the CR2 domain was mutated. One possible explanation for this observation is that the interaction of Unp is stronger with pRb than with p130, in which case, only when Unp is unable to bind pRb (in the CR2 mutant) can it bind p130. An alternate explanation would be that the interaction with pRb is not necessarily stronger but the observation is due to the presence of a higher concentration of pRb than p130 in the protein lysate. This observation was usually made when using sub-confluent, growing, NIH3T3 cells for generating the cell lysates. Most experiments were performed with confluent dishes of cells or a mix of confluent and sub-confluent dishes, and often with cells having some degree of contact inhibition. In cell cycle arrested cells, the most abundant pocket protein is p130 (Smith et al., 1996; Smith et al., 1998). On the other hand, neither motifs were required for binding to p107 protein since all fusion proteins maintained the

ability to interact with it. The region(s) of Unp required for binding with p107 remain unknown. Binding of p107 has been demonstrated for other proteins including cMyc (Beijersbergen et al., 1994b), B-Myb (Sala et al., 1996), and PR59 (a regulatory subunit of protein phosphatase 2A) (Voorhoeve et al., 1999) in addition to E2F-4 (Ginsberg et al., 1994). None of these proteins contain the CR1 or CR2 motifs. HBP1, which contains a CR2 domain is able to bind both pRb and p130, but not p107 (Tevosian et al., 1997). Furthermore, no mutation of E1A's CR1 and CR2 was able to abolish p107 binding (Corbeil and Branton, 1994). There is no obvious motif conserved between these p107 interacting proteins that would be indicative of a possible binding domain. This may be the result of several existing p107 binding motifs, or this motif may only be formed by the three-dimensional structure of the interacting proteins. However, it is still a possibility that Unp binds p107 through either the CR1 or CR2 domain. In this study only point mutations were made in these domains, perhaps a full deletion of a CR domain is required for abolition of the interaction.

Although the Unp interacting proteins in this system are from an endogenous source, from the lysates of untransfected cells, the Unp component is purified from a bacterial expressing system. Although this was necessary to study interaction with Unp mutants, it would be useful to study interactions in a more endogenous setting. It was possible to co-immunoprecipitate endogenous Unp and pRb complexes from H1299 cell lysates. Co-immunoprecipitation was observed when precipitating with both the Unp antibody as well as with a pRb antibody, although not all pRb antibodies were proficient. The fact that not all antibodies were able to co-immunoprecipitate is not surprising. The antigen motif recognized by an antibody, especially with monoclonal antibodies, may be hidden by the

interacting protein of the complex. The signal detected in co-immunoprecipitation was stronger when cells were transfected with both Unp and pRb, as expected for a complex of low abundance. Both the hyperphosphorylated and hypophosphorylated forms of pRb were detected in the coIP experiments, while control IP with E2F-1 only detected the hypophosphorylated form. Only the hypophosphorylated form of pRb was expected to interact with E2F-1 since phosphorylation of pRb is the signal that causes it to release E2F-1 at the end of the G1 phase. The fact that Unp is able to bind both forms of pRb indicates that the interaction is phosphorylation independent thus significantly different from the E2F-1 interaction.

In addition to Unp, other proteins have been demonstrated to bind all three pocket proteins, HDAC-1 (Ferreira et al., 1998), TFIIB (Sutcliffe et al., 1999) and prohibitin (Wang et al., 1999). These proteins are all implicated in the repression of E2F mediated transcription.

In pull-down experiments performed with metabolically labelled proteins for the detection of all interacting proteins, other bands were reproducibly detected in addition to the approximately 100 KDa band. This suggest that other Unp interacting proteins exist. At this point in time thought, the identity of these proteins is unknown.

Phosphorylation status

Unp is detected as a doublet by western blot analysis. This doublet cannot be due to alternative splicing since it is also detected with an anti myc antibody after transfection of a myc-tagged cDNA in cells. Another possibility is that Unp is modified by phosphorylation. This hypothesis was supported by the finding that Unp is phosphorylated

on serine residue(s), detected by a tryptic/phospho-amino acid analysis (see Figure 1 in appendix 2). In that analysis no phosphorylation of threonine or tyrosine residues was detected. Phosphorylation of Unp was confirmed with immunoprecipitation-western experiments with phospho-amino acid antibodies. Phosphorylation was consistently detected with anti phospho-serine antibody whether it was used in the immunoprecipitation (detected with anti Unp) or in the western detection (IP with anti Unp). These experiments do not rule out the possibility of phosphorylation on either threonine or tyrosine. In fact, in some instances positive signals were obtained, but these results were not consistent. The possibility exists that phosphorylation occurs on these residues, but present in lower stoichiometry than with serine phosphorylation, at the limit of detection.

Although Unp is a phosphoprotein, phosphorylation does not account for the appearance of the doublet. In fact both bands can be detected on a western blot with an antibody directed against the phospho-serine epitope. In addition, following treatment with a general phosphatase (CIP), the signal with the phospho-serine antibody is lost, while the doublet is still present when the western blot is probed with anti Unp antibody.

The identity of the modification that results in this doublet is still unknown. In addition to phosphorylation, we have eliminated the possibility of it being a conjugation to ubiquitin and to a ubiquitin like molecule, SUMO-1 (data not shown). Conjugation to SUMO-1 was considered since it has been shown that its conjugation on certain proteins targets them to the nuclear membrane or nuclear pores (Matunis et al., 1998; Saitoh et al., 1998). Since Unp is detected as a ring surrounding the nucleus, it was plausible that conjugation with SUMO-1 was responsible for this localization.

Although the phosphorylation does not account for the presence of the doublet

observed on western blots, it is not without effect. The deubiquitinating activity of Unp was shown to be dependent on its phosphorylation status. Treatment with the CIP phosphatase completely eliminated its activity when assayed by the *in vitro* activity test, while not changing its molecular mass. Whether the phosphorylation event necessary for regulating Unp's activity is on a serine residue or on a hard to detect threonine/tyrosine residue is still unknown. It is also still unknown which serine residue(s) are phosphorylated in Unp.

The possibility that a deubiquitinating enzyme is regulated by phosphorylation is very interesting. It would be the first case of such regulation with the deubiquitinating aspect of the ubiquitin pathway. The ubiquitination part of the pathway has been demonstrated to be regulated in some instances by phosphorylation. Many proteins involved in the G1 phase of the cell cycle are degraded in a phosphorylation dependent manner. These include cyclin E (Clurman et al., 1999; Won and Reed, 1996) and the Cdk inhibitor p27 (Tsvetkov et al., 1999). These proteins are recognized by the SCF type of E3 enzymes. To date, all proteins ubiquitinated with help of the SCF E3s have been shown to require phosphorylation for recognition. The only possible exception is E2F-1 for which it is still unknown if phosphorylation is required, although it is expected that it will be. Regulation of Unp's activity by kinases and phosphatases may be a quick way for the cell to respond to a changing environment. If phosphorylation by a kinase is sufficient for Unp's activation following a specific signal, it could quickly result in the stabilization of its substrate(s). See manuscript in Appendix 2 for a more comprehensive analysis of the phosphorylation status of Unp.

Model and speculation

As more deubiquitinating enzymes are discovered, it is becoming clear that they are not solely involved in the recycling of ubiquitin monomers as originally thought, but have important roles in regulating specific pathways. Some of these Ubps are expressed only in certain conditions and in certain tissues. For example, the murine Dub1 (Zhu et al., 1996) and Dub2 (Zhu et al., 1997) proteins are only expressed in hematopoietic cells following specific cytokine induction. Their overexpression induces cell cycle arrest and thus they seem to provide a checkpoint for proper induction of cycling. The Ubps are now viewed as critical regulators of protein degradation. The half life of a particular protein is probably mediated by the balance of the activity of ubiquitin conjugating and cleaving enzymes, where a change in the level of activity of either could change the stability of the substrate. This type of regulation would permit a rapid temporal or spatial change of a protein's abundance by modifying the activity of either enzymes. This is of particular interest in the cell cycle where a protein's stability suddenly decreases in a particular stage of the cycle.

The questions that are now arising in the deubiquitinating field are dealing with discovering the identity of Ubp substrates and the modes of regulation of these enzymes. For most of these enzymes, including Unp, these answers are still unknown.

When overexpressed, Unp acts as an oncogene (Gupta et al., 1994), and its human homologue (USP4) has been shown to be involved in certain types of human lung cancers (Gray et al., 1995). It is therefore a likely possibility that at least one of its substrates is involved in the control of the cell cycle. Although Unp is capable of binding the three pocket proteins, it is unlikely that these are actual substrates of Unp. There are two reasons for this. Due to their transient nature, an enzyme-substrate interaction is not expected to be strong

enough for detection by the type of assay used. But more important, with the exception of p130 (Smith et al., 1998), these proteins have not been shown to be conjugated by ubiquitin and degraded by the ubiquitin pathway. It is more probable that the effect of their binding is the regulation of Unp's activity in some manner. One interesting possibility is that these pocket proteins serve as scaffold proteins to bring Unp in contact with its substrate(s).

Since Unp is oncogenic when overexpressed, it is not unreasonable to postulate that at least one of its substrates is a proto-oncogene. The overexpression of Unp is expected to result in the stabilization of its substrate(s) by the shortening of their polyubiquitin chain. It would therefore produce the same effect as overexpressing these substrates, including this possible proto-oncogene. In fact, this effect of overexpression has been demonstrated with the *Drosophila* deubiquitinating enzyme Faf, the first Ubp for which a substrate has been identified. An increased expression of its substrate, liquid facet (lqf), was found to be sufficient to compensate for the effects caused by the absence of functional faf proteins (Cadavid et al., 2000).

The E2F family of transcription factors have many characteristics that make them good candidates for Unp's substrates. First, many of its members have proven oncogenic properties (Xu et al., 1995). E2F-1 in particular has been shown to be sufficient to induce DNA replication (Johnson et al., 1993; Kowalik et al., 1995; Qin et al., 1994; Shan and Lee, 1994), and to cooperate with activated Ras to transform cells and form tumours in nude mice (Johnson et al., 1994a; Jooss et al., 1995; Singh et al., 1994). The activity of the E2Fs is tightly regulated at many levels. In addition to their temporally regulated transcription (Johnson et al., 1994b; Neuman et al., 1994; Slansky et al., 1993), the activity of the proteins is modulated negatively by binding to pocket proteins, their DNA binding activity is

regulated by phosphorylation (by cyclinA/Cdk2)(Dymlacht et al., 1994; Krek et al., 1995; Leone et al., 1998), and the protein levels are regulated by ubiquitin mediated degradation. This degradation has been demonstrated for E2F-1, 3, and 4 (Flores et al., 1998; Hateboer et al., 1996; Hofmann et al., 1996), suggesting that it is a general mechanism to regulate E2F protein abundance. Interestingly, the ubiquitin mediated degradation of E2F1 and E2F4 occurs only with the “free” form of E2Fs (free of pocket proteins, but in a dimerized form with DP). Binding of E2F with a pocket protein protects it from degradation (Hateboer et al., 1996; Hofmann et al., 1996; Martelli and Livingston, 1999). To date, this protection effect of the pocket proteins has not been fully explained. It is thought to be necessary to assure that in G0 and G1 phases of the cycle, enough E2F/pocket protein complexes are present in the cell to fully repress E2F responsive genes, while the excessive E2F, which is not bound to a pocket protein would be degraded. Since most of the free E2F occurs in S phase while the pocket-protein bound E2F occurs mostly in G0-G1 phase, the protection of E2F by pocket proteins results in phase dependent different half-life of E2F. Expression of large T antigen which abolishes E2F/pRb complexes also eliminates this cycle dependent difference in half life (Martelli and Livingston, 1999).

Our model is that the E2Fs are substrates of Unp, that their protection from degradation obtained by binding to pocket proteins is mediated by the action of Unp, see Figure 4.1. In effect, the function of Unp's binding to the pocket proteins would be to allow Unp access to its substrates, the E2Fs, but only in G0, G1 phases when these proteins need to be stabilized. Unp ability to bind p107 and p130 in addition to pRb would result from the fact that E2F-4 and E2F-5 binds preferentially to them.

Although the binding of viral CR2 containing proteins to pRb results in the dissociation of pRb from the E2Fs, this is not a direct consequence of the CR2 mediated binding. This effect most likely arises from a steric hindrance caused by other parts of the viral proteins. Binding of HDAC-1 to pRb through a similar CR2 domain does not disrupt its interaction with the E2Fs. In fact it depends on these interactions to assume its function which is the repression of E2F-responsive genes (Ferreira et al., 1998). In the same manner, binding of Unp to pocket proteins is not expected to disrupt the pocket/E2F interactions.

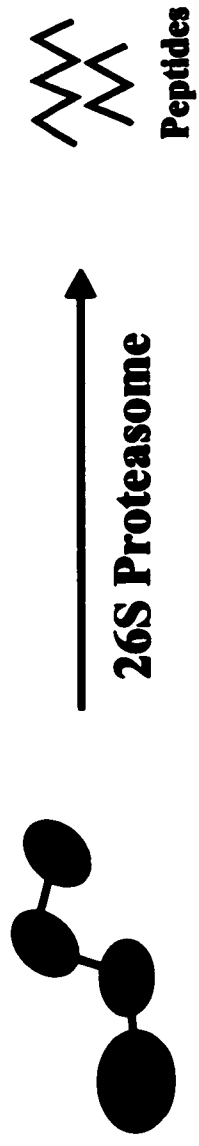
The CR2 binding site in pRb is highly conserved across species which suggests that this structure is critical to the normal function of pRb (Lee et al., 1998). Many pRb interacting proteins have been discovered, and among them at least 19 (in addition to Unp) have the LXCXE or related sequence (Dick et al., 2000). A key component of pRb's properties as a cell cycle regulator is thought to be the regulation of E2F-dependent transcription (Dyson, 1998), and it has been shown that the repression of E2F target genes is sufficient to induce a cell cycle arrest (Sellers et al., 1995). The elimination of the LXCXE binding site in pRb does not affect pRb's ability to bind the E2Fs (Dick et al., 2000). Surprisingly, this mutant retained the ability to actively repress the transcription of E2F-responsive promoters and efficiently arrest pRb-deficient cells in G1 (Dick et al., 2000). As a consequence, that mutant generates a pRb arrest that is resistant to the inactivating effects of viral proteins. Thus, the evolutionary conservation of the LXCXE binding motif does not have the purpose of binding cellular proteins to affect its cell cycle arrest properties. It must therefore be conserved to bind cellular LXCXE containing proteins whose function is independent of cell cycle arrest. Binding to Unp may be one of these functions. Unp's

Figure 4.1 Model of Unp's function

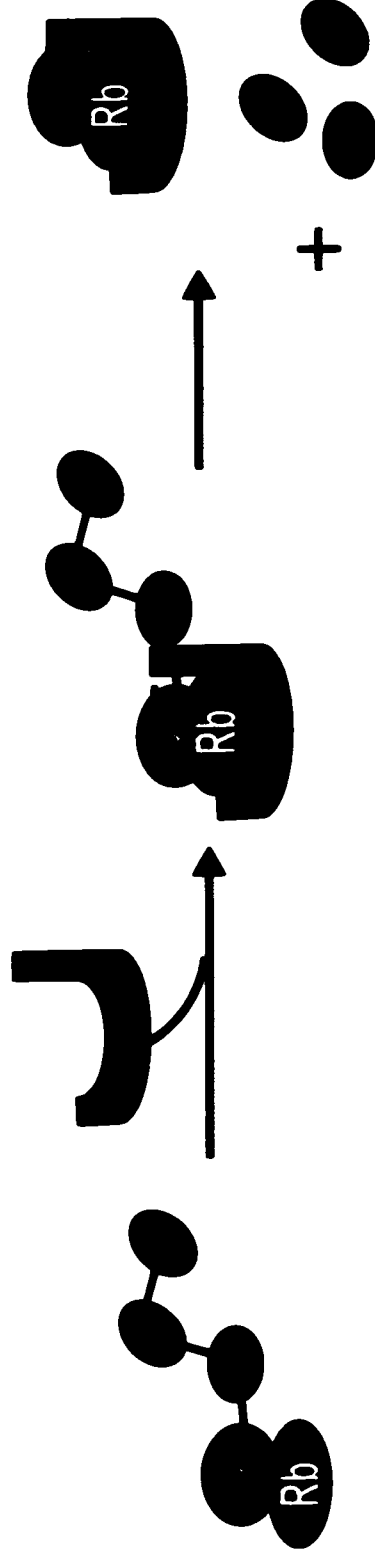
A Cells in S phase. In this phase, the E2Fs are not in complex with pocket proteins. In this free form, they are ubiquitinated and efficiently degraded by the proteasome.

B Cells in G0 (quiescence) or early G1. In these phases, the E2Fs are bound to pocket proteins (here shown with pRb). Although ubiquitination still occurs, the chains are cleaved by Unp which have access to the E2Fs through its interaction with the pocket proteins. As a result, the E2F proteins possess a longer half-life.

A



B



binding with pocket proteins and the stabilization of E2Fs may assure that enough E2F is present at the restriction point to promote an efficient transition from G1 to S phase.

A more direct piece of evidence to support this model of Unp was obtained from *in vitro* experiments in which the synthesis of a polyubiquitin chain on E2F-1 was reduced by a factor of two in the presence of Unp (Pagano, personal communication).

Although the *in vivo* experiments did not demonstrate any significant differences on the stability of E2Fs in Unp transfected versus untransfected cells, this may simply be due to an inappropriate system used in these experiments. The ideal system would be a Unp negative cell line versus a Unp expressing cell line. Since Unp expression is ubiquitous to all cells, a knock-out cell line would be required. Unfortunately, all the attempts made to generate knock-out mice were unsuccessful. So the next best approach was also tried. ES cells in which one allele had been targeted by the knock-out vector were grown in 10 times the concentration of hygromycin to select for colonies in which the second allele was converted to the targeted allele. Although tried with many ES clones, this approach was also unsuccessful. This failure to generate a double knock-out ES cell line may be due to technical difficulties (location of Unp's gene) or to the possibility that the absence of Unp is lethal to the cells.

This model may also help explain the difficulties encountered when attempts were made in establishing stable cell lines expressing Unp. E2F-1 has been shown to have tumour-suppressor capabilities in addition to its oncogenic ability (Field et al., 1996; Yamasaki et al., 1996) when functionally overexpressed. This function of E2F-1 is mediated through its ability to induce apoptosis in a p53 dependent manner. Thus, cells overexpressing Unp may as a consequence also be overexpressing E2F-1. This would result

in an activation of p53 followed by either apoptosis or cell cycle arrest. In either case, the transfected cells would not be able to grow sufficiently to produce colonies. Interestingly, the two cell lines in which it was possible to obtain stable colonies, p19 and H1299, have been confirmed to be deficient in the expression of p53 (Boivin et al., 1999; Schmidt-Kastner et al., 1998). In the original tumorigenicity experiment, Unp was transfected in NIH3T3 cells and injected in nude mice. Mice receiving Unp-transfected cells produce tumours whereas mice receiving untransfected cells did not. However it has not been possible to establish Unp stable cell lines with NIH3T3 cells. At most these colonies express for a few passages before losing expression. This apparent discrepancy can be resolved when taking into consideration that for the nude mice experiment, Unp transfected cells were selected only for a brief time before they were pooled and injected in mice. Whereas in the stable experiments individual colonies were selected, thus requiring long term stability of expression. The conditions of growth are also very different in tissue culture compared to growth in the mouse organism. Therefore a gene whose expression is selected against in an *in vitro* culture, would not necessary be selected against in an *in vivo* culture, or as rapidly.

Although the model proposes E2Fs as substrates, these are most likely not the only ones, especially since only a small fraction of Unp seems to localize to the nucleus. Other substrates of Unp may be found to co-localize at the nuclear envelope or at the endoplasmic reticulum (ER).

Unp has previously been demonstrated to be an oncogene. The human homologue, USP4, has been implicated in lung cancers, in small cell lung cancer and adenocarcinomas of the lung (Gray et al., 1995), in which it was consistently overexpressed. USP4 has been

localized to chromosome 3p21 (Gray et al., 1995), a region that is frequently rearranged in lung and breast tumors (Ali et al., 1989; Kok et al., 1987). This suggests a mechanism of action for the induction of Unp's overexpression in lung tumor.

Interestingly, Eymin et al. have recently shown that E2F1 is upregulated in the majority of small cell lung cancer (Eymin et al., 2001). This upregulation of E2F1 was not due to amplification of the gene, but to increase levels of mRNA. The E2F1 was functional, determined by elevated levels of cyclin A and E, cdc2 and DHFR, and they found no correlation between the status of E2F1 and pRb in the cells. The authors were unable to explain the elevated expression of E2F1 in these tumors except to mention the observation that E2F1 has been reported to stimulate its own transcription. Since E2F1 is known to be a tumor suppressor as well as an oncogene, they have measured the ratio of Bcl2 and Bax and the KI67 apoptotic index and determined that SCLC are less susceptible to apoptosis than NSCLC (non small cell lung cancer), which correlated with the levels of E2F1 between these two groups. Therefore, they conclude that in SCLC, overexpression of E2F1 would lead to increase proliferation. One possible explanation of these results, in the light of our model is that the overexpression of Unp seen in this type of tumors may protect E2F1 from degradation which would in return transactivate its own promoter to result in a drastic increase in the E2F1 levels and promote proliferation.

It is now demonstrated that Unp is a deubiquitinating enzyme capable of interacting with the three pocket proteins. Since these proteins are involved in the control of the cell cycle, it is a likely possibility that overexpression of an enzymatically active Unp affect their regulation. As a consequence, interfering with Unp's enzymatic activity would be a possible approach in restoring the cell cycle control that is disrupted in these tumors.

It will be very interesting to find out what the phenotype of mice null for Unp will be. The targeting vector will be used with a different system for generating knock-outs that has been recently established in the Cancer Research Group. Their approach use the newer aggregation technique combine with a different ES cell line, (R1 cells) instead of the traditional blastocyst injection method. The recent cloning of two human genes, USP15 and USP11 (Baker et al., 1999; Swanson et al., 1996), with high homology with USP4 suggest the presence of Unp related genes in the mouse genome. Whether these proteins have the same localization and specificity than Unp, and if they have redundancy remains to be determined, but Unp null mutant mice would also address that issue.

References

- Alesse, E., Zazzeroni, F., Angelucci, A., Giannini, G., Di Marcotullio, L., and Gulino, A. (1998). The growth arrest and downregulation of c-myc transcription induced by ceramide are related events dependent on p21 induction, Rb underphosphorylation and E2F sequestering. *Cell Death Differ* 5, 381-9.
- Ali, I. U., Lidereau, R., and Callahan, R. (1989). Presence of two members of c-erbA receptor gene family (c-erbA beta and c-erbA2) in smallest region of somatic homozygosity on chromosome 3p21- p25 in human breast carcinoma [see comments]. *J Natl Cancer Inst* 81, 1815-20.
- Amerik, A., Swaminathan, S., Krantz, B. A., Wilkinson, K. D., and Hochstrasser, M. (1997). In vivo disassembly of free polyubiquitin chains by yeast Ubp14 modulates rates of protein degradation by the proteasome. *Embo J* 16, 4826-38.
- Amon, A., Imiger, S., and Nasmyth, K. (1994). Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell* 77, 1037-50.
- Amon, A., Tyers, M., Futcher, B., and Nasmyth, K. (1993). Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. *Cell* 74, 993-1007.
- Baker, R. T., Wang, X. W., Woollatt, E., White, J. A., and Sutherland, G. R. (1999) Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. *Genomics* 59, 264-74.
- Baker, R. T., Tobias, J. W., and Varshavsky, A. (1992). Ubiquitin-specific proteases of *Saccharomyces cerevisiae*. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. *J Biol Chem* 267, 23364-75.
- Bast, R. C., Kufe, D. W., Pollock, R. E., Weichselbaum, R. R., Holland, J. F., and Frei, E. (2000). *Holland-Frei Cancer Medicine-5*. B.C. Decker, Inc. 2546 pages.
- Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998). The proteasome: paradigm of a self-compartmentalizing protease. *Cell* 92, 367-80.
- Beijersbergen, R. L., Kerkhoven, R. M., Zhu, L., Carlee, L., Voorhoeve, P. M., and Bernards, R. (1994a). E2F-4, a new member of the E2F gene family, has oncogenic activity and associates with p107 in vivo. *Genes Dev* 8, 2680-90.
- Beijersbergen, R. L., Hijmans, E. M., Zhu, L., and Bernards, R. (1994b). Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. *Embo J* 13, 4080-6.

Boivin, D., Morrison, M. R., Marcellus, R. C., Querido, E., and Branton, P. E. (1999). Analysis of synthesis, stability, phosphorylation, and interacting polypeptides of the 34-kilodalton product of open reading frame 6 of the early region 4 protein of human adenovirus type 5. *J Virol* *73*, 1245-53.

Bonifacino, J. S., and Weissman, A. M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annu Rev Cell Dev Biol* *14*, 19-57.

Bortolato, M., Besson, F., and Roux, B. (1999). Role of metal ions on the secondary and quaternary structure of alkaline phosphatase from bovine intestinal mucosa. *Proteins* *37*, 310-8.

Braun, B. C., Glickman, M., Kraft, R., Dahlmann, B., Kloetzel, P. M., Finley, D., and Schmidt, M. (1999). The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nat Cell Biol* *1*, 221-6.

Breitschopf, K., Bengal, E., Ziv, T., Admon, A., and Ciechanover, A. (1998). A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. *Embo J* *17*, 5964-73.

Brook, F. A., and Gardner, R. L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci U S A* *94*, 5709-12.

Brooks, P., Fuertes, G., Murray, R. Z., Bose, S., Knecht, E., Rechsteiner, M. C., Hendil, K. B., Tanaka, K., Dyson, J., and Rivett, J. (2000). Subcellular localization of proteasomes and their regulatory complexes in mammalian cells. *Biochem J* *346 Pt 1*, 155-61.

Brown, V. D., Phillips, R. A., and Gallie, B. L. (1999). Cumulative effect of phosphorylation of pRB on regulation of E2F activity. *Mol Cell Biol* *19*, 3246-56.

Cadavid, A. L., Ginzel, A., and Fischer, J. A. (2000). The function of the *Drosophila* Fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis. *Development* *127*, 1727-1736.

Cai, S. Y., Babbitt, R. W., and Marchesi, V. T. (1999). A mutant deubiquitinating enzyme (Ubp-M) associates with mitotic chromosomes and blocks cell division. *Proc Natl Acad Sci U S A* *96*, 2828-33.

Canadian Cancer Statistics, LCDC, Health Canada (2000). Canadian Cancer Society, National Cancer Institute of Canada, Statistics Canada, and Health Canada).

Chau, V., Tobias, J. W., Bachmair, A., Marriott, D., Ecker, D. J., Gonda, D. K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* *243*, 1576-83.

Chellappan, S. P., Hiebert, S., Mudryj, M., Horowitz, J. M., and Nevins, J. R. (1991). The

E2F transcription factor is a cellular target for the RB protein. *Cell* 65, 1053-61.

Chen, Z., Hagler, J., Palombella, V. J., Melandri, F., Scherer, D., Ballard, D., and Maniatis, T. (1995). Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes Dev* 9, 1586-97

Chow, K. N. and Dean, D. C. (1996). Domains A and B in the Rb pocket interact to form a transcriptional repressor motif. *Mol Cell Biol* 16, 4862-8.

Chow, K. N., Starostik, P., and Dean, D. C. (1996). The Rb family contains a conserved cyclin-dependent-kinase-regulated transcriptional repressor motif. *Mol Cell Biol* 16, 7173-81.

Chung, C. H., and Baek, S. H. (1999). Deubiquitinating enzymes: their diversity and emerging roles. *Biochem Biophys Res Commun* 266, 633-40.

Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *Embo J* 17, 7151-60.

Ciechanover, A., Orian, A., and Schwartz, A. L. (2000). Ubiquitin-mediated proteolysis: biological regulation via destruction. *Bioessays* 22, 442-51.

Ciechanover, A., Orian, A., and Schwartz, A. L. (2000). The ubiquitin-mediated proteolytic pathway: Mode of action and clinical implications. *J Cell Biochem* 77, 40-51.

Clarke, A. R., Maandag, E. R., van Roon, M., van der Lugt, N. M., van der Valk, M., Hooper, M. L., Berns, A., and te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development [see comments]. *Nature* 359, 328-30.

Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., and Roberts, J. M. (1996). Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdk2 binding and cyclin phosphorylation. *Genes Dev* 10, 1979-90.

Cobrinik, D., Lee, M. H., Hannon, G., Mulligan, G., Bronson, R. T., Dyson, N., Harlow, E., Beach, D., Weinberg, R. A., and Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev* 10, 1633-44.

Cook, W. J., Jeffrey, L. C., Kasperek, E., and Pickart, C. M. (1994). Structure of tetraubiquitin shows how multiubiquitin chains can be formed. *J Mol Biol* 236, 601-9.

Corbeil, H. B., and Branton, P. E. (1994). Functional importance of complex formation between the retinoblastoma tumor suppressor family and adenovirus E1A proteins as determined by mutational analysis of E1A conserved region 2. *J Virol* 68, 6697-709.

D'Andrea, A., and Pellman, D. (1998). Deubiquitinating enzymes: a new class of biological regulators. *Crit Rev Biochem Mol Biol* 33, 337-52.

Davis, N. G., Horecka, J. L., and Sprague, G. F., Jr. (1993). Cis- and trans-acting functions required for endocytosis of the yeast pheromone receptors. *J Cell Biol* 122, 53-65.

Day, E. S., Wen, D., Garber, E. A., Hong, J., Avedissian, L. S., Rayhorn, P., Shen, W., Zeng, C., Bailey, V. R., Reilly, J. O., Roden, J. A., Moore, C. B., Williams, K. P., Galdes, A., Whitty, A., and Baker, D. P. (1999). Zinc-dependent structural stability of human Sonic hedgehog. *Biochemistry* 38, 14868-80.

Day, M. L., Foster, R. G., Day, K. C., Zhao, X., Humphrey, P., Swanson, P., Postigo, A. A., Zhang, S. H., and Dean, D. C. (1997). Cell anchorage regulates apoptosis through the retinoblastoma tumor suppressor/E2F pathway. *J Biol Chem* 272, 8125-8.

Debbas, M., and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* 7, 546-54.

DeCaprio, J. A., Furukawa, Y., Ajchenbaum, F., Griffin, J. D., and Livingston, D. M. (1992). The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression. *Proc Natl Acad Sci USA* 89, 1795-8.

DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* 54, 275-83.

Defeo-Jones, D., Huang, P. S., Jones, R. E., Haskell, K. M., Vuocolo, G. A., Hanobik, M. G., Huber, H. E., and Oliff, A. (1991). Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. *Nature* 352, 251-4.

DeGregori, J., Kowalik, T., and Nevins, J. R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes [published erratum appears in *Mol Cell Biol* 1995 Oct;15(10):5846-7]. *Mol Cell Biol* 15, 4215-24.

DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc Natl Acad Sci U S A* 94, 7245-50.

Denu, J. M., Stuckey, J. A., Saper, M. A., and Dixon, J. E. (1996). Form and function in protein dephosphorylation. *Cell* 87, 361-4.

Deshaies, R. J., Chau, V., and Kirschner, M. (1995). Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *Embo J* 14, 303-12.

Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998). SUMO-1 modification of I κ B α inhibits NF- κ B activation. *Mol Cell* 2, 233-9.

Di Fruscio, M., Gilchrist, C. A., Baker, R. T., and Gray, D. A. (1998). Genomic structure of Unp, a murine gene encoding a ubiquitin-specific protease. *Biochim Biophys Acta* 1398, 9-

17.

Dick, F. A., Sailhamer, E., and Dyson, N. J. (2000). Mutagenesis of the pRB pocket reveals that cell cycle arrest functions are separable from binding to viral oncoproteins. *Mol Cell Biol* 20, 3715-27.

Diehl, J. A., Zindy, F., and Sherr, C. J. (1997). Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. *Genes Dev* 11, 957-72.

Draetta, G., Luca, F., Westendorf, J., Brizuela, L., Ruderman, J., and Beach, D. (1989). Cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. *Cell* 56, 829-38.

Dynlacht, B. D., Flores, O., Lees, J. A., and Harlow, E. (1994). Differential regulation of E2F transactivation by cyclin/cdk2 complexes. *Genes Dev* 8, 1772-86.

Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes Dev* 12, 2245-62.

Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* 243, 934-7.

Evans, T., Rosenthal, E. T., Youngblom, J., Distel, D., and Hunt, T. (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33, 389-96.

Eymin, B., Gazzeri, S., Brambilla, C., Brambilla, E. (2001). Distinct pattern of E2F1 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma. *Oncogene* 20, 1678-87.

Ewen, M. E., Sluss, H. K., Sherr, C. J., Matsushime, H., Kato, J., and Livingston, D. M. (1993). Functional interactions of the retinoblastoma protein with mammalian D- type cyclins. *Cell* 73, 487-97.

Ewen, M. E., Xing, Y. G., Lawrence, J. B., and Livingston, D. M. (1991). Molecular cloning, chromosomal mapping, and expression of the cDNA for p107, a retinoblastoma gene product-related protein. *Cell* 66, 1155-64.

Fattaey, A. R., Harlow, E., and Helin, K. (1993). Independent regions of adenovirus E1A are required for binding to and dissociation of E2F-protein complexes. *Mol Cell Biol* 13, 7267-77.

Fehling, H. J., Swat, W., Laplace, C., Kuhn, R., Rajewsky, K., Muller, U., and von Boehmer, H. (1994). MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* 265, 1234-7.

Ferreira, R., Magnaghi-Jaulin, L., Robin, P., Harel-Bellan, A., and Trouche, D. (1998). The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. *Proc Natl Acad Sci U S A* 95, 10493-8.

Field, S. J., Tsai, F. Y., Kuo, F., Zubiaga, A. M., Kaelin, W. G., Jr., Livingston, D. M., Orkin, S. H., and Greenberg, M. E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* 85, 549-61.

Finley, D., Bartel, B., and Varshavsky, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* 338, 394-401.

Finley, D., Sadis, S., Monia, B. P., Boucher, P., Ecker, D. J., Crooke, S. T., and Chau, V. (1994). Inhibition of proteolysis and cell cycle progression in a multiubiquitination-deficient yeast mutant. *Mol Cell Biol* 14, 5501-9.

Fischer-Vize, J. A., Rubin, G. M., and Lehmann, R. (1992). The fat facets gene is required for Drosophila eye and embryo development. *Development* 116, 985-1000.

Flores, A. M., Kassatly, R. F., and Cress, W. D. (1998). E2F-3 accumulation is regulated by polypeptide stability. *Oncogene* 16, 1289-98.

Francke, U. (1976). Retinoblastoma and chromosome 13. *Cytogenet Cell Genet* 16, 131-4

Frederick, A., Rolfe, M., and Chiu, M. I. (1998). The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines [published erratum appears in *Oncogene* 1998 Apr 30;16(17):2293]. *Oncogene* 16, 153-65.

Freedman, D. A., and Levine, A. J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. *Mol Cell Biol* 18, 7288-93.

Fritsche, M., Haessler, C., and Brandner, G. (1993). Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents [published erratum appears in *Oncogene* 1993 Sep;8(9):2605]. *Oncogene* 8, 307-18.

Fuchs, S. Y., Adler, V., Buschmann, T., Yin, Z., Wu, X., Jones, S. N., and Ronai, Z. (1998). JNK targets p53 ubiquitination and degradation in nonstressed cells. *Genes Dev* 12, 2658-63.

Funa, K., Steinholtz, L., Nou, E., and Bergh, J. (1987). Increased expression of N-myc in human small cell lung cancer biopsies predicts lack of response to chemotherapy and poor prognosis. *Am J Clin Pathol* 88, 216-20.

Galan, J. M., Moreau, V., Andre, B., Volland, C., and Haguenaer-Tsapis, R. (1996). Ubiquitination mediated by the Np1p/Rsp5p ubiquitin-protein ligase is required for endocytosis of the yeast uracil permease. *J Biol Chem* 271, 10946-52.

Ghislain, M., Dohmen, R. J., Levy, F., and Varshavsky, A. (1996). Cdc48p interacts with Ufd3p, a WD repeat protein required for ubiquitin-mediated proteolysis in *Saccharomyces cerevisiae*. *Embo J* 15, 4884-99.

Giaccia, A. J., and Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* 12, 2973-83.

Gilchrist, C. A., Gray, D. A., and Baker, R. T. (1997). A ubiquitin-specific protease that efficiently cleaves the ubiquitin-proline bond. *J Biol Chem* 272, 32280-5.

Ginsberg, D., Vairo, G., Chittenden, T., Xiao, Z. X., Xu, G., Wydner, K. L., DeCaprio, J. A., Lawrence, J. B., and Livingston, D. M. (1994). E2F-4, a new member of the E2F transcription factor family, interacts with p107. *Genes Dev* 8, 2665-79.

Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998). The regulatory particle of the *Saccharomyces cerevisiae* proteasome. *Mol Cell Biol* 18, 3149-62.

Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway [see comments]. *Nature* 349, 132-8.

Gong, L., Kamitani, T., Millas, S., and Yeh, E. T. (2000). Identification of a novel isopeptidase with dual specificity for ubiquitin- and NEDD8-conjugated proteins. *J Biol Chem* 275, 14212-6.

Gostissa, M., Hengstermann, A., Fogal, V., Sandy, P., Schwarz, S. E., Scheffner, M., and Del Sal, G. (1999). Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1. *Embo J* 18, 6462-71.

Gottesman, S., Wickner, S., and Maurizi, M. R. (1997). Protein quality control: triage by chaperones and proteases. *Genes Dev* 11, 815-23.

Govers, R., ten Broeke, T., van Kerkhof, P., Schwartz, A. L., and Strous, G. J. (1999). Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor. *Embo J* 18, 28-36.

Gray, D. A., Inazawa, J., Gupta, K., Wong, A., Ueda, R., and Takahashi, T. (1995). Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. *Oncogene* 10, 2179-83.

Groettrup, M., Soza, A., Eggers, M., Kuehn, L., Dick, T. P., Schild, H., Rammensee, H. G., Koszinowski, U. H., and Kloetzel, P. M. (1996). A role for the proteasome regulator PA28alpha in antigen presentation. *Nature* 381, 166-8.

Groll, M., Ditzel, L., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997). Structure of 20S proteasome from yeast at 2.4 Å resolution [see comments]. *Nature* 386, 463-71.

- Gupta, K., Chevrette, M., and Gray, D. A. (1994). The Unp proto-oncogene encodes a nuclear protein. *Oncogene* 9, 1729-31.
- Gupta, K., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Gray, D. A. (1993). Unp, a mouse gene related to the tre oncogene. *Oncogene* 8, 2307-10.
- Haas, A. L., and Siepmann, T. J. (1997). Pathways of ubiquitin conjugation. *Faseb J* 11, 1257-68.
- Haas, A. L., Warms, J. V., Hershko, A., and Rose, I. A. (1982). Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. *J Biol Chem* 257, 2543-8.
- Hagan, I., Hayles, J., and Nurse, P. (1988). Cloning and sequencing of the cyclin-related *cdc13+* gene and a cytological study of its role in fission yeast mitosis. *J Cell Sci* 91, 587-95.
- Hall, P. A., McKee, P. H., Menage, H. D., Dover, R., and Lane, D. P. (1993). High levels of p53 protein in UV-irradiated normal human skin. *Oncogene* 8, 203-7.
- Hannon, G. J., Demetrick, D., and Beach, D. (1993). Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes Dev* 7, 2378-91.
- Harbour, J. W., Lai, S. L., Whang-Peng, J., Gazdar, A. F., Minna, J. D., and Kaye, F. J. (1988). Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* 241, 353-7.
- Harbour, J. W., Luo, R. X., Dei Santi, A., Postigo, A. A., and Dean, D. C. (1999). Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell* 98, 859-69.
- Hartwell, L. H., and Kastan, M. B. (1994). Cell cycle control and cancer. *Science* 266, 1821-8.
- Hasty, P., Rivera-Perez, J., and Bradley, A. (1991). The length of homology required for gene targeting in embryonic stem cells. *Mol Cell Biol* 11, 5586-91.
- Hateboer, G., Kerkhoven, R. M., Shvarts, A., Bernards, R., and Beijersbergen, R. L. (1996). Degradation of E2F by the ubiquitin-proteasome pathway: regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev* 10, 2960-70.
- Hatfield, P. M., Callis, J., and Vierstra, R. D. (1990). Cloning of ubiquitin activating enzyme from wheat and expression of a functional protein in *Escherichia coli*. *J Biol Chem* 265, 15813-7.
- Hatfield, P. M., and Vierstra, R. D. (1992). Multiple forms of ubiquitin-activating enzyme E1 from wheat. Identification of an essential cysteine by *in vitro* mutagenesis. *J Biol Chem* 267, 14799-803.

- Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* 387, 296-9.
- Hayashi, S., Murakami, Y., and Matsufuji, S. (1996). Ornithine decarboxylase antizyme: a novel type of regulatory protein. *Trends Biochem Sci* 21, 27-30.
- Heissmeyer, V., Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1999). NF-kappaB p105 is a target of IkappaB kinases and controls signal induction of Bcl-3-p50 complexes. *Embo J* 18, 4766-78.
- Helin, K. (1998). Regulation of cell proliferation by the E2F transcription factors. *Curr Opin Genet Dev* 8, 28-35.
- Hendil, K. B., Khan, S., and Tanaka, K. (1998). Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes. *Biochem J* 332, 749-54.
- Hermeking, H. and Eick, D. (1994) Mediation of c-Myc-induced apoptosis by p53. *Science* 5181, 2091-3.
- Hibi, K., Takahashi, T., Yamakawa, K., Ueda, R., Sekido, Y., Ariyoshi, Y., Suyama, M. Takagi, H., and Nakamura, Y. (1992). Three distinct regions involved in 3p deletion in human lung cancer. *Oncogene* 7, 445-9.
- Hicke, L., and Riezman, H. (1996). Ubiquitination of a yeast plasma membrane receptor signals its ligand- stimulated endocytosis. *Cell* 84, 277-87.
- Hinds, P. W., Mittnacht, S., Dulic, V., Arnold, A., Reed, S. I., and Weinberg, R. A (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 70, 993-1006.
- Hochstrasser, M. (1996). Ubiquitin-dependent protein degradation. *Annu Rev Genet* 30, 405-39.
- Hofmann, F., Martelli, F., Livingston, D. M., and Wang, Z. (1996). The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. *Genes Dev* 10, 2949-59.
- Hofmann, R. M., and Pickart, C. M. (1999). Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* 96, 645-53.
- Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. *FEBS Lett* 420, 25-7.
- Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994). Database of p53 gene somatic

mutations in human tumors and cell lines. *Nucleic Acids Res* 22, 3551-5.

Hori, T., Osaka, F., Chiba, T., Miyamoto, C., Okabayashi, K., Shimbara, N., Kato, S., and Tanaka, K. (1999). Covalent modification of all members of human cullin family proteins by NEDD8. *Oncogene* 18, 6829-34.

Huang, Y., Baker, R. T., and Fischer-Vize, J. A. (1995). Control of cell fate by a deubiquitinating enzyme encoded by the fat facets gene. *Science* 270, 1828-31.

Humbert, P. O., Rogers, C., Ganiatsas, S., Landsberg, R. L., Trimarchi, J. M., Dandapani, S., Brugnara, C., Erdman, S., Schrenzel, M., Bronson, R. T., and Lees, J. A. (2000). E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Mol Cell* 6, 281-91.

Ikeda, M. A., Jakoi, L., and Nevins, J. R. (1996). A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. *Proc Natl Acad Sci U S A* 93, 3215-20.

Jacks, T., Fazeli, A., Schmitt, E. M., Bronson, R. T., Goodell, M. A., and Weinberg, R. A. (1992). Effects of an Rb mutation in the mouse [see comments]. *Nature* 359, 295-300.

Jensen, D. E., Proctor, M., Marquis, S. T., Gardner, H. P., Ha, S. I., Chodosh, L. A., Ishov, A. M., Tommerup, N., Vissing, H., Sekido, Y., Minna, J., Borodovsky, A., Schultz, D. C., Wilkinson, K. D., Maul, G. G., Barlev, N., Berger, S. L., Prendergast, G. C., and Rauscher, F. J., 3rd (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* 16, 1097-112.

Jentsch, S., and Pyrowolakis, G. (2000). Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol* 10, 335-42.

Johnson, D. G., Cress, W. D., Jakoi, L., and Nevins, J. R. (1994a). Oncogenic capacity of the E2F1 gene. *Proc Natl Acad Sci U S A* 91, 12823-7.

Johnson, D. G., Ohtani, K., and Nevins, J. R. (1994b). Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression. *Genes Dev* 8, 1514-25.

Johnson, D. G., Schwarz, J. K., Cress, W. D., and Nevins, J. R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* 365, 349-52.

Johnson, E. S. and Gupta, A. A. (2001). An E3-like factor that promotes SUMO conjugation to the yeast septins. *Cell* 106, 735-44.

Johnston, S. C., Larsen, C. N., Cook, W. J., Wilkinson, K. D., and Hill, C. P. (1997). Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 Å resolution. *Embo J* 16, 3787-96.

Jooss, K., Lam, E. W., Bybee, A., Girling, R., Muller, R., and La Thangue, N. B. (1995). Proto-oncogenic properties of the DP family of proteins. *Oncogene* 10, 1529-36.

Kaelin, W. G., Jr. (1997). Recent insights into the functions of the retinoblastoma susceptibility gene product. *Cancer Invest* 15, 243-54.

Kahyo, T., Nishida, T., and Yasuda, H. (2001). Involvement of PIAS1 in the sumoylation of tumor suppressor p53. *Mol Cell* 8, 713-8.

Kalderon, D., Roberts, B. L., Richardson, W. D., and Smith, A. E. (1984). A short amino acid sequence able to specify nuclear location. *Cell* 39, 499-509.

Kamura, T., Conrad, M. N., Yan, Q., Conaway, R. C., and Conaway, J. W. (1999). The Rbx1 subunit of SCF and VHL E3 ubiquitin ligase activates Rub1 modification of cullins Cdc53 and Cul2. *Genes Dev* 13, 2928-33.

Kato, J., Matsushime, H., Hiebert, S. W., Ewen, M. E., and Sherr, C. J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev* 7, 331-42.

Kessel, M., Maurizi, M. R., Kim, B., Kocsis, E., Trus, B. L., Singh, S. K., and Steven, A. C. (1995). Homology in structural organization between *E. coli* ClpAP protease and the eukaryotic 26 S proteasome. *J Mol Biol* 250, 587-94.

King, R. W., Deshaies, R. J., Peters, J. M., and Kirschner, M. W. (1996). How proteolysis drives the cell cycle. *Science* 274, 1652-9.

Kisselev, A. F., Akopian, T. N., Woo, K. M., and Goldberg, A. L. (1999). The sizes of peptides generated from protein by mammalian 26 and 20 S proteasomes. Implications for understanding the degradative mechanism and antigen presentation. *J Biol Chem* 274, 3363-71.

Klingmuller, U., Lorenz, U., Cantley, L. C., Neel, B. G., and Lodish, H. F. (1995). Specific recruitment of SH-PTP1 to the erythropoietin receptor causes inactivation of JAK2 and termination of proliferative signals. *Cell* 80, 729-38.

Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999). A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* 96, 635-44.

Kok, K., Osinga, J., Carritt, B., Davis, M. B., van der Hout, A. H., van der Veen, A. Y., Landsvater, R. M., de Leij, L. F., Berendsen, H. H., Postmus, P. E., and et al. (1987). Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature* 330, 578-81.

Kovesdi, I., Reichel, R., and Nevins, J. R. (1987). Role of an adenovirus E2 promoter

- binding factor in E1A-mediated coordinate gene control. *Proc Natl Acad Sci U S A* **84**, 2180-4.
- Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J Virol* **69**, 2491-500.
- Krek, W., Xu, G., and Livingston, D. M. (1995). Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. *Cell* **83**, 1149-58.
- Kursula, P., Merilainen, G., Lehto, V. P., and Heape, A. M. (1999). The small myelin-associated glycoprotein is a zinc-binding protein. *J Neurochem* **73**, 2110-8.
- Lam, Y. A., Xu, W., DeMartino, G. N., and Cohen, R. E. (1997). Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. *Nature* **385**, 737-40.
- Laney, J. D., and Hochstrasser, M. (1999). Substrate targeting in the ubiquitin system. *Cell* **97**, 427-30.
- Larsen, C. N., Price, J. S., and Wilkinson, K. D. (1996). Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. *Biochemistry* **35**, 6735-44.
- Layfield, R., Franklin, K., Landon, M., Walker, G., Wang, P., Ramage, R., Brown, A., Love, S., Urquhart, K., Muir, T., Baker, R., and Mayer, R. J. (1999). Chemically synthesized ubiquitin extension proteins detect distinct catalytic capacities of deubiquitinating enzymes. *Anal Biochem* **274**, 40-9.
- Lazar, G. A., Desjarlais, J. R., and Handel, T. M. (1997). De novo design of the hydrophobic core of ubiquitin. *Protein Sci* **6**, 1167-78.
- LeCouter, J. E., Kablar, B., Hardy, W. R., Ying, C., Megeney, L. A., May, L. L., and Rudnicki, M. A. (1998a). Strain-dependent myeloid hyperplasia, growth deficiency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. *Mol Cell Biol* **18**, 7455-65.
- LeCouter, J. E., Kablar, B., Whyte, P. F., Ying, C., and Rudnicki, M. A. (1998b). Strain-dependent embryonic lethality in mice lacking the retinoblastoma-related p130 gene. *Development* **125**, 4669-79.
- Lee, E. Y., Chang, C. Y., Hu, N., Wang, Y. C., Lai, C. C., Herrup, K., Lee, W. H., and Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis [see comments]. *Nature* **359**, 288-94.
- Lee, J. O., Russo, A. A., and Pavletich, N. P. (1998). Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature* **391**, 859-65.

Lee, M. H., Williams, B. O., Mulligan, G., Mukai, S., Bronson, R. T., Dyson, N., Harlow, E., and Jacks, T. (1996). Targeted disruption of p107: functional overlap between p107 and Rb. *Genes Dev* 10, 1621-32.

Lees, J. A., Saito, M., Vidal, M., Valentine, M., Look, T., Harlow, E., Dyson, N., and Helin, K. (1993). The retinoblastoma protein binds to a family of E2F transcription factors. *Mol Cell Biol* 13, 7813-25.

Leone, G., DeGregori, J., Yan, Z., Jakoi, L., Ishida, S., Williams, R. S., and Nevins, J. R. (1998). E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. *Genes Dev* 12, 2120-30.

Levine, A. J., Momand, J., and Finlay, C. A. (1991). The p53 tumour suppressor gene. *Nature* 351, 453-6.

Li, Q., Hariharan, I. K., Chen, F., Huang, Y., and Fischer, J. A. (1997). Genetic interactions with Rap1 and Ras1 reveal a second function for the fat facets deubiquitinating enzyme in *Drosophila* eye development. *Proc Natl Acad Sci U S A* 94, 12515-20.

Li, Y., Graham, C., Lacy, S., Duncan, A. M., and Whyte, P. (1993). The adenovirus E1A-associated 130-kD protein is encoded by a member of the retinoblastoma gene family and physically interacts with cyclins A and E. *Genes Dev* 7, 2366-77.

Liakopoulos, D., Busgen, T., Brychzy, A., Jentsch, S., and Pause, A. (1999). Conjugation of the ubiquitin-like protein NEDD8 to cullin-2 is linked to von Hippel-Lindau tumor suppressor function. *Proc Natl Acad Sci U S A* 96, 5510-5.

Liang, S. H., and Clarke, M. F. (1999). A bipartite nuclear localization signal is required for p53 nuclear import regulated by a carboxyl-terminal domain. *J Biol Chem* 274, 32699-703.

Lin, H., Keriell, A., Morales, C. R., Bedard, N., Zhao, Q., Hingamp, P., Lefrancois, S., Combaret, L., and Wing, S. S. (2000). Divergent N-terminal sequences target an inducible testis deubiquitinating enzyme to distinct subcellular structures. *Mol Cell Biol* 20, 6568-78.

Lin, H., Yin, L., Reid, J., Wilkinson, K. D., and Wing, S. S. (2001). Divergent N-terminal sequences of a deubiquitinating enzyme modulate substrate specificity. *J Biol Chem* 276, 20357-63.

Lin, L., and Ghosh, S. (1996). A glycine-rich region in NF-kappaB p105 functions as a processing signal for the generation of the p50 subunit. *Mol Cell Biol* 16, 2248-54.

Little, C. D., Nau, M. M., Carney, D. N., Gazdar, A. F., and Minna, J. D. (1983). Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature* 306, 194-6.

Lipinski, M. M., and Jacks, T. (1999). The retinoblastoma gene family in differentiation and

development. *Oncogene 18*, 7873-82.

Lowe, J., Stock, D., Jap, B., Zwickl, P., Baumeister, W., and Huber, R. (1995). Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4 Å resolution. *Science 268*, 533-9.

Lowe, S. W. and Ruley, H. E. (1994). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev 7*, 535-45.

Lucero, P., Penalver, E., Vela, L., and Lagunas, R. (2000). Monoubiquitination is sufficient to signal internalization of the maltose transporter in *Saccharomyces cerevisiae*. *J. Bacteriol 182*, 241-3.

Lukas, J., Herzinger, T., Hansen, K., Moroni, M. C., Resnitzky, D., Helin, K., Reed, S. I., and Bartek, J. (1997). Cyclin E-induced S phase without activation of the pRb/E2F pathway. *Genes Dev 11*, 1479-92.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell 88*, 97-107.

Martelli, F., and Livingston, D. M. (1999). Regulation of endogenous E2F1 stability by the retinoblastoma family proteins. *Proc Natl Acad Sci U S A 96*, 2858-63.

Matsui, S. I., Seon, B. K., and Sandberg, A. A. (1979). Disappearance of a structural chromatin protein A24 in mitosis: implications for molecular basis of chromatin condensation. *Proc Natl Acad Sci U S A 76*, 6386-90.

Matunis, M. J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol 135*, 1457-70.

Matunis, M. J., Wu, J., and Blobel, G. (1998). SUMO-1 modification and its role in targeting the Ran GTPase-activating protein, RanGAP1, to the nuclear pore complex. *J Cell Biol 140*, 499-509.

McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991). UBA 1: an essential yeast gene encoding ubiquitin-activating enzyme. *Embo J 10*, 227-36.

Miller, H. I., Henzel, W. J., Ridgway, J. B., Kuang, W.-J., Chisholm, V. and Liu, C. C. (1989). *BioTechnology 7*, 698-704.

Miller, M., Park, M. K., and Hanover, J. A. (1991). Nuclear pore complex: structure, function, and regulation. *Physiol Rev 71*, 909-49.

Moazed, D., and Johnson, D. (1996). A deubiquitinating enzyme interacts with SIR4 and

regulates silencing in *S. cerevisiae*. *Cell* 86, 667-77.

Moberg, K., Starz, M. A., and Lees, J. A. (1996). E2F-4 switches from p130 to p107 and pRB in response to cell cycle reentry. *Mol Cell Biol* 16, 1436-49.

Morgan, D. O. (1995). Principles of CDK regulation. *Nature* 374, 131-4.

Morkel, M., Wenkel, J., Bannister, A. J., Kouzarides, T., and Hagemeyer, C. (1997). An E2F-like repressor of transcription [letter]. *Nature* 390, 567-8.

Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I., and Smith, A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc Natl Acad Sci U S A* 91, 4303-7.

Muda, M., Manning, E. R., Orth, K., and Dixon, J. E. (1999). Identification of the human YVH1 protein-tyrosine phosphatase orthologue reveals a novel zinc binding domain essential for in vivo function. *J Biol Chem* 274, 23991-5.

Mueller, R. D., Yasuda, H., Hatch, C. L., Bonner, W. M., and Bradbury, E. M. (1985). Identification of ubiquitinated histones 2A and 2B in *Physarum polycephalum*. Disappearance of these proteins at metaphase and reappearance at anaphase. *J Biol Chem* 260, 5147-53.

Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K., and Ichihara, A. (1992). Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* 360, 597-9.

Nakamura, T., Hillova, J., Mariage-Samson, R., Onno, M., Huebner, K., Cannizzaro, L. A., Boghosian-Sell, L., Croce, C. M., and Hill, M. (1992). A novel transcriptional unit of the *trc* oncogene widely expressed in human cancer cells. *Oncogene* 7, 733-41.

Naylor, S. L., Johnson, B. E., Minna, J. D., and Sakaguchi, A. Y. (1987). Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. *Nature* 329, 451-4.

Neuman, E., Flemington, E. K., Sellers, W. R., and Kaelin, W. G., Jr. (1994). Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter [published erratum appears in *Mol Cell Biol* 1995 Aug;15(8):4660]. *Mol Cell Biol* 14, 6607-15.

Opresko, L. K., Chang, C. P., Will, B. H., Burke, P. M., Gill, G. N., and Wiley, H. S. (1995). Endocytosis and lysosomal targeting of epidermal growth factor receptors are mediated by distinct sequences independent of the tyrosine kinase domain. *J Biol Chem* 270, 4325-33.

Orian, A., Whiteside, S., Israel, A., Stancovski, I., Schwartz, A. L., and Ciechanover, A. (1995). Ubiquitin-mediated processing of NF-kappa B transcriptional activator precursor p105. Reconstitution of a cell-free system and identification of the ubiquitin-carrier protein,

E2, and a novel ubiquitin-protein ligase, E3, involved in conjugation. *J Biol Chem* 270, 21707-14.

Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994). The ubiquitin-proteasome pathway is required for processing the NF- κ B1 precursor protein and the activation of NF- κ B. *Cell* 78, 773-85.

Papa, F. R., and Hochstrasser, M. (1993). The yeast DOA4 gene encodes a deubiquitinating enzyme related to a product of the human *trc-2* oncogene. *Nature* 366, 313-9.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. *Science* 246, 603-8.

Park, K. C., Choi, E. J., Min, S. W., Chung, S. S., Kim, H., Suzuki, T., Tanaka, K., and Chung, C. H. (2000). Tissue-specificity, functional characterization and subcellular localization of a rat ubiquitin-specific processing protease, UBP109, whose mRNA expression is developmentally regulated. *Biochem J* 349, 443-453.

Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993). Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci U S A* 90, 8392-6.

Pickart, C. M. (1997). Targeting of substrates to the 26S proteasome. *Faseb J* 11, 1055-66.

Pickart, C. M., Kasperk, E. M., Beal, R., and Kim, A. (1994). Substrate properties of site-specific mutant ubiquitin protein (G76A) reveal unexpected mechanistic features of ubiquitin-activating enzyme (E1). *J Biol Chem* 269, 7115-23.

Pickart, C. M. and Rose, I. A. (1985). Ubiquitin carboxyl-terminal hydrolase acts on ubiquitin carboxyl-terminal amides. *J Biol Chem* 260, 7903-10.

Pines, J. (1999). Four-dimensional control of the cell cycle. *Nat Cell Biol* 1, E73-9.

Plempner, R. K., and Wolf, D. H. (1999). Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem Sci* 24, 266-70.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78, 59-66.

Podust, V. N., Brownell, J. E., Gladysheva, T. B., Luo, R. S., Wang, C., Coggins, M. B., Pierce, J. W., Lightcap, E. S., and Chau, V. (2000). A Nedd8 conjugation pathway is essential for proteolytic targeting of p27Kip1 by ubiquitination. *Proc Natl Acad Sci U S A* 97, 4579-84.

Qin, X. Q., Livingston, D. M., Kaelin, W. G., Jr., and Adams, P. D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc Natl Acad Sci U S A* 91, 10918-22.

- Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W. G., Conaway, R. C., Conaway, J. W., and Branton, P. E. (2001). Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* 15, 3104-17.
- Rao-Naik, C., Chandler, J. S., McArdle, B., and Callis, J. (2000). Ubiquitin-specific proteases from *Arabidopsis thaliana*: cloning of AtUBP5 and analysis of substrate specificity of AtUBP3, AtUBP4, and AtUBP5 using *Escherichia coli* in vivo and in vitro assays. *Arch Biochem Biophys* 379, 198-208.
- Rechsteiner, M. (1998). The 26S proteasome. In *Ubiquitin and the biology of the cell*, J.-M. Peters, J. R. Harris and D. J. Finley, eds. (New-York: Plenum Press), pp. 147-190.
- Rempel, R. E., Saenz-Robles, M. T., Storms, R., Morham, S., Ishida, S., Engel, A., Jakoi, L., Melhem, M. F., Pipas, J. M., Smith, C., and Nevins, J. R. (2000). Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Mol Cell* 6, 293-306.
- Rodenhuis, S., and Slebos, R. J. (1990). The ras oncogenes in human lung cancer. *Am Rev Respir Dis* 142, S27-30.
- Rodriguez, M. S., Desterro, J. M., Lain, S., Midgley, C. A., Lane, D. P., and Hay, R. T. (1999). SUMO-1 modification activates the transcriptional response of p53. *Embo J* 18, 6455-61.
- Rose, I. A. and Warms, J. V. (1983). An enzyme with ubiquitin carboxy-terminal esterase activity from reticulocytes. *Biochemistry* 22, 4234-7.
- Ross, J. F., Liu, X., and Dynlacht, B. D. (1999). Mechanism of transcriptional repression of E2F by the retinoblastoma tumor suppressor protein. *Mol Cell* 3, 195-205.
- Roth, A. F., and Davis, N. G. (1996). Ubiquitination of the yeast a-factor receptor. *J Cell Biol* 134, 661-74.
- Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001). PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev* 15, 3088-103.
- Saitoh, H., Sparrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998). Ubc9p and the conjugation of SUMO-1 to RanGAP1 and RanBP2. *Curr Biol* 8, 121-4.
- Sala, A., De Luca, A., Giordano, A., and Peschle, C. (1996). The retinoblastoma family member p107 binds to B-MYB and suppresses its autoregulatory activity. *J Biol Chem* 271, 28738-40.
- Sardet, C., Vidal, M., Cobrinik, D., Geng, Y., Onufryk, C., Chen, A., and Weinberg, R. A.

(1995). E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. *Proc Natl Acad Sci USA* **92**, 2403-7.

Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993). The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75**, 495-505.

Schmidt-Kastner, P. K., Jardine, K., Cormier, M., and McBurney, M. W. (1998). Absence of p53-dependent cell cycle regulation in pluripotent mouse cell lines. *Oncogene* **16**, 3003-11.

Schneider, P. M., Hung, M. C., Chiocca, S. M., Manning, J., Zhao, X. Y., Fang, K., and Roth, J. A. (1989). Differential expression of the c-erbB-2 gene in human small cell and non-small cell lung cancer. *Cancer Res* **49**, 4968-71.

Sellers, W. R., Rodgers, J. W., and Kaelin, W. G., Jr. (1995). A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. *Proc Natl Acad Sci U S A* **92**, 11544-8.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593-602.

Shan, B., and Lee, W. H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol Cell Biol* **14**, 8166-73.

Shao, Z., Siegert, J. L., Ruppert, S., and Robbins, P. D. (1997). Rb interacts with TAF(II)250/TFIID through multiple domains. *Oncogene* **15**, 385-92.

Shaulsky, G., Goldfinger, N., Ben-Ze'ev, A., and Rotter, V. (1990). Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Mol Cell Biol* **10**, 6565-77.

Sherr, C. J. (1994). G1 phase progression: cycling on cue [see comments]. *Cell* **79**, 551-5.

Sherr, C. J. (1998). Tumor surveillance via the ARF-p53 pathway. *Genes Dev* **12**, 2984-91.

Shih, S. C., Sloper-Mould, K. E., and Hicke, L. (2000). Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *Embo J* **19**, 187-98.

Simanis, V. and Nurse, P. (1986). The cell cycle control gene *cdc2+* of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45**, 261-8.

Singer, J. D., Gurian-West, M., Clurman, B., and Roberts, J. M. (1999). Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells. *Genes Dev* **13**, 2375-87.

Singer, J. D., Manning, B. M., and Formosa, T. (1996). Coordinating DNA replication to produce one copy of the genome requires genes that act in ubiquitin metabolism. *Mol Cell Biol* 16, 1356-66.

Singh, P., Wong, S. H., and Hong, W. (1994). Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *Embo J* 13, 3329-38.

Sionov, R. V., and Haupt, Y. (1999). The cellular response to p53: the decision between life and death. *Oncogene* 18, 6145-57.

Slansky, J. E., Li, Y., Kaelin, W. G., and Farnham, P. J. (1993). A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter [published erratum appears in *Mol Cell Biol* 1993 Nov;13(11):7201]. *Mol Cell Biol* 13, 1610-8.

Slebos, R. J., Kibbelaar, R. E., Dalesio, O., Kooistra, A., Stam, J., Meijer, C. J., Wagenaar, S. S., Vanderschueren, R. G., van Zandwijk, N., Mooi, W. J., et al. (1990). K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 323, 561-5.

Sloper-Mould, K. E., Eyre, H. J., Wang, X. W., Sutherland, G. R., and Baker, R. T. (1999). Characterization and chromosomal localization of USP3, a novel human ubiquitin-specific protease. *J Biol Chem* 274, 26878-84.

Smith, E. J., Leone, G., and Nevins, J. R. (1998). Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ* 9 297-303.

Smith, E. J., Leone, G., DeGregori, J., Jakoi, L., and Nevins, J. R. (1996). The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 cell state. *Mol Cell Biol* 16, 6965-76.

Sommer, T., and Wolf, D. H. (1997). Endoplasmic reticulum degradation: reverse protein flow of no return. *Faseb J* 11, 1227-33.

Song, X., von Kampen, J., Slaughter, C. A., and DeMartino, G. N. (1997). Relative functions of the alpha and beta subunits of the proteasome activator, PA28. *J Biol Chem* 272, 27994-8000.

Spence, J., Sadis, S., Haas, A. L., and Finley, D. (1995). A ubiquitin mutant with specific defects in DNA repair and multiubiquitination. *Mol Cell Biol* 15, 1265-73.

Staub, O., Gautschi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997). Regulation of stability and function of the epithelial Na⁺ channel (ENaC) by ubiquitination. *Embo J* 16, 6325-36.

Statistics_Canada. (1999). Health Indicators: Health Statistics Division.

Stephen, A. G., Trausch-Azar, J. S., Handley-Gearhart, P. M., Ciechanover, A., and Schwartz, A. L. (1997). Identification of a region within the ubiquitin-activating enzyme required for nuclear targeting and phosphorylation. *J Biol Chem* 272, 10895-903.

Stewart, Z. A., Leach, S. D., and Pietsenpol, J. A. (1999). p21(Waf1/Cip1) inhibition of cyclin E/Cdk2 activity prevents endoreduplication after mitotic spindle disruption. *Mol Cell Biol* 19, 205-15.

Stock, D., Nederlof, P. M., Seemuller, E., Baumeister, W., Huber, R., and Lowe, J. (1996). Proteasome: from structure to function. *Curr Opin Biotechnol* 7, 376-85.

Sutcliffe, J. E., Cairns, C. A., McLees, A., Allison, S. J., Tosh, K., and White, R. J. (1999). RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. *Mol Cell Biol* 19, 4255-61.

Swanson, D. A., Freund, C. L., Ploder, L., McInnes, R. R., and Valle, D. (1996). A ubiquitin C-terminal hydrolase gene on the proximal short arm of the X chromosome: implications for X-linked retinal disorders. *Hum Mol Genet* 5, 533-8.

Takahashi, T., Nau, M. M., Chiba, I., Birrer, M. J., Rosenberg, R. K., Vinocour, M., Levitt, M., Pass, H., Gazdar, A. F., and Minna, J. D. (1989). p53: a frequent target for genetic abnormalities in lung cancer. *Science* 246, 491-4

Takeshima, H., Izawa, I., Lee, P. S., Safdar, N., Levin, V. A., and Saya, H. (1994). Detection of cellular proteins that interact with the NF2 tumor suppressor gene product. *Oncogene* 9, 2135-44.

Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H., and Hay, R. T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem* 276, 35368-74.

Taya, S., Yamamoto, T., Kano, K., Kawano, Y., Iwamatsu, A., Tsuchiya, T., Tanaka, K., Kanai-Azuma, M., Wood, S. A., Mattick, J. S., and Kaibuchi, K. (1998). The Ras target AF-6 is a substrate of the fam deubiquitinating enzyme. *J Cell Biol* 142, 1053-62.

Taya, Y., Yasuda, H., Kamijo, M., Nakaya, K., Nakamura, Y., Ohba, Y., and Nishimura, S. (1989). In vitro phosphorylation of the tumor suppressor gene RB protein by mitosis-specific histone H1 kinase. *Biochem Biophys Res Commun* 164, 580-6.

Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998). A function for monoubiquitination in the internalization of a G protein- coupled receptor. *Mol Cell* 1, 193-202.

Tevosian, S. G., Shih, H. H., Mendelson, K. G., Sheppard, K. A., Paulson, K. E., and Yee, A. S. (1997). HBP1: a HMG box transcriptional repressor that is targeted by the retinoblastoma family. *Genes Dev* 11, 383-96.

- Thomas, K. R., Deng, C., and Capecchi, M. R. (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. *Mol Cell Biol* *12*, 2919-23.
- Thrower, J. S., Hoffman, L., Rechsteiner, M., and Pickart, C. M. (2000). Recognition of the polyubiquitin proteolytic signal. *Embo J* *19*, 94-102.
- Tibbo, E. (1997). M.Sc. Thesis, Departement of Biochemistry, University of Ottawa.
- Tobias, J. W., and Varshavsky, A. (1991). Cloning and functional analysis of the ubiquitin-specific protease gene UBP1 of *Saccharomyces cerevisiae*. *J Biol Chem* *266*, 12021-8.
- Tomoda, K., Kubota, Y., and Kato, J. (1999). Degradation of the cyclin-dependent-kinase inhibitor p27Kip1 is instigated by Jab1 [see comments]. *Nature* *398*, 160-5.
- Travis, W. D., Travis, L. B., and Devesa, S. S. (1995). Lung cancer. *Cancer* *75*, 191-202.
- Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. (1999). p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* *9*, 661-4.
- Vairo, G., Livingston, D. M., and Ginsberg, D. (1995). Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. *Genes Dev* *9*, 869-81.
- Van Kaer, L., Ashton-Rickardt, P. G., Eichelberger, M., Gaczynska, M., Nagashima, K., Rock, K. L., Goldberg, A. L., Doherty, P. C., and Tonegawa, S. (1994). Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity* *1*, 533-41.
- van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996). The multiubiquitin-chain-binding protein Mub1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. *Mol Cell Biol* *16*, 6020-8.
- Vijay-Kumar, S., Bugg, C. E., and Cook, W. J. (1987). Structure of ubiquitin refined at 1.8 Å resolution. *J Mol Biol* *194*, 531-44.
- Vogelstein, B., and Kinzler, K. W. (1993). The multistep nature of cancer. *Trends Genet* *9*, 138-41.
- Voges, D., Zwickl, P., and Baumeister, W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. *Annu Rev Biochem* *68*, 1015-68.
- Voorhoeve, P. M., Hijmans, E. M., and Bernards, R. (1999). Functional interaction between a novel protein phosphatase 2A regulatory subunit, PR59, and the retinoblastoma-related p107 protein. *Oncogene* *18*, 515-24.

- Wagner, A. J., Kokontis, J. M., and Hay, N. (1994). Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. *Genes Dev* 8, 2817-30.
- Wang, S., Nath, N., Adlam, M., and Chellappan, S. (1999). Prohibitin, a potential tumor suppressor, interacts with RB and regulates E2F function. *Oncogene* 18, 3501-10.
- Watanabe, S., Sato, H., Komiyama, N., Kanda, T., and Yoshiike, K. (1992). The E7 functions of human papillomaviruses in rat 3Y1 cells. *Virology* 187, 107-14.
- Waterman, H., Levkowitz, G., Alroy, I., and Yarden, Y. (1999). *J Biol Chem* 274, 22151-4.
- Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* 81, 323-30.
- Whang-Peng, J., Bunn, P. A., Jr., Kao-Shan, C. S., Lee, E. C., Carney, D. N., Gazdar, A., and Minna, J. D. (1982a). A nonrandom chromosomal abnormality, del 3p(14-23), in human small cell lung cancer (SCLC). *Cancer Genet Cytogenet* 6, 119-34.
- Whang-Peng, J., Kao-Shan, C. S., Lee, E. C., Bunn, P. A., Carney, D. N., Gazdar, A. F., and Minna, J. D. (1982b). Specific chromosome defect associated with human small-cell lung cancer; deletion 3p(14-23). *Science* 215, 181-2.
- Wickner, S., Gottesman, S., Skowrya, D., Hoskins, J., McKenney, K., and Maurizi, M. R. (1994). A molecular chaperone, ClpA, functions like DnaK and DnaJ. *Proc Natl Acad Sci U S A* 91, 12218-22.
- Wilkinson, K. D. (1997). Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. *Faseb J* 11, 1245-56.
- Wilkinson, K. D., and Hochstrasser, M. (1997). The deubiquitinating enzymes. In *Ubiquitin*, D. J. Finley and J.-M. Peters, eds. (New York: Plenum Press).
- Wilkinson, K. D., Tashayev, V. L., O'Connor, L. B., Larsen, C. N., Kasperek, E., and Pickart, C. M. (1995). Metabolism of the polyubiquitin degradation signal: structure, mechanism, and role of isopeptidase T. *Biochemistry* 34, 14535-46.
- Won, K. A., and Reed, S. I. (1996). Activation of cyclin E/CDK2 is coupled to site-specific autophosphorylation and ubiquitin-dependent degradation of cyclin E. *Embo J* 15, 4182-93.
- Woo, S. K., Lee, J. I., Park, I. K., Yoo, Y. J., Cho, C. M., Kang, M. S., Ha, D. B., Tanaka, K., and Chung, C. H. (1995). Multiple ubiquitin C-terminal hydrolases from chick skeletal muscle. *J Biol Chem* 270, 18766-73.
- Wu, X. and Levine, A. J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc Natl Sci USA* 91, 3602-6.

- Xu, G., Livingston, D. M., and Krek, W. (1995). Multiple members of the E2F transcription factor family are the products of oncogenes. *Proc Natl Acad Sci U S A* 92, 1357-61.
- Yaglom, J., Linskens, M. H., Sadis, S., Rubin, D. M., Futcher, B., and Finley, D. (1995). p34Cdc28-mediated control of Cln3 cyclin degradation. *Mol Cell Biol* 15, 731-41.
- Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., and Dyson, N. J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* 85, 537-48.
- Yee, A. S., Reichel, R., Kovetski, I., and Nevins, J. R. (1987). Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. *Embo J* 6, 2061-8.
- Yew, P. R., and Berk, A. J. (1992). Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein. *Nature* 357, 82-5.
- Yokota, J., Wada, M., Shimosato, Y., Terada, M., Sugimura, T. (1987). Loss of heterozygosity on chromosomes 3, 13, and 17 in small-cell carcinoma and on chromosome 3 in adenocarcinoma of the lung. *Proc Natl Acad Sci U S A* 84, 9252-6.
- Zhan, Q., Carrier, F., and Fornace, A. J., Jr. (1993). Induction of cellular p53 activity by DNA-damaging agents and growth arrest [published erratum appears in *Mol Cell Biol* 1993 Sep;13(9):5928]. *Mol Cell Biol* 13, 4242-50.
- Zhu, Y., Carroll, M., Papa, F. R., Hochstrasser, M., and D'Andrea, A. D. (1996). DUB-1, a deubiquitinating enzyme with growth-suppressing activity. *Proc Natl Acad Sci U S A* 93, 3275-9.
- Zhu, Y., Lambert, K., Corless, C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and D'Andrea, A. D. (1997). DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes. *J Biol Chem* 272, 51-7.

Appendix 1



SHORT REPORTS

Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130

Paola Blanchette¹, Catherine A Gilchrist^{2,3}, Rohan T Baker² and Douglas A Gray^{*1}

¹Centre for Cancer Therapeutics, Ottawa Regional Cancer Centre, 503 Smyth Road, Ottawa, Canada K1H 1C4; ²Molecular Genetics Group, John Curtin School of Medical Research, Australian National University, GPO Box 334 (Mills Road), Canberra ACT 2601 Australia

The murine *Unp* gene encodes a widely expressed ubiquitin-specific protease. The predicted sequence of the UNP protein features motifs common to viral oncoproteins through which these proteins interact with the retinoblastoma gene product pRb, as well as the related 'pocket proteins' p107 and p130. We have explored the possibility that UNP interacts with pocket proteins, and report here that such associations can be detected *in vitro* and in cells. Associations of UNP and pocket proteins are sensitive to site-directed mutations in a manner directly analogous to those documented in viral oncoproteins. We conclude that within cells UNP does physically associate with pRb, and can also associate with p107 and p130. *Oncogene* (2001) 20, 5533–5537.

Keywords: ubiquitin; Ubp; deubiquitinating enzyme; retinoblastoma; pocket protein

The pRb tumor suppressor protein plays a central role in the regulation of the G1 to S phase transition of cell cycle, its function mediated through interactions with several cellular proteins (reviewed in Grana *et al.*, 1998; Lipinski and Jacks, 1999). Binding of associated proteins to pRb can occur in different binding domains of pRb, but most proteins bind the 'pocket' region, which is composed of two domains, A and B, separated by a spacer region (Lipinski and Jacks, 1999). Proteins binding the pocket tend to interact through CR domains, first discovered in the viral oncoproteins adenovirus E1A (Whyte *et al.*, 1989), SV40 large T antigen (DeCaprio *et al.*, 1988), and HPV E7 (Dyson *et al.*, 1989) but subsequently identified in cellular proteins, including RBP1 (Fattaey *et al.*, 1993), RBP2 (Fattaey *et al.*, 1993), and RIZ (Buyse *et al.*, 1995). Cell cycle transitions are rendered irreversible by the destruction of key molecules through ubiquitin-mediated proteolysis (reviewed in Hoyt, 1997; Koepp *et al.*, 1999). Relevant proteins regulated by this pathway include the cyclins (Diehl *et al.*, 1997; Glotzer

et al., 1991), Cdk inhibitors (Carrano *et al.*, 1999) and p130 (Smith *et al.*, 1998), a member of the pRb family. Under normal conditions pRb does not appear to be a substrate of ubiquitin/proteasome pathway, but it may be directed into the proteolytic pathway by viral proteins (Boyer *et al.*, 1996). Some pRb-binding proteins are known substrates of the ubiquitin proteolytic system, including *c-myc* (Gregory and Hann, 2000), E2F-1 (Hofmann *et al.*, 1996) and E2F-4 (Hateboer *et al.*, 1996). *Unp* encodes a ubiquitin-specific protease (Ubp) that in some circumstances can promote oncogenesis (Gupta *et al.*, 1994). As is the case for most of the ubiquitin-specific proteases, the number and identity of UNP's substrates and the mode of its regulation are unknown. In an effort to identify UNP interacting proteins (which could be either substrates or regulators) binding studies were undertaken. Having previously noted the presence of sequences related to viral oncoprotein CR domains in the UNP protein sequence (*ibid.*, Figure 1a), we were particularly interested in the possibility that UNP physically interacts with pRb, and/or the related pocket proteins p107 and p130.

GST fusions of *myc*-tagged UNP and mutants thereof were generated, purified from a bacterial expression system and incubated with metabolically-labeled NIH3T3 cell lysates for GST pull-down experiments. Consistent UNP-interacting bands were observed (Figure 1c) for both wild-type and catalytically inactive UNP, demonstrating that associations exist that do not require catalytic activity. One such band was detected at approximately 100 kDa. The apparent molecular mass of this band together with the presence of the LxCxE motif in UNP (Gupta *et al.*, 1994) suggested that it might be pRb. The pull-down experiment was repeated with non-labeled cell lysates and the interacting proteins were probed with an anti-pRb antibody (Figure 1e). As seen in Figure 1e, the interaction of UNP with pRb was found to require the LxCxE motif. Substitution of the central cysteine residue in this motif abolished pRb association as had been previously demonstrated for the analogous residue in the adenovirus E1A LxCxE motif. This association was not specific to the mouse or to NIH3T3 cells in particular; identical results were obtained in lysates from murine p19 teratocarcinoma cells, human A431 epidermoid carcinoma cells, and

*Correspondence: DA Gray,

³Current address: Department of Biological Sciences, Stanford University, Stanford CA 94305-5020 USA

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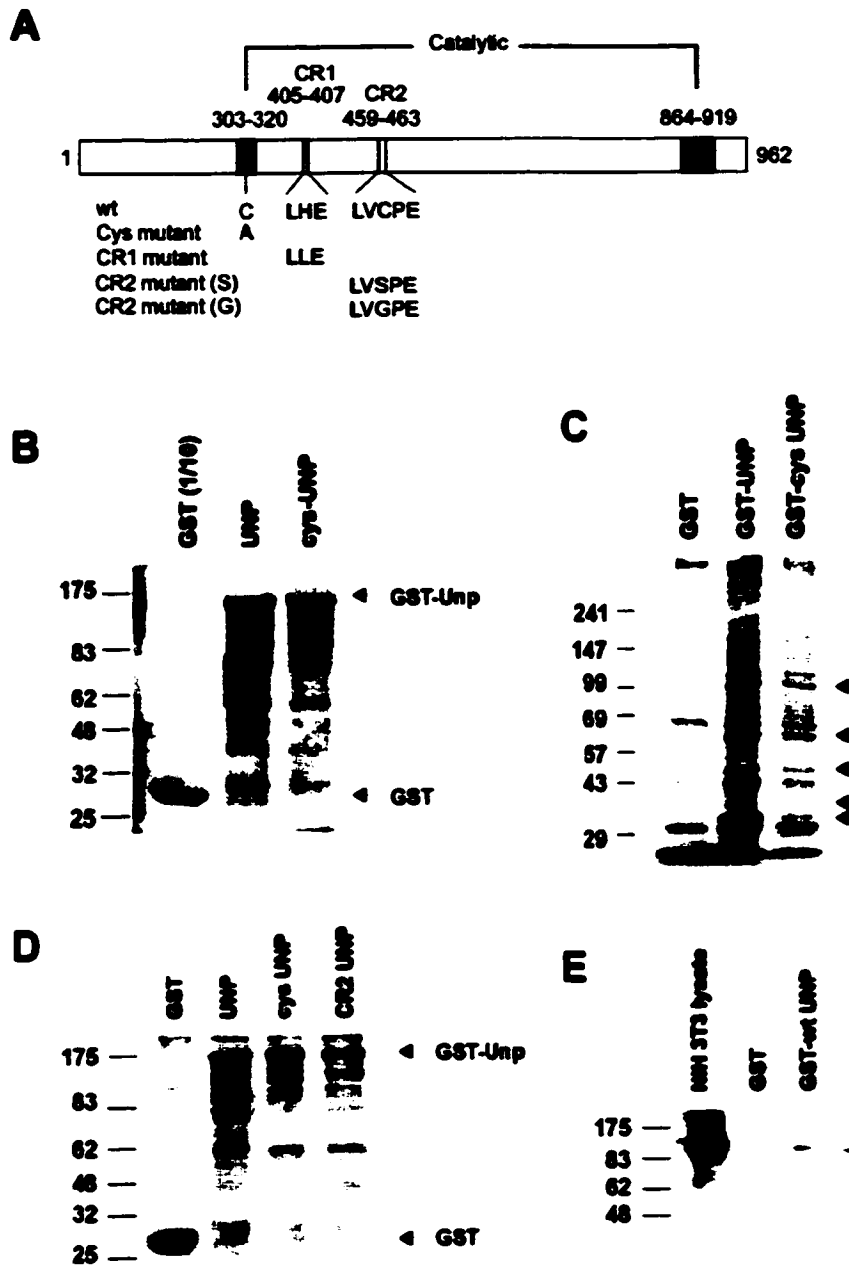


Figure 1 Association of UNP with pRb. (a) Schematic of UNP, with the positions of the putative interaction domains (CR1 and CR2) indicated. The shaded boxes represent highly conserved catalytic domains common to Ubp enzymes. (b) Production of recombinant GST fusions. GST fusions of *myc*-tagged wild type UNP, the Cys domain mutant of UNP, and the CR2 mutant of UNP were expressed in the TG1 strain of *E. coli*, induced at room temperature with 30 mM IPTG for 4 h, lysed in PBS+1% Triton + inhibitors and purified with GSH agarose beads (Sigma). Expression levels were verified by silverstaining an SDS-PAGE gel. The GST-UNP band was detected at its predicted molecular mass of 170 kDa. (c) GST pull-downs of metabolically labeled NIH3T3 cells. 100 mm dishes of ~80% confluent cells were labeled with 200 mCi/ml of ³⁵S methionine/cysteine (Promix, Amersham) for 5 h at 37°C and were lysed in lysis buffer (20 mM Tris pH7.5, 150 mM NaCl, 2 mM DTT, 1% Triton X-100, 5% glycerol, 2 mM each of NaF and NaPPI, 500 mM sodium vanadate, 200 mM PMSF, 5 mg/ml leupeptin, and 2 mg/ml aprotin). Following an overnight pre-clearing of the lysates with GST-beads, they were incubated with fusion-beads for 2 h at 4°C. The fusion beads were then extensively washed and binding proteins migrated on an SDS-PAGE and visualized by autoradiography. Arrows to the right indicate bands that are consistently seen in the UNP fusion lanes but absent in the GST alone control lane. (d) Verification of recombinant GST fusions, as per b. (e) GST pull-downs as per b, but performed in the absence of metabolic labeling. The unlabelled interacting proteins were transferred to a nitrocellulose membrane and probed with a mouse anti-pRb antibody, G3-245 (Pharmingen, 1/200 dilution)

human H1299 non-small cell lung cancer cells (data not shown). Further, in an independent body of work in the laboratory of Dr Michele Pagano, the *in vivo*,

LxCxE-dependent association of UNP and pRb was detected in a distinct set of human cell lines (described in the accompanying report of DeSalle *et al.*, 2001).

Our unpublished work suggests that like pRb, UNP can be phosphorylated *in vivo* (Raynal and Blanchette, unpublished). It is unlikely that the association of UNP

and pRb requires post-translational modification of either partner, because it could be detected when both proteins were produced in bacteria (PhD thesis of C

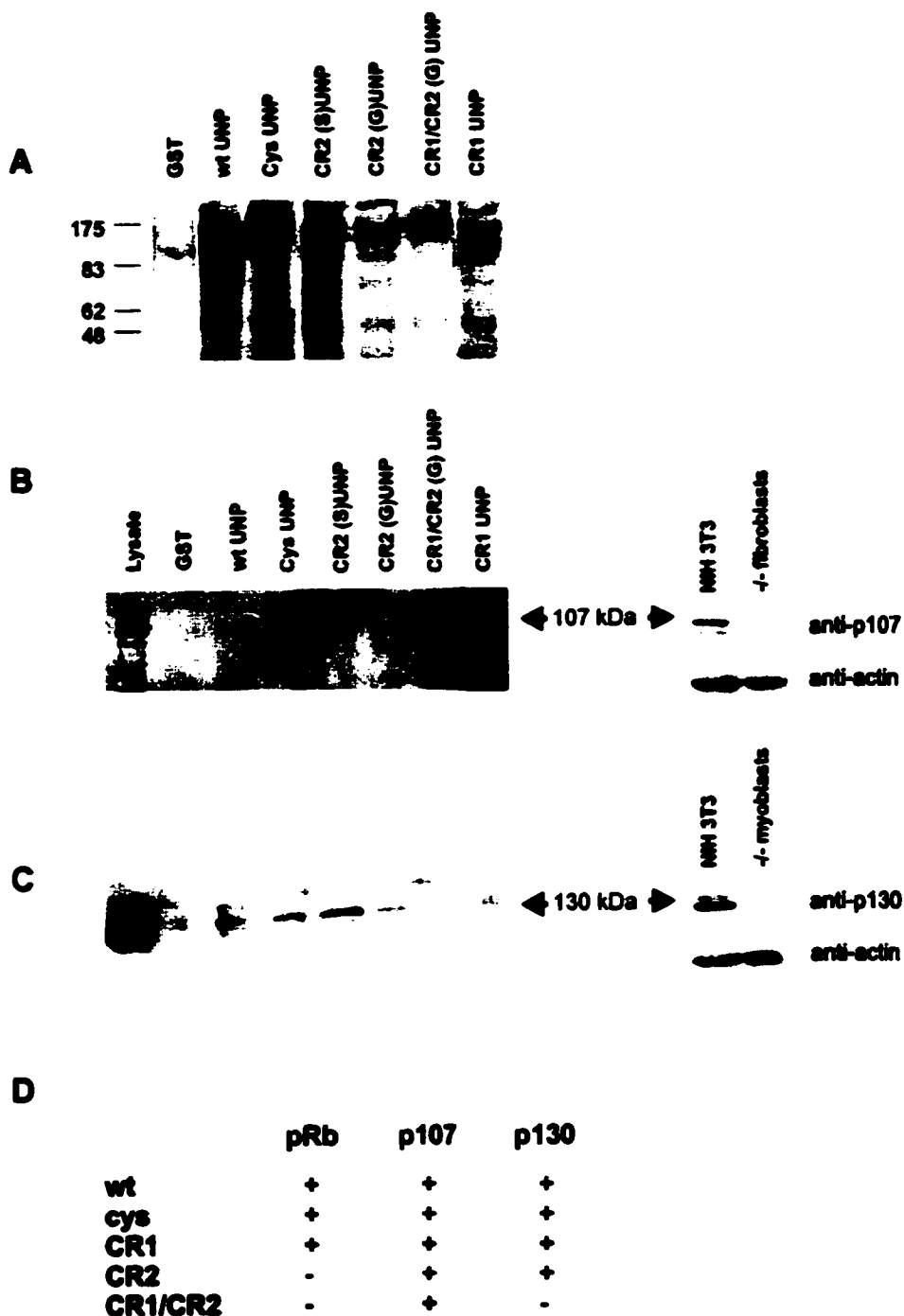


Figure 2 Association of UNP with p107 and p130. (a) GST-UNP fusions were created containing site-directed mutations of the CR1 domain as well as a CR1/CR2 double mutation. These fusions were expressed in bacteria, and expression levels were verified by silverstaining of an SDS-PAGE gel. (b) Left panel: GST pull-down as per 1d, probed with an anti-p107 rabbit polyclonal antibody (C-17, Santa Cruz). Right panel: Western blot confirming the specificity of the antibody for p107. No signal was detected in a lysate of primary fibroblasts from a p107 homozygous null mouse (LeCouter *et al.*, 1998a). (c) Left panel: GST pull-down as per 1d, probed with an anti-p130 rabbit polyclonal antibody (C-20, Santa Cruz). Right panel: Western blot confirming the specificity of the antibody for p130. No signal was detected in a lysate of primary myoblasts from a p130 homozygous null mouse (LeCouter *et al.*, 1998b). Antibody dilutions for panels b and c were 1/100. (d) Summary of all GST pull-down results for the interactions of the three pocket proteins with UNP mutants

Gilchrist, Australian National University, 1998). Association of bacterially produced UNP and pRb was abolished by mutation of the UNP LxCxE motif (*ibid*).

Because pRb belongs to a family of proteins that includes p107 and p130, we tested for possible interaction of Unp with these pocket proteins. As seen in Figure 2, both p107 (b) and p130 (c) interact with UNP, although with different requirements for the CR domain LxCxE motif than pRb. Interactions of mutant UNP with the pocket proteins are summarized in D. No interactions were detected with either E2F-1 or cyclin D1 (data not shown).

The UNP-pRb interaction occurs in an endogenous complex, but not an abundant one. A weak interaction was detected in co-immunoprecipitations of untransfected H1299 cell lysates with an anti-UNP antibody (Figure 3a, long exposure), or with one of two anti-

pRb antibodies tested (b, goat polyclonal lane). As expected from a low abundance complex, exogenous transfection of plasmids for these proteins increased the signal obtained (c, lane 3 *versus* lane 2).

It has been reported that only the hypophosphorylated form of pRb binds to E2F-1 (Helin *et al.*, 1992; Shan *et al.*, 1992), a finding that has been incorporated into the popular model of pRb's function in the G1-S transition (reviewed in Nevins, 1998). We have observed that UNP can interact with both hypo- and hyperphosphorylated forms of pRb as indicated by the doublet in Figure 3a (long exposure). These data suggest a fundamental difference in the interactions of UNP or E2F-1 with pRb. There are also major differences in the manners in which the pocket proteins interact with UNP. The interaction of pRb requires only the CR2 domain LxCxE motif, whereas p130 can utilize either the CR1 or the CR2 domain. Our data suggest that p107 requires neither. The region of UNP involved in binding p107 is still unknown, but it is not the first protein to which p107 binds outside the CR domains (Beijersbergen *et al.*, 1994; Sala *et al.*, 1996; Voorhoeve *et al.*, 1999; Ginsberg *et al.*, 1994).

It is clear that Ubp enzymes do not function solely to recycle the cell's ubiquitin pools, with regulation of the ubiquitin pathway left entirely to the ubiquitin conjugating enzymes and ligases; it is now becoming apparent that Ubps also have roles in specific regulation of the pathway. Recent results from studies of the *Drosophila* Fat facet Ubp strongly suggest a role of maintaining higher levels of specific substrates by protecting those substrates from formation of a ubiquitin chain of threshold length for proteasomal targeting (Cadavid *et al.*, 2000). One can imagine a scenario in which the ubiquitin conjugating machinery and the deubiquitinating enzymes would possess opposing activities, resulting in an equilibrium that determines the net half-life of specific proteins. If a particular substrate is a protein involved in the cell cycle control, a change of abundance or activity of either the chain forming or cleaving activities might be sufficient to stabilize an oncoprotein or inactivate a tumor-suppressor protein. We have demonstrated that UNP interacts with all three pocket proteins, but we think it unlikely that they are substrates of UNP. Rather we speculate that the pocket proteins serve as a bridge to bring UNP in contact with one or more proteins that also interact with the pocket proteins. Given the central role of pRb in cell cycle regulation it is conceivable that through such interactions abnormally high levels of UNP could significantly affect the levels of critical protein substrates, whose increased abundance could lead to accelerated proliferation. In light of the pRb association we report here and our previous observation of elevated expression of UNP in primary human lung cancer samples (Gray *et al.*, 1995) we believe this model deserves further exploration. The identity of UNP substrates amongst pRb-associated proteins is the focus of our current research.

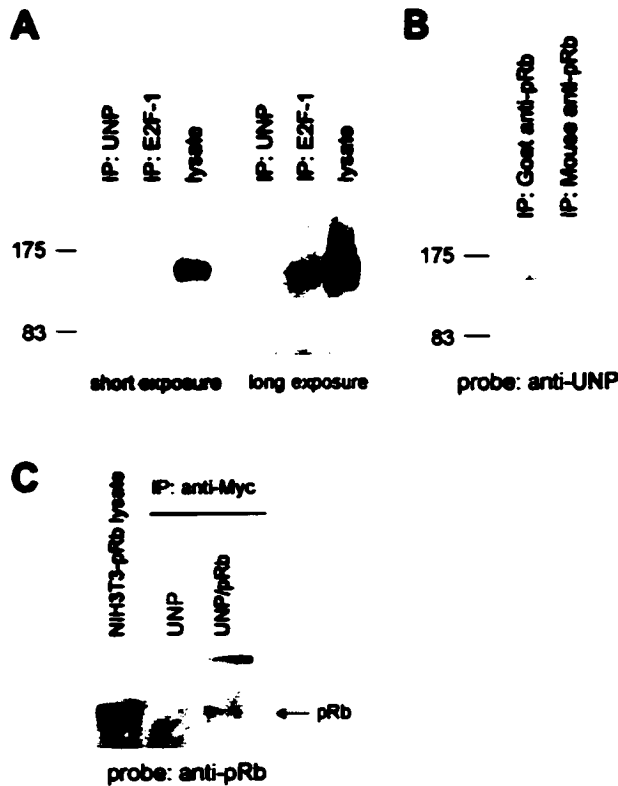


Figure 3 *In vivo* interaction of UNP and pRb as detected by co-immunoprecipitation. (a) H1299 cells were lysed in the same buffer as in Figure 1c and immunoprecipitated with an anti-UNP rabbit polyclonal antibody (CSM-11, Zymed, 1/100 dilution) or an anti-E2F-1 rabbit polyclonal antibody (C-20, Santa Cruz, 1/125 dilution), then probed with an antibody to pRb. (b) Reciprocal experiment. H1299 cells were lysed, immunoprecipitated with a goat anti-pRb polyclonal antibody (C-15 goat, Santa Cruz, 1/100 dilution) or a murine anti-pRb monoclonal antibody (G3-245, Pharmingen, 1/100), then probed with the anti-UNP polyclonal antibody (Zymed CSM-11, 1/500). (c) Co-immunoprecipitation of UNP and pRb in transfected murine cells. NIH3T3 cells were transfected with a *myc*-tagged UNP plasmid with or without a pRb expression plasmid. UNP was immunoprecipitated using the mouse monoclonal antibody 9E10 against the *myc* epitope tag, and the precipitated proteins were probed with the anti-pRb monoclonal G3-245

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References

- Beijersbergen RL, Hijmans EM, Zhu L and Bernards R. (1994). *EMBO J.*, **13**, 4080–4086.
- Boyer SN, Wazer DE and Band V. (1996). *Cancer Res.*, **56**, 4620–4624.
- Buyse IM, Shao G and Huang S. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 4467–4471.
- Cadavid AL, Ginzler A and Fischer JA. (2000). *Development*, **127**, 1727–1736.
- Carrano AC, Eytan E, Hershko A and Pagano M. (1999). *Nat. Cell. Biol.*, **1**, 193–199.
- DeCaprio JA, Ludlow JW, Figge J, Shew JY, Huang CM, Lee WH, Marsilio E, Paucha E and Livingston DM. (1988). *Cell*, **54**, 275–283.
- DeSalle LM, Latres E, Lin D, Graner E, Montagnoli A, Baker RT, Pagano M and Loda M. (2001). *Oncogene*, **20**, 5538–5542.
- Diehl JA, Zindy F and Sherr CJ. (1997). *Genes Dev.*, **11**, 957–972.
- Dyson N, Howley PM, Munger K and Harlow E. (1989). *Science*, **243**, 934–937.
- Fattaey AR, Helin K, Dembski MS, Dyson N, Harlow E, Vuocolo GA, Hanobik MG, Haskell KM, Oliff A, Defeo-Jones D and Jones RE. (1993). *Oncogene*, **8**, 3149–3156.
- Ginsberg D, Vairo G, Chittenden T, Xiao ZX, Xu G, Wydner KL, DeCaprio JA, Lawrence JB and Livingston DM. (1994). *Genes Dev.*, **8**, 2665–2679.
- Glotzer M, Murray AW and Kirschner MW. (1991). *Nature*, **349**, 132–138.
- Grana X, Garriga J and Mayol X. (1998). *Oncogene*, **17**, 3365–3383.
- Gray DA, Inazawa J, Gupta K, Wong A, Ueda R and Takahashi T. (1995). *Oncogene*, **10**, 2179–2183.
- Gregory MA and Hann SR. (2000). *Mol. Cell. Biol.*, **20**, 2423–2435.
- Gupta K, Chevrette M and Gray DA. (1994). *Oncogene*, **9**, 1729–1731.
- Hateboer G, Kerkhoven RM, Shvarts A, Bernards R and Beijersbergen RL. (1996). *Genes Dev.*, **10**, 2960–2970.
- Helin K, Lees JA, Vidal M, Dyson N, Harlow E and Fattaey A. (1992). *Cell*, **70**, 337–350.
- Hofmann F, Martelli F, Livingston DM and Wang Z. (1996). *Genes Dev.*, **10**, 2949–2959.
- Hoyt MA. (1997). *Cell*, **91**, 149–151.
- Koepp DM, Harper JW and Elledge SJ. (1999). *Cell*, **97**, 431–434.
- LeCouter JE, Kablar B, Hardy WR, Ying C, Megeney LA, May LL and Rudnicki MA. (1998a). *Mol. Cell. Biol.*, **18**, 7455–7465.
- LeCouter JE, Kablar B, Whyte PF, Ying C and Rudnicki MA. (1998b). *Development*, **125**, 4669–4679.
- Lipinski MM and Jacks T. (1999). *Oncogene*, **18**, 7873–7882.
- Nevins JR. (1998). *Cell Growth Differ.*, **9**, 585–593.
- Sala A, De Luca A, Giordano A and Peschle C. (1996). *J. Biol. Chem.*, **271**, 28738–28740.
- Shan B, Zhu X, Chen PL, Durfee T, Yang Y, Sharp D and Lee WH. (1992). *Mol. Cell Biol.*, **12**, 5620–5631.
- Smith EJ, Leone G and Nevins JR. (1998). *Cell Growth Differ.*, **9**, 297–303.
- Voorhoeve PM, Hijmans EM and Bernards R. (1999). *Oncogene*, **18**, 515–524.
- Whyte P, Williamson NM and Harlow E. (1989). *Cell*, **56**, 67–75.

Appendix 2

Phosphorylation of the UNP ubiquitin-specific protease

**Lelia Raynal^{1*}, Paola Blanchette^{1,2*}, Claire Bemben^{1,2}, Emma Tibbo¹,
and Douglas A. Gray^{1,3,#}**

1. Ottawa Regional Cancer Centre, 501 Smyth Rd., Ottawa, Ontario, Canada K1H 8L6
2. Department of Biochemistry, Microbiology & Immunology, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario, Canada K1H 8M5
3. Department of Medicine, University of Ottawa, 451 Smyth Rd., Ottawa, Ontario, Canada K1H 8M5

***these authors have contributed equally to the work**

corresponding author

**mailing address: Ottawa Regional Cancer Centre, 501 Smyth Rd., Ottawa, ON K1H 8L6
telephone: 613-737-7700 ext.6896
fax: 613-247-3524
email: Doug.Gray@orcc.on.ca**

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SUMMARY

The mouse *Unp* gene and its human ortholog USP4 encode ubiquitin-specific proteases that have been implicated in oncogenic transformation. Although the *in vivo* substrates of the UNP and USP4 are not yet known, the enzymes contain structural motifs common to viral oncoproteins whose function is the deregulation of cell cycle control. We have investigated the possibility that UNP enzyme activity is regulated by phosphorylation, a central element mechanism of cell cycle regulation and of signal transduction modulation. We report here that UNP is a phosphoprotein in which phosphoserine is the predominant phosphoamino acid. The ability of UNP isolated from mammalian cells to cleave a synthetic substrate could be totally eliminated by treatment with a phosphatase. Alternative splicing and differential phosphorylation were excluded as the source of two observed isoforms of UNP, and both isoforms were found to be sensitive to inactivation by phosphatase treatment. These data suggest that UNP may be regulated by phosphorylation *in vivo*.

INTRODUCTION

Ubiquitin-mediated proteolysis is the principal cellular mechanism for the regulated degradation of proteins (for recent reviews see [1, 2]). The enzymatic components of this pathway are able to recognize and designate specific proteins for destruction, while ignoring the constellation of other proteins present in the cell. The basis of the exquisite specificity of ubiquitin-mediated proteolysis is not yet fully understood, but it is thought that the necessary complexity is derived from the presence of multiple ubiquitin conjugating (E2) enzymes and ubiquitin ligase (E3) enzymes, that may act combinatorially.

There are many occasions during which cellular substrates are degraded temporally or in response to extracellular signals, and it is not at all surprising that the selectivity of the ubiquitin system is brought to bear in such circumstances. A classic example of temporally regulated proteolysis is the destruction of mitotic cyclins occurring with each cell cycle [3]. The proteolytic degradation of cyclins is ubiquitin-mediated, and the enzymology of the process is reasonably well understood [4]. In signaling pathways, it is now apparent that not only are many of the 'immediate early' effector molecules of short half-life because of ubiquitin-mediated degradation [5, 6], but many of the upstream molecules – even some membrane-bound receptors – have their activities delimited by ubiquitin-mediated proteolysis or ubiquitin-mediated trafficking [7-9]. Because so much of cellular regulation (including the cell cycle and signal transduction pathways) is accomplished through the action of kinases, it seems likely that many intersections must exist between the systems that mediate the phosphorylation and ubiquitination of proteins. It is reasonable to suppose that phosphorylation is used to inform the ubiquitin

pathway that certain substrates should be degraded, and it would seem that this hypothesis is supported by recent findings in the degradation of p27, for example [10]. In this case phosphorylation of the substrate is used to attract the attention of the ubiquitination machinery, but one could equally suppose that phosphorylation of the ubiquitin conjugation or ligation enzymes themselves could regulate their activities. The multiprotein complex that targets mitotic cyclins for destruction (anaphase-promoting complex, also known as the cyclosome) seems to contain such enzymes [11].

There is another side to ubiquitination that is perhaps underappreciated, represented by the activities of the ubiquitin carboxyterminal hydrolases (UCH enzymes) and ubiquitin-specific proteases (Ubp enzymes), enzymes which serve to cleave ubiquitin peptide or isopeptide bonds. By reducing the lengths of ubiquitin chains or by cleaving them off a substrate such enzymes might preclude formation of the threshold-length ubiquitin chain that serves to target the substrate to the proteasome [12]. This might explain why there are so many Ubp enzymes in the cell (16 in yeast, and an unknown but probably large number in metazoans). It may be that the net stability of a protein substrate can be finely tuned by the opposing activities of enzymes that are actively lengthening and shortening the ubiquitin chain. Were both activities subject to kinase-mediated regulation, the system could respond rapidly to a variety of signals to degrade or stabilize the substrate in question.

We have previously reported the identification of a mammalian Ubp enzyme encoded by a gene we designated *Unp* ([13]; the human ortholog is now designated USP4, but was formerly designated *Unph* [14]. We have investigated the phosphorylation status of the UNP enzyme, and have found that it is phosphorylated and that its activity is utterly

dependent on phosphorylation. We believe this to be the first report of potential regulation of a ubiquitin-cleaving enzyme by phosphorylation.

EXPERIMENTAL PROCEDURES

Cell lines, bacterial strains, and antibodies. Cos-1 cells were obtained from Dr. John Bell (Ottawa Regional Cancer Centre), and were used for the metabolic labeling. HeLa cells were obtained from the ATCC. Endogenous UNP was analyzed in H1299 cells, the gift of Dr. Philip Branton (McGill University). GST-Unp was produced in the TG1 strain of *E. coli*. The UNP antibody (CSM-11) was purchased from Zymed, and was used at a dilution of 1 in 100 for immunoprecipitations and 1 in 500 for western blots. The RGSHis antibody was purchased from Qiagen. The antibody to phosphoserine was purchased from Sigma, and was used at a dilution of 1 in 100. The antibody to pRb was purchased from Pharmingen and used at a dilution of 1 in 100.

Constructs used in the experiments. Construct pDG50 encodes amino acids 169-945 of murine UNP, fused to a C-terminal 6 x myc tag. Plasmid pDG104 encodes residues 1-945 fused to a C-terminal 6 x myc tag. The analogous hexahistidine-tagged Unp construct pDG280 was produced by replacement of the myc tag of pDG104 with the RGSHis6 sequence. The bacterial GST-Unp fusion construct pDG284 was created by subcloning the myc-tagged Unp cDNA from pDG104 in frame in the pGEX4T-3 vector (Pharmacia). Bacteria expressing the GST-ubiquitin fusion were obtained from Dr. Rohan Baker (Australian National University, Canberra). The plasmid pDG288 encoding the smaller isoform of UNP was generated from plasmid pDG280 (encoding UNP with the C-terminal 6x His tag) as follows. An RT-PCR fragment spanning the position of the alternatively spliced exon in Unp [15, 16] was obtained from NIH 3T3

RNA using the forward oligonucleotide primer GAGTCAGACCTTGAAGG and the reverse primer GCTTCTTCTTTACGCGG. The RT-PCR product was digested with Cla I and Nhe I, and was used to replace the corresponding Cla I/Nhe I fragment of plasmid pDG280. The resulting plasmid pDG288 was sequenced to verify the spliced exon was absent, that the reading frame had been maintained across the junction, and that no mutations were introduced into the Unp sequence during the RT-PCR amplification.

Protein extracts, immunoprecipitation and purification of Unp. Eukaryotic recombinant Unp was obtained from HeLa cells transiently transfected using Fugene 6 transfection reagent (Roche Molecular Biochemicals). Epitope-tagged Unp (24 hours post-transfection) or endogenous Unp protein was harvested from confluent 100mm culture dishes with 500 μ l of lysis buffer containing protease and phosphatase inhibitors (20mM Tris pH 7.5, 150mM NaCl, 2mM EDTA, 2mM DTT, 1% Triton X-100, 5% glycerol, 10mM NaF, 4mM NaPPi, 50 μ M β -glycerophosphate, 500 μ M Na-vanadate, 200 μ g/ml PMSF, 5 μ g/ml leupeptin, and 2 μ g/ml aprotinin). After 30 min on ice, soluble proteins were recovered in the supernatant after a 10 min. centrifugation at 4 C to pellet cell debris. Each extract was incubated overnight with the appropriate antibody. Antibody-antigen complexes were recovered by binding to protein G sepharose beads (Gammabind G Sepharose, Amersham Pharmacia).

Phosphoamino acid analysis. COS-1 cells transiently transfected with pDG50 (encoding myc-tagged Unp) were placed in phosphate-free α -DMEM medium for 1h at 37 C. 500 μ M Na-Vanadate and 200 μ Ci/ml 32 P orthophosphate (Amersham) were added and cells were incubated 4h at 37°C. After 5 washes with PBS, radiolabeled soluble proteins were prepared as for non-radioactive protein extracts. The epitope-tagged protein was

recovered by addition of the 9E10 monoclonal antibody followed by rabbit anti-mouse antibody bound to protein A sepharose beads. The beads were washed 3 times in lysis buffer prior to separation by SDS-PAGE and transfer to an Immobilon-P membrane (Millipore). ^{32}P -labeled proteins were detected by conventional autoradiography. The exposed film and membrane were aligned and the Unp bands were excised from the membrane and hydrolysed directly in 6N double-distilled HCL for 75 minutes at 110 C. The hydrolysate was lyophilized, mixed with authentic phosphoamino acids, and separated by two dimensional thin layer chromatography using standard procedures.

Ubiquitin cleavage assays. GST-UNP was produced in bacteria and purified by glutathione affinity chromatography using standard methodologies. The measurement of UNP activity against the ubiquitin-GST substrate has been described previously [17]. For activity assays using UNP from mammalian cells, the enzyme was isolated by immunoprecipitation. Each immunoprecipitate was divided into two portions, and one portion was treated with calf intestinal alkaline phosphatase (NEB) for 1 hour prior to incubation with the substrate at 37 C for 1 hour. Samples were separated by SDS-PAGE (10% gels) and were analyzed using a PhosphorImager SI instrument (Molecular Dynamics).

RESULTS

UNP is a phosphoprotein. We have frequently observed that UNP appears either as a single band of approximately 110 kDa or as a closely migrating doublet on western blots. One possible explanation for the appearance of a second band is post-translational modification, and we sought to determine if the enzyme was modified by phosphorylation. COS-1 cells were transfected with an expression plasmid encoding

myc-tagged UNP, and were metabolically labeled with ^{32}P -orthophosphate. UNP protein was immunoprecipitated using an antibody specific for the epitope tag, and was resolved by SDS-PAGE. Autoradiography (Figure 1A) revealed a doublet centered at approximately 90 kDa, consistent with the predicted size of the amino terminally truncated UNP (the endogenous doublet is typically detected at 110 kDa on western blots, not shown). Detection of the doublet by autoradiography indicated that whatever their source, both isoforms of UNP were phosphorylated, and both bands were excised from the gel and pooled for phosphoaminoacid analyses. Complete digestion of UNP and resolution of amino acids by two-dimensional thin-layer chromatography revealed that the predominant phosphoaminoacid in both isoforms of UNP was phosphoserine (Figure 1B). These data do not exclude phosphorylation of threonine or tyrosine, but if these residues were phosphorylated they were below the level of detection in our system. Preliminary data from preliminary tryptic peptide analyses were consistent with phosphorylation on multiple sites, but the number of phosphorylation sites and their positions could not be determined from these data, and the data are not shown.

UNP activity is sensitive to phosphorylation state. It has been previously reported that UNP has ubiquitin-specific protease activity, and is able to cleave synthetic ubiquitin-protein fusions *in vitro* [17, Gilchrist and Baker, in press]. The UNP in these experiments was produced in bacteria, so we first sought to confirm that UNP isolated from mammalian cells had comparable activity. As reported in Figure 2, immunoprecipitated UNP had activity comparable to the recombinant protein in the *in vitro* cleavage assay. To determine if the phosphorylation of UNP was of any significance with respect to its activity we tested the ability of the enzyme to cleave the synthetic test substrate before

and after its treatment with the nonspecific calf intestinal alkaline phosphatase (CIP). We observed that upon CIP treatment both immunoprecipitated endogenous UNP and FPLC purified his-tagged UNP isolated from transfected cells was inactive (Figure 3), showing background activity comparable to a previously reported (17) site-directed, catalytically inactive mutant version of the enzyme [17]. Immunoprecipitated UNP catalytic activity was also found to be sensitive to protein phosphatase 1 (PP1; data not shown), but there was evidence of proteolytic degradation of UNP in the presence of PPI that could not be inhibited with the specific phosphatase inhibitor I-2 (unlike CIP, which did not seem to contain contaminating protease activity, *vide infra*). Bacterially-produced UNP was also found to be sensitive to CIP, but for unknown reasons the results showed more variability than was observed with UNP of mammalian origin.

Elimination of phosphoserine immunoreactivity by CIP treatment. To ensure that the observed inactivation of UNP activity by CIP treatment was due to dephosphorylation as opposed to contaminating activities (such as proteolysis), aliquots of the UNP enzyme were analyzed by western blotting following CIP treatment. Similar UNP protein doublets were observed before and after incubation with CIP, eliminating the possibility that loss of activity was due to proteolysis (Figure 4A). These data also suggested that the two bands did not arise from alternative phosphorylation. The phosphatase activity of CIP was confirmed in parallel experiments in which the slower migrating, hyperphosphorylated form of the retinoblastoma protein was eliminated by treatment with CIP (Figure 4B), as expected. The observed loss of UNP activity ubiquitin-specific protease activity upon CIP treatment (Figure 3) was accompanied by a total loss of phosphoserine immunoreactivity (Figure 4C).

The alternatively spliced exon of UNP is not required for its enzymatic activity. Both human USP4 [15] and mouse *Unp* (Gray, unpublished) are alternatively spliced to generate two mRNA isoforms. The alternatively spliced exon in the *Unp* gene is exon 7 ([16]; Gray, unpublished) which encodes residues 233-279 of the UNP protein, a region containing a high density of serine residues (16 serines out of 47 residues). To determine if the residues encoded by the alternatively spliced exon were required for the phosphatase-sensitive cleavage activity of the UNP enzyme, we generated an epitope-tagged version of the shorter isoform by RT-PCR. Plasmids encoding the short or long isoforms of UNP were transfected into 293T cells, and the enzymes were isolated by immunoprecipitation using the antibody to the hexahistidine tag. Cleavage activities of the two isoforms were then tested in the *in vitro* assay. As reported in Figure 5, there was no apparent difference in the activities of the two isoforms as evidenced by production of the cleaved product. Further, the short and long isoforms showed the same sensitivity to phosphatase treatment.

DISCUSSION

Western blotting analysis of UNP has frequently revealed the presence of two closely migrating isoforms. In other systems multiple isoforms can be explained by the existence of one or more closely related genes, alternative splicing of mRNA from a single gene, or various post-translational modifications of a single gene product. Both the human USP4 [15] and the murine *Unp* gene (Gray, unpublished) display alternative splicing of the seventh exon, but this cannot explain the appearance of the two protein isoforms because an UNP protein doublet can be detected in transfected cells using an antibody specific for an epitope tag (Figure 1A). We therefore think it most likely that

the two UNP isoforms are the products of one or more post-translational modifications. The data presented herein confirm that phosphorylation comprises at least one such modification: UNP can be metabolically labeled with ^{32}P orthophosphate (Figure 1A), can be detected using an antibody specific for phosphoserine (Figure 4C), and is sensitive to a nonspecific phosphatase (Figure 3). Metabolic labeling revealed that UNP was predominantly phosphorylated on serine residues (Figure 1B), but it is possible that phosphorylation of other hydroxyamino acids occurs at lower stoichiometric levels. It is also possible that phosphorylation of the endogenous UNP protein could occur on threonine or tyrosine residues at the amino or carboxy terminus of UNP, both of which were removed in the generation of pDG50, which was used for metabolic labeling. No difference has been detected in the cleavage activities of full-length or truncated UNP produced in bacteria (Gilchrist and Baker, submitted; Blanchette, unpublished) and we have no evidence for other functional roles for the termini, but we currently cannot exclude the possibility that phosphorylation of N or C terminal threonine or tyrosine occurs at some level *in vivo*. Whether or not this is the case the endogenous protein is clearly phosphorylated on serine residues (Figure 4C), and we conclude from the available data that at least some of the UNP present in cells exists as a phosphoprotein, and that phosphorylation is essential for the activity of the enzyme. The phosphorylation of UNP cannot account for the appearance of two closely migrating bands on western blots, however, because both bands persist following phosphatase treatment (Figure 4A) when phosphoserine immunoreactivity is lost (4C). It is therefore likely that UNP is subject to other post-translational modifications in addition to phosphorylation. The nature of these modifications is currently unknown.

UNP contains a large number of phosphorylation consensus sites as revealed by computerized motif searching. Without additional information there is no basis on which to predict which of these sites, if any, is actually utilized in cells. An examination of the predicted amino acid sequence of the protein revealed several regions rich in serine residues, most notably the region encoded by the alternatively spliced exon 7. Were the residues encoded by exon 7 targets for phosphorylation, an attractive model of UNP regulation could be envisaged in which alternative splicing would generate isoforms of differential sensitivity to regulation by phosphorylation. Elimination of the exon in a shorter version of transfected, epitope-tagged UNP did not alter cleavage activity *in vitro* or sensitivity to CIP (Figure 5) indicating that the residues encoded by exon 7 are dispensable for cleavage activity, and irrelevant with respect to phosphatase sensitivity. These data do not exclude that phosphorylation of residues encoded by exon 7 occurs, but the activity of the enzyme cannot be dependent on such phosphorylation events. The critical phosphorylation sites must therefore reside elsewhere in the protein. The location and identity of these residues is a focus of ongoing research.

What purpose could be served by the phosphorylation-dependent cleavage activity of UNP? There are a constellation of kinases and phosphatases in the cell that are responsive to temporal and signalling pathways. It may be that the stability of UNP's cellular substrate (or substrates) can be rapidly adjusted through the phosphorylation or dephosphorylation of UNP in response to cellular signals. The net stability of cellular proteins may, in fact, be determined by the opposing activities of ubiquitin conjugating and deconjugating activities, each poised to interpret phosphorylation-mediated signals. Given the responsiveness of the proposed mechanism in fine-tuning substrate stability,

we predict that UNP will be but the first of many deubiquitinating enzymes to show dependence on phosphorylation status.

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REFERENCES

- [1] A. Hershko and A. Ciechanover. (1998) *Annu Rev Biochem* 67, 425-79.
- [2] M. Hochstrasser. (1996) *Annu Rev Genet* 30, 405-39.
- [3] T. Evans, E.T. Rosenthal, J. Youngblom, D. Distel and T. Hunt. (1983) *Cell* 33, 389-96.
- [4] D.M. Koepp, J.W. Harper and S.J. Elledge. (1999) *Cell* 97, 431-4.
- [5] M. Treier, L.M. Staszewski and D. Bohmann. (1994) *Cell* 78, 787-98.
- [6] C. Tsurumi, N. Ishida, T. Tamura, A. Kakizuka, E. Nishida, E. Okumura, T. Kishimoto, M. Inagaki, K. Okazaki, N. Sagata and et al. (1995) *Mol Cell Biol* 15, 5682-7.
- [7] S. Mori, C.H. Heldin and L. Claesson-Welsh. (1992) *J Biol Chem* 267, 6429-34.
- [8] S. Mori, L. Claesson-Welsh, Y. Okuyama and Y. Saito. (1995) *Biochem Biophys Res Commun* 213, 32-9.
- [9] K. Miyazawa, K. Toyama, A. Gotoh, P.C. Hendrie, C. Mantel and H.E. Broxmeyer. (1994) *Blood* 83, 137-45.

- [10] A. Montagnoli, F. Fiore, E. Eytan, A.C. Carrano, G.F. Draetta, A. Hershko and M. Pagano. (1999) *Genes Dev* 13, 1181-9.
- [11] V. Sudakin, D. Ganoth, A. Dahan, H. Heller, J. Hershko, F.C. Luca, J.V. Ruderman and A. Hershko. (1995) *Mol Biol Cell* 6, 185-97.
- [12] S. Taya, T. Yamamoto, M. Kanai-Azuma, S.A. Wood and K. Kaibuchi. (1999) *Genes Cells* 4, 757-67.
- [13] K. Gupta, N.G. Copeland, D.J. Gilbert, N.A. Jenkins and D.A. Gray. (1993) *Oncogene* 8, 2307-10.
- [14] D.A. Gray, J. Inazawa, K. Gupta, A. Wong, R. Ueda and T. Takahashi. (1995) *Oncogene* 10, 2179-83.
- [15] A. Frederick, M. Rolfe and M.I. Chiu. (1998) *Oncogene* 16, 153-65.
- [16] M. Di Fruscio, C.A. Gilchrist, R.T. Baker and D.A. Gray. (1998) *Biochim Biophys Acta* 1398, 9-17.
- [17] C.A. Gilchrist, D.A. Gray and R.T. Baker. (1997) *J Biol Chem* 272, 32280-5.

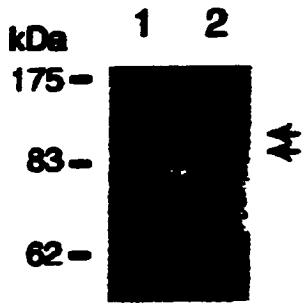
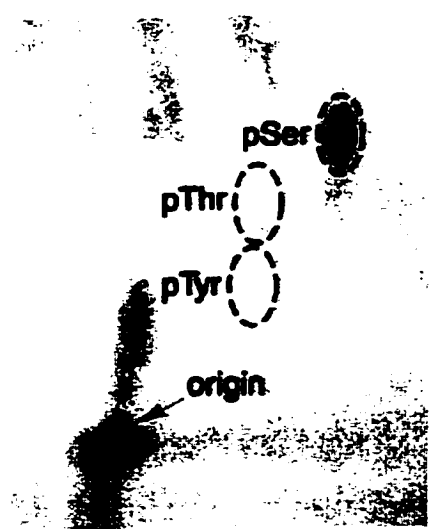
Figure 1. Phosphorylation of UNP as detected by metabolic labeling. A) Autoradiogram of SDS PAGE of immunoprecipitated UNP protein from COS-1 cells transfected with myc-tagged UNP. Lane 1: Secondary antibody control (9E10 primary α myc antibody omitted); Lane 2: UNP immunoprecipitated using 9E10 monoclonal antibody followed by rabbit α mouse/protein A sepharose. The position of the UNP doublet is indicated by the arrows.

Figure 2. In vitro cleavage activity of UNP. Bacterially produced ^{35}S -labeled ubiquitin-GST fusion protein was exposed to bacterially produced GST-UNP (lane 1), immunoprecipitated wild-type mammalian UNP (lane 2), or immunoprecipitated C311A mutant UNP, which is catalytically inactive (lane 3). The upper band corresponds to the intact fusion protein, and the arrow indicated the position of cleaved GST (the ubiquitin component was allowed to run off the bottom of the gel to increase the separation of the fusion protein and GST). Mammalian UNP was found to have cleavage activity equivalent to that of the recombinant protein.

Figure 3. Effect of phosphatase treatment on UNP cleavage activity. UNP was isolated from mammalian cells and divided into two aliquots, one of which was treated with calf intestinal alkaline phosphatase (CIP). Sample 1: Transfected UNP isolated by immunoprecipitation using an antibody to the epitope tag. Sample 2: Endogenous UNP isolated by immunoprecipitation using an anti-UNP antibody. Sample 3: Transfected UNP isolated by nickel matrix FPLC purification. In all cases the cleaved GST (bracket) was not detectable following CIP treatment, indicating that UNP had been inactivated.


Figure 4. Western blot analysis of phosphatase-treated UNP. A) Immunoprecipitate of endogenous UNP untreated (-) or treated with CIP (+). In both cases the UNP doublet was detectable, and there was no evidence of proteolysis. B) Immunoprecipitate of pRb, treated as in panel A. C) Endogenous UNP immunoprecipitate treated as in panel A, and probed with an α phosphoserine antibody. CIP treatment eliminated phosphoserine immunoreactivity.

Figure 5. Cleavage activity of the alternatively spliced UNP isoforms. A) Schematic representation of UNP showing the position and sequence of the residues encoded by the alternatively spliced exon. Note the preponderance of serine residues. B) Cleavage assays. Lane 1: GST-UNP (positive control); lane 2: glutathione beads (negative control); lane 3: long isoform of UNP; lane 4: long isoform of UNP treated with CIP; lane 5: Δ 233-279 isoform of UNP; Δ 233-279 isoform of UNP treated with CIP. The position of cleaved GST is indicated by the arrow. The enzymatic activities of both isoforms were found to be sensitive to phosphatase treatment.

A**B**

1 2 3



	1		2		3	
CIP	-	+	-	+	-	+
						

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