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Mutational Analysis of Mammalian Ubiquitin

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Mutational Analysis of Mammalian Ubiquitin

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ABSTRACT

Much of what is known about the ubiquitin/proteasome pathway has been deduced from mutational analysis performed in the yeast model system. From the high level of conservation between yeast and mammalian ubiquitin it would be expected that expression of analogous ubiquitin isoforms in higher eukaryotes would result in similar phenotypes. A site directed mutagenesis approach was employed to investigate the phenotypes of expression of mutant ubiquitin in higher eukaryotes and in the *in vivo* setting of novel ubiquitin transgenic mice. It was found that Ub-EGFP fusion proteins are efficiently recognized and processed by ubiquitin specific proteases both in mammalian cells and in transgenic mice; the transgene-derived ubiquitin moiety was found to substitute for endogenous ubiquitin in poly-Ub chain assembly and ubiquitinated conjugates were recovered using standard purification methodologies. The expression of chain-terminating ubiquitin derivatives (K48R and K63R) predisposed cells to the toxic effects of misfolded proteins and sensitized cells to DNA damaging agents. In transgenic mice, the expression of K48R mutant ubiquitin was found to confer protective effects and delay the deterioration of Purkinje neurons in a mouse model of SCA-1. The neuroprotective effect of K48R mutant ubiquitin may be mediated though stabilization of key transcription factors whose loss figured in the normal course of the SCA1 disease.

The expression of C-terminal variants in yeast has been proposed to have profound effects on ubiquitin metabolism. A mechanistically related mechanism has been proposed to contribute to the pathogenesis of Alzheimer's disease wherein transcriptional frameshifting of the ubiquitin B mRNA generates an aberrant ubiquitin protein (termed UBB+1) with an altered C-terminus. To investigate the constraints with regard to processing/conjugation and recycling of ubiquitin in higher eukaryotes a plethora of C-terminal ubiquitin variants were generated and introduced in mammalian cells as linear fusions with EGFP. Mutations that inactivate yeast ubiquitin did not abolish the function of ubiquitin in higher eukaryotes; C-terminal ubiquitin variants were processed by deubiquitinating enzymes and in some cases were found to conjugate to cellular proteins. The tolerance of mammalian cells to mutant ubiquitin may be attributable to loosened constraints that exist at the C-terminus due to mechanisms that couple deubiquitination, targeting and destruction of Ub-EGFP

fusion proteins. Preliminary data suggest that prolonged exposure of cells of neuronal lineage to C-terminal ubiquitin variant as assessed in transgenic mice may result in perturbed ubiquitin homeostasis, a feature observed in the pathogenesis of Alzheimer's disease.

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I am also eternally grateful to my family and mostly to my mother for her persistent encouragement, guidance, understanding and support.

The great tragedy of science - the slaying of a beautiful hypothesis by an ugly fact.

Thomas Henry Huxley

This thesis is dedicated to my father who continues to stand beside me in spirit.
Dad, I love and miss you dearly.
This one is for you.

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
Amp	Ampicillin
ATP	Adenosine triphosphate
Atx-1	Ataxin-1
cAMP	Cyclic adenosine monophosphate
Cdk	Cyclin dependent kinase
cDNA	Complementary dioxy-ribonucleotide
CHX	Cycloheximide
CMV	Cytomegalovirus
CRE	Cyclin AMP response element
CREB	cyclic AMP response element binding protein
C-terminal	Carboxy-terminal
Cys	Cysteine
DNA	Dioxyribonucleic acid
DUB	De-ubiquitinating enzymes
EDTA	Ethyl diamine-tetra-acetic acid
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ENaC	Epithelial sodium channel
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ES	Embryonic stem cell
FBS	Fetal bovine serum
FCS	Fetal calf serum
GHR	Growth hormone receptor
GST	Glutathione S-transferase
HAT	Histone acetyl-transferase
HDAC	Histone de-acetylase
HD	Huntington's disease
HECT	Homology to E6-AP carboxy-terminus
His	Histidine
HMW	High molecular weight
HSP	Heat shock protein
IAP	Inhibitor of apoptosis
Kan	Kanamycin
Kb	kilobase
kDa	kilodalton
LB	Luria broth
LPS	Lipopolysaccharide
MEM	Minimal essential medium
mRNA	Messenger RNA

NaCl	Sodium chloride
NAF	Sodium Fluoride
NaPPi	Sodium pyrophosphate
NFT	Neurofibrillary tangle
NLS	Nuclear localization sequence
NP-40	Nonidet P-40
N-terminal	Amino-terminal
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Proteasome inhibitor
PKC	Protein kinase C
PMSF	Phenylmethylsulphonyl fluoride
Poly Q	Polyglutamine
RING	Really Important New Gene
RGS	Arginine-Glycine-Serine
SCA-1	Spinocerebellar ataxia 1
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SV40	Simian virus 40
TBST	Tris buffered saline with Tween-20
TNF	Tumor necrosis factor
TRIS	Tris (hydrozymethyl) aminomethane
Ub	Ubiquitin
UbC	Ubiquitin C
UbH	Ubiquitin-homology
UBL	Ubiquitin-like
UBP	Ubiquitin specific protease
UCH	Ubiquitin carboxy-terminal hydrolase
UFD	Ubiquitin fusion degradation pathway
UPP	Ubiquitin proteasome pathway
Wt	Wild-type

Chapter 1
Introduction

1.1 Intracellular degradation; a historical perspective

Progress on the understanding of intracellular protein degradation pathways has accelerated over the years with the identification of molecular and cellular events that regulate protein destruction. The observation by Christian de Duve in the 1950's that the activity of certain enzymes isolated from fractionated liver extracts increased in a time dependent manner resulted in the postulate that these enzymes may be normally compartmentalized in a distinct organelle (reviewed in (Bowers, 1998)). The investigation of this compartment led to the discovery of the lysosome (otherwise referred to as the suicide-bag), formerly recognized as the sole mechanism mediating the degradation of protein substrates in eukaryotes. Lysosomal-mediated-proteolysis is now viewed as the slow component that accounts for only a minor proportion of protein degradation and serves in the destruction of numerous membrane-associated proteins, proteins that enter the endocytic pathway and the non-selective degradation of intracellular proteins under stressed conditions (reviewed in (Cuervo and Dice, 1998)).

The existence of an ATP-dependent proteolytic system in reticulocyte lysates was originally proposed by Etlinger and Goldberg (Etlinger and Goldberg, 1977) when in the early 70's and 80's Hershko, Cichanover and Rose (three investigators who won the 2004 nobel prize in Chemistry for their elegant work in deciphering the ubiquitin proteolytic pathway) demonstated that protein degradation in reticulocyte lysates (devoid of lysosomes) is dependent on the presence of two previously isolated fractions (Fraction I and II). The initial observation that Fraction II was lacking the ATP-dependent proteolytic activity which can be re-stored upon addition of Fraction I suggested that the former

contained a component with the ability to stimulate the ATP-dependent process. The active component in fraction I was identified as ubiquitin (Ciechanover et al., 1980) whereas fraction II was found to be enriched in ATP (Ciechanover et al., 1981). Paralleling this finding was the identification of a novel protein from cattle's thymus in early 70's by Goldstein *et al* while investigating the biochemical mechanism of *myasthenia gravis* (Goldstein et al., 1975). It was originally proposed to act as a thymic hormone (referred to as lymphocyte differentiation promoting factor) involved in the differentiation of both B and T lymphocyte populations (Goldstein et al., 1975) only later to be identified as ubiquitin. The association of a non-histone component in H2A had long been appreciated when in 1977 this component of the nuclear protein (refered to as A24) was identifed as ubiquitin (Hunt and Dayhoff, 1977). It was found to be connected by an isopeptide bond between its epsilon amino group of Lys^{119} to the C-teminal G76 residue of ubiquitin (Goldknopf and Busch, 1977). This suggested a novel role for ubiquitin and established that it could be conjugated to other proteins in the presence of ATP. The identification of the components associated with the UPP (ubiquitin proteasome pathway) were slowly discovered and the ubiquitin pathway was acknowledged as the main non-lysosomal proteolytic system in eukaryotic cells. The UPP which is operative in the cytoplasmic and nuclear compartments serves as the primary system for the rapid and regulated destruction of cellular protein substrates by directing them for destruction to the 26S proteasome, an ATP-dependent multi-protease (reviewed in (Glickman and Ciechanover, 2002)). Ubiquitin, the central player in proteolysis, serves as a molecular tag to efficiently target proteins for elimination.

1.2 The UPP pathway; a system's overview

The UPP is required in the maintenance of normal cellular homeostasis typified by the degradation of cell cycle cyclins (reviewed in (Irniger, 2002)), the degradation of short and long lived proteins under normal metabolic conditions and in the endoplasmic reticulum associated degradation pathway (ERAD) where it is involved in the retro-translocation and degradation of misfolded ER proteins (reviewed in (Hampton, 2002)). The pathway can mechanistically be simplified in a three step process schematically portrayed in Figure 1: the activation and conjugation of ubiquitin followed by the assembly of a polyubiquitin chain on the target protein and its degradation by the 26S proteasome. Briefly, ubiquitin is activated by an E1 ubiquitin activating enzyme whose role is to elevate ubiquitin to a higher energetic state that, with combinatorial association with E2 conjugases (enzymes that assemble ubiquitin chains) and E3 ligases (substrate specificity factors), permits the formation of a covalent isopeptide bond between the C-terminal glycine residue of ubiquitin and an internal lysine residue of a protein substrate. The initial ubiquitin molecule can serve as a substrate for the attachment of succeeding ubiquitin moieties whose C-termini are attached to the lysine at position 48 within the preceding ubiquitin. Successive rounds of ubiquitination serve to assemble a poly-ubiquitin chain of a threshold length of four ubiquitin subunits arranged in a zigzag topology, the structural unit recognized by the 26S proteasome (Piotrowski et al., 1997). The substrate is then directed to the 26S proteasome, unfolded and translocated through the interior of the 20S core, where it is degraded by a combination of resident proteases; the ubiquitin chain is cleaved from the residual peptide of the substrate and then disassembled to yield the monomeric form of ubiquitin. The conjugating system, the enzymatic activities responsible for the processing and recycling of ubiquitin are discussed below.

1.3 The UPP components

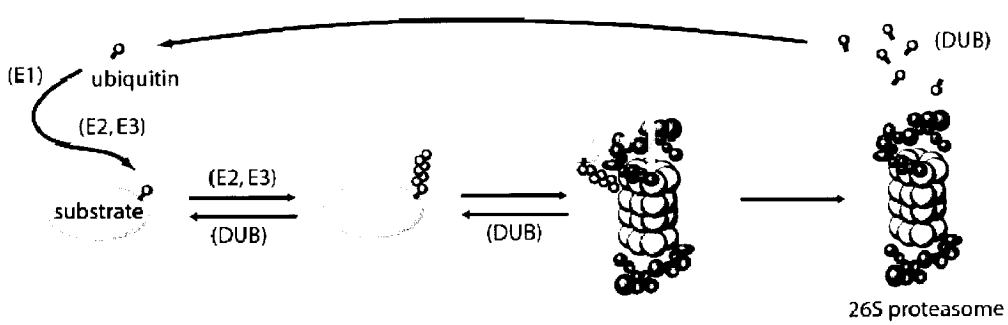
1.3.1 Ubiquitin, the central player in proteolysis

Evolutionary conservation of ubiquitin and organization of ubiquitin genes

Ubiquitin is a relatively small, ubiquitously expressed 76 amino acid globular polypeptide with an unparalleled high evolutionary conservation between eukaryotes (Ozkaynak et al., 1984) with yeast ubiquitin differing by only three conservative amino acids (position 19 Ser→Pro, position 24 Asp→Glu and position 28 Ser→Ala) from mammalian ubiquitin and yeast ubiquitin differing by only two amino acids from Barley, Oat, Soya-bean and Arabidopsis ubiquitin (position 28 Ser→Ala and position 57 Ser→Ala)(sequence alignment is shown in Figure 2). The replacement of yeast ubiquitin amino acids Ser19, Asp24 and Ser28 by the amino acids Pro, Glu and Ala respectively (found in human ubiqutin) have not been found to alter the function of yeast ubiquitin as assessed by in vitro degradation assays in reticulocyte lysates (Ecker et al., 1987b). The conservation of ubiquitin is highly suggestive of an essential role for each residue in the folding, stability and/or function of ubiquitin in mammalian systems. Prokaryotes are largely devoid of ubiquitin with the exception of *Anabconyaena variabilis*, a cynobacterium and archaebacteria which possess it ((Wolf et al., 1993) and Durner and Böger, 1995)); initial biochemical characterization of the former ubiquitin isoform demonstrates a remarkable similarity to eukaryotic ubiquitin with respect to conjugation (Durner and Böger, 1995). Ubiquitin genes exist as a multi-gene family consisting of three genes: UBA, UBB and UBC. The first arrangement encoded by the UBB and UBC genes consist of tandem repeats of an ubiquitin coding sequence in a spacerless head to tail arrangement with the terminal ubiquitin extended by one amino acid (Tyr in chicken, Val in human, (Dworkin-Rastl et al., 1984; Ozkaynak et al., 1984)).

Figure 1. Schematic representation of the UPP

Subsequent to the generation of a high-energy C-terminal thioester bond by an E1 enzyme, ubiquitin is covalently attached to a substrate protein (either a normal protein destined for degradation or a damaged or abnormally folded protein). Substrate recognition and subsequent chain assembly are mediated through the activities of E3 and E2 enzymes, respectively. Upon reaching the threshold length, ubiquitin chains direct the substrate to the proteasome where it is unfolded and directed into the central channel of the proteasome. Proteolytic activities lining the inner rings of the proteasome cleave the substrate into short peptides. DUBs (deubiquitinating enzymes) disassemble the ubiquitin chains to yield monomers and may also oppose trafficking to the proteasome by shortening ubiquitin chains or cleaving them off of substrates.



Gray et al, SAGEKE, 2003

Figure 2. Primary amino acid sequence alignment of the eukaryotic ubiquitin genes

The three amino acids differing between yeast and human ubiquitin are colored in blue. Amino acids varying between yeast and other ubiquitin isoforms are colored in green. The serine at position 57 in yeast and mammalian ubiquitin which is replaced by Alanine in other organism is underlined.

Human Ubiquitin: MQIFVKTLTGKTITLEVEPSDTIENVKA
Yeast Ubiquitin: MQIFVKTLTGKTITLEVESSDTIDNVKS
Barley Ubiquitin: MQIFVKTLTGKTITLEVESSDTIDNVK
Oat Ubiquitin: MQIFVKTLTGKTITLEVESSDTIDNVK
Soya-bean Ubiquitin: MQIFVKTLTGKTITLEVESSDTIDNVK
Arabidopsis Ubiquitin:MQIFVKTLTGKTITLEVESSDTIDNVK

Human Ubiquitin: KIQDKEGIPPDQQRLIFAGKQLEDGRTL
Yeast Ubiquitin: KIQDKEGIPPDQQRLIFAGKQLEDGRTL
Barley Ubiquitin: KIQDKEGIPPDQQRLIFAGKQLEDGRTL
Oat Ubiquitin: KIQDKEGIPPDQQRLIFAGKQLEDGRTL
Soya-bean Ubiquitin: KIQDKEGIPPDQQRLIFAGKQLEDGRTL
Arabidopsis Ubiquitin:KIQDKEGIPPDQQRLIFAGKQLEDGRTL

Human Ubiquitin: SDYNIQKESTLHLVLRLRGG
Yeast Ubiquitin: SDYNIQKESTLHLVLRLRGG
Barley Ubiquitin: ADYNIQKESTLHLVLRLRGG
Oat Ubiquitin: ADYNIQKESTLHLVLRLRGG
Soya-bean Ubiquitin: ADYNIQKESTLHLVLRLRGG
Arabidopsis Ubiquitin ADYNIQKESTLHLVLRLRGG

Polyubiquitin genes are universally expressed although the numbers of repeats vary between species; a constant number of three ubiquitin coding sequences encoded by the UBB gene in all mammals and a varying number of repeats encoded by the UBC gene (6 to 11 for human, 10 to 12 for chimpanzee, 8 for gorilla, and 10 for orangutan). The UBA transcripts belong to the second arrangement consisting of linear fusions of N-terminal ubiquitin proteins to ribosomal subunits 50 and 80 respectively ((Redman and Rechsteiner, 1989), Redman and Rechsteiner, 1988; Baker and Board, 1991)). The functional relevence of this fusion is poorly defined; one hypotheses stipulates that the attached ubiquitin moiety may serve as a molecular chaperone in the folding of the ribosomal subunits (the rapid folding of the ubiquitin moiety may prevent the illicit degradation of the ribosomal subunit, (Finley et al., 1989)). The expression of the ribosomal subunit independly of ubiquitin results in a reduced growth rate of yeast and in ribosomal derangements despite the short lifetime of the fusion protein (Finley et al., 1989). N-terminal linear fusions of ubiquitin with actin (an integral cytoskeletal protein) and the acidic ribosomal protein P1 have been identified in the *Chlorarachniophyte* algae (Archibald et al., 2003). Again, the functional significance of these fusions remains poorly defined; it has been speculated that ubiquitin may be involved in the proper assembly of ribosomes (much like in higher eukaryotes) or in an indirect role in cytoskeleton rearrangements (ubiquitin may serve in activating the actin monomers thereby regulating the available pools for microfilament assembly (Archibald et al., 2003). Whereas ribosomal fusions are constitutively expressed as part of the housekeeping genes in all cells, the tandem repeats of ubiquitin provided by the UBB and UBC genes can be induced in response to starvation and elevated temperatures attributed to the presence of an upstream region with strong homology to the consensus

'heat shock box' nucleotide sequence found in the promoters of many stress inducible eukaryotic genes (Ozkaynak et al., 1987). Not surprisingly, one of the best promoters identified to date for the tissue-independent transcription of transgenes is derived from the human UBC gene (Schorpp et al., 1996). Both types of fusions are subject to co-translational proteolytic processing by enzymes with ubiquitin cleaving activity, UBPs (Baker et al., 1992) and UCHs (Larsen et al., 1998) yielding functional ubiquitin moieties that can participate in cellular processes (discussed in further detail below).

The functional constraints of the ubiquitin monomer

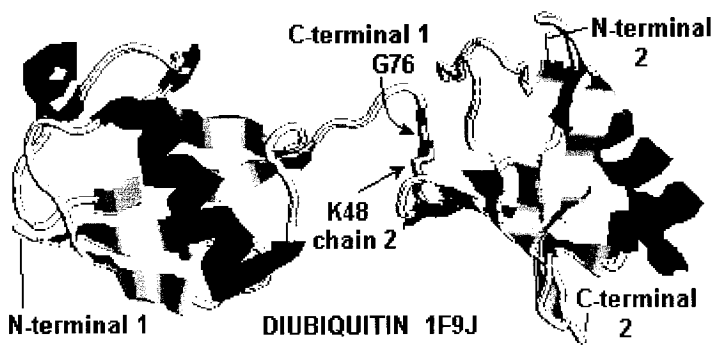
Crystallographic data on the ubiquitin monomer resolved at 1.8Å (Vijay-Kumar et al., 1987) have revealed that the protein can functionally be separated into two major domains; the globular and highly compact N-terminal domain which encompasses amino acids 1-69 and the more flexible and protruding C-terminus consisting of only six amino acids (70-76) (depicted in Figure 3). The structure of the ubiquitin monomer is reminiscent to a lollipop in cartoon form and as such is often referred to as the "ball and stick" protein. Ubiquitin has a mixed α/ß structure, with 5 ß-strands and an amphipatic 3.5-turn α-helix intercalated across the ß-sheet (Vijay-Kumar et al., 1987). Seven reverse turns and a short 3_{10} helix represent approximately one third of the total secondary structure required for the arrangement of the hydrophobic core (Vijay-Kumar et al., 1987). The majority (~90%) of the polypeptide chain is involved in hydrogen bonding interactions that maintain secondary structure providing a structural basis for ubiquitin's uncommonly high chemical and thermal stability. Ubiquitin is extremely heat resistant and difficult to unfold even in the presence of strong reducing agents; as such it has become standard procedure to autoclave membranes that are subject to analysis with ubiquitin specific antibodies

Figure 3. Schematic representation of ubiquitin

A) The structure reveals a compact N-terminus composed of a mixture of α-helices and β-strands (depicted by ribbons) and the protruding flexible C-terminus which lacks a distinct conformation. B) Schematic diagram of a di-ubiquitin chain assembled through lysine 48 of one ubiquitin moiety to the glycine 76 residue of the other. The N-and C-terminal domains of both ubiquitin moieties are indicated by 1 and 2 respectively. Lys48 (K48) and Gly76 (G76) residues partaking in the conjugation step are also indicated.

A

B



http://www.chembio.uoguelph.ca/educmat/chm736/degradat.htm

especially those pertaining to tissues (Mimnaugh et al., 1999). The N-terminus of ubiquitin can be extensively modified without drastically impairing the biological activity. This attribute has been used advantageously in appending epitope tags to ubiquitin with the objective of facilitating the detection and/or recovery of ubiquitin and ubiquitinated proteins. Ubiquitin has been produced with an amino-terminally fused HA tag (Treier et al., 1994), myc tag (Ellison and Hochstrasser, 1991; Ward et al., 1995), hexahistidine tag ((Treier et al., 1994), (Ward et al., 1995), (Beers and Callis, 1993)) as well as with glutathione S-transferase (GST, (Scheffner et al., 1993); it is rather remarkable that the GST moiety, considerably larger size than ubiquitin itself, does not interfere with its conjugation.

The N-terminal globular domain

The acidic and basic amino acid side chains are found on the surface of ubiquitin including those of ubiquitin's seven lysine residues (K6, K11, K27, K29, K33, K48, K63) two of which (K48 and K63) have been subject to intensive investigation. Physiological roles have been attributed to K29, K48 and K63 while roles for the remaining lysines have only been suggested based on information extrapolated from studied in an in vitro artificial setting (*in vitro* assembly of poly-Ub chains). Paradoxically, most roles of ubiquitin are dependent on the assembly of polyubiquitin chains (rather than mono-ubiquitin) of either a zigzag topology conferred by G76-K48 linkages or of a non-conventional linear topology assembled through K63 of ubiquitin. The most characterized function of ubiquitin is in intracellular protein degradation, a process in which ubiquitin behaves as a molecular tag to target cellular proteins for elimination. Site directed mutation of the K48 of ubiquitin has been shown to affect cellular growth in yeast (growth arrested in late G2 or M phase of the cell cycle) and leads in lethality when representing the sole source of ubiquitin (Finley et

al., 1994) highlighting the importance of K48 linkages in the efficient targeting of cellular proteins. Yeast cells expressing K48R chain-terminating ubiquitin exhibit a deficit in the turnover of short-lived cellular proteins and are sensitive to protein damaging agents presumably by a mechanism involving the interference of exogenous ubiquitin with the assembly of targeting signals on key proteins ((Arnason and Ellison, 1994) and (Finley et al., 1994)). The expression of K63R mutant ubiquitin results in defects in the error prone DNA repair pathway and in an enhanced sensitivity to UV induced stress ((Arnason and Ellison, 1994) and (Spence et al., 1995)). The crystal structure of the ubiquitin monomer reveals that the side chains of K6, K33 and K63 are solvent-exposed, while the side chains of K11, K27, K29 and K48 participate in hydrogen bonds (Vijay-Kumar et al., 1987). K27 is the least exposed Lys residue on the surface of the ubiquitin monomer and analogously to K33 has not been implicated in ubiquitin-ubiquitin conjugation ((Arnason and Ellison, 1994) and (Spence et al., 1995)). Yeast cells expressing K27R or K33R as the sole source of ubiquitin exhibit a diminished growth rate (Spence et al., 1995); the basis for this deficiency is unclear. The expression of K6R and K11R has not been shown to affect viability in yeast (Spence et al., 1995) or engage in the formation of polyubiquitin chains (Arnason and Ellison, 1994). Both lysines have been shown however to be sites of ubiquitin attachment in vitro (Baboshina and Haas, 1996) and K6 linked polyubiquitin chains have recently been reported in mammalian cells where they serve a role in DNA repair (Morris and Solomon, 2004). Lys 29 polyubiquitin chains have been noted in yeast which serve in proteolytic targeting of substrates to the 26S proteasome (Johnson et al., 1995) by utilizing an alternative system referred to as the ubiquitin fusion degradation pathway (UFD will be discussed below). Such K29 polyubiquitin chains are not as

abundant (Spence et al., 1995) as the previously described G76-K48 linkages however the expression of K29R as the sole source of ubiquitin in yeast results in growth defects (Spence et al., 1995). Again, the basis for this deficiency is currently unclear. The globular domain of the ubiquitin monomer contains two highly conserved residues (H68 and Y59) of undefined importance; the hydroxyl group of Y59 participates in hydrogen bonding and confers stability to the molecule (Vijay-Kumar et al., 1987). A mutation of both these residues independently has been shown to result in a decrease in solubility (Y59F and H68K) of the ubiquitin monomer with defective degradation abilities (70% and 30% of wt ubiquitin respectively) as assessed *in vitro* (Ecker et al., 1987b). The expression of H68K mutant ubiquitin in yeast as the sole source of ubiquitin results in impaired growth rate presumably due to the loss of contact with the E1 ubiquitin activating enzyme (Ecker et al., 1987b).

Three hydrophobic side chains (L8, I44 and V70) are exposed at the surface of the ubiquitin monomer; they form a patch on the surface of functional significance for proteolytic targeting and mutations in these residues (L8A or L8A/I44A) abrogates their recognition by the proteasome (despite their ability to form a K48-linked polyubiquitin chain, (Beal et al., 1996)). The acidic and basic side chains of ubiquitin's surface are largely confined to distinct patches of the molecule whose functional significance remains unclear for the acidic residues (Vijay-Kumar et al., 1987). A subset of the basic residues restricted to these distinct patches (R54 and R42) have been shown to be of importance for the recognition of ubiquitin by the E1 activating enzyme as assessed by in vitro conjugation assays with R54L and R42L mutants (Burch and Haas, 1994).

The C-terminus of ubiquitin

The protruding C-terminus of ubiquitin consisting of only six amino acids (LRLRGG) is a more extended and flexible domain containing information pertaining to the attachment of ubiquitin to target proteins. The highly conserved C-terminal G76 residue of ubiquitin is indispensable for signaling by mono- and poly-ubiquitin chain assembly and yeast cells expressing C-terminal ubiquitin variants are growth impaired (Hodgins et al., 1992; Hicke and Dunn, 2003). The expression of ubiquitin derivatives either substituted at position 76 (G76A, (Butt et al., 1988) and (Hodgins et al., 1992)) or GG deletion (desGG, cited in (Arnason and Ellison, 1994) mutant results in severe growth defects reminiscent to the ones observed in cells expressing K48 chain-terminating version of ubiquitin. Scanning alanine mutagenesis of yeast ubiquitin revealed the existence of severe sequence constraints on the carboxyterminal region with most alterations resulting in conjugation-deficient ubiquitin derivatives that have lost their ability to contact components of the UPP (Sloper-Mould et al., 2001). *In vitro* activation and degradation assays performed with variants of yeast ubiquitin have revealed that site directed mutations/deletions in the stick region of ubiquitin (R72S, R72A, DL73) and in the region upstream of the tail (L67D, L69D) resulted in the loss of interaction with the E1 enzyme and the ability of mutant ubiquitin to participate in degradation of test substrates ((Ecker et al., 1987b) and (Burch and Haas, 1994)).

1.3.2 The conjugating system

In addition to the requirement for poly-ubiquitin chain assembly in the efficient targeting and degradation of protein substrates ancillary factors associated with the UPP have been identified and currently referred to as the "the components of the UPP". The targeting and

degradation of cellular proteins is a hierarchical multi-step cascade of enzymatic reactions, some of which require the hydrolysis of ATP.

E1 ubiquitin-activating enzyme

The initial reaction involves the activation of ubiquitin by an E1 ubiquitin activating enzyme on the highly conserved G76 residue of ubiquitin in a reaction that requires the hydrolysis of ATP and proceeds via an E1-ubiquitin-adenylate intermediate which serves as a donor of ubiquitin to an active sulfhydryl group of E1 to form a thiol ester bond (Ciechanover et al., 1981). The E1 enzyme can accommodate two distinct ubiquitin moieties which presumably ensure the activation of multiple ubiquitin derivatives by a single E1. The E1 enzyme in eukaryotes exists in two alternatively spliced isoforms encoded by a single gene with the shorter form (E1A) residing in the cytoplasmic compartment and the longer isoform (E1B) in the nucleus (Shang et al., 2001). It has recently been proposed that the nuclear localization of E1B is dependent upon its prior phosphorylation (Stephen et al., 1997). The degradation of cellular proteins has long been believed to occur exclusively in the cytoplasm however the concept of nuclear degradation has been recently entertained with the identification of proteins whose destruction takes place in the nuclear compartment (typified by the degradation of the differentiation factor MyoD, (Floyd et al., 2001)). Deletion studies in yeast have demonstrated the indispensability of the E1 enzyme in ubiquitin metabolism; lethality of yeast ensues upon elimination of E1 ((McGrath et al., 1991) and (Dohmen et al., 1995)) although temperature sensitive mutants have been generated in both yeast and higher eukaryotes and have served admirably in deciphering the enzymology of the UPP and the effects of E1 depletion (Ciechanover et al., 1984; Finley et al., 1984; Ciechanover et al., 1985).

E2 ubiquitin conjugating enzymes

The activation of ubiquitin is followed by the conjugation to an internal lysine of a protein substrate, a function requiring the activity of an E2 ubiquitin conjugase, in a transthiolation reaction involving the transfer of the activated ubiquitin moiety from the active cysteine in E1 to a conserved cysteine in E2. Unlike the E1 enzyme, a growing family of conjugases have been identified all bearing a highly conserved catalytic domain of about 160 amino acids (the UBC domain) which contains an active site cysteine (reviewed in (Jentsch et al., 1990)). This cysteine accepts ubiquitin from E1 to form a thiol ester and mutational analysis has demonstated that the substitution of this cysteine abolishes E2 conjugase activity ((Sung et al., 1991), (Sommer and Jentsch, 1993), (Seufert et al., 1995)). A suggested motif rich in basic residues found at the N-terminus of the UBC domain has been proposed to serve as a binding site for the E1 enzyme. E2 conjugases can be of the simple (class I) or complex type (class II or class III); in the former, the UBC domain is the only active motif and such conjugases are inefficient in transferring ubiquitin to protein substrates but rather require the presence of an E3 ubiquitin ligase. The *S. cerevisiae* UBC4 and UBC5 as well as UBC1 conjugases of *Arabidopsis thaliana* are examples of this class of E2s, and are known for their important role in the ubiquitination of many short-lived and abnormal proteins ((Seufert and Jentsch, 1990) and (Seufert et al., 1990)). In the latter, the E2 conjugases contain, in addition to the UBC domain, a C-or N-terminal extension with the former consisting of a stretch of acidic amino acids. The UBC2 conjugase (otherwise referred to as RAD6) of S.cerevesiase appears to mediate interaction with basic histones via its C-terminal extension (Sullivan and Vierstra, 1991). The putative role of histone ubiquitination by RAD6 is poorly defined but has been speculated to result

in DNA repair rather than proteolysis ((Hoege et al., 2002), (de Padula et al., 2004), (Xiao et al., 2000)). Other C-terminal extensions appear to be involved in E2 localization as exemplified by UBC6 of S. cerevisiae (Yang et al., 1997). The 95 amino acid C-terminal extension includes a hydrophobic anchor-signal that enables the enzyme to incorporate into the ER membrane where it's involved in the endoplasmic reticulum protein degradation pathway (ERAD pathway, (Yang et al., 1997)). A unique amino-terminal extension of approximately 40 amino-acids has been identified in UbcH6 (Kimura et al., 1997) and more recently in UbcH9, (Ito et al., 1999) a human E2 conjugase with high structural homology to human UbcH4 and UbcH5 and yeast UBC4 conjugases. The functional significance of this extension is presently unknown.

E3 ubiquitin ligases

The most diversified class of enzymes associated with the UPP are the E3 ubiquitin ligases which are thought to confer substrate specificity to the UPP pathway. They either assist E2 conjugases in the final step in the UPP pathway in which the activated ubiquitin is transferred from E2 to the ε-amino group of a lysine residue in the potential substrate thus forming an isopeptide bond (RING/U box containing E3 ligases and complex E3s) or they form a thiol ester directly with ubiquitin (HECT domain E3 ligases). The mechanism by which E3 ligases confer such selectivity is largely unknown however they employ protein interaction domains to impart substrate specificity.

RING/U box and complex E3 ligases

This subtype of E3 ligases provide a scaffold to direct proper interaction of E2 conjugases with the target substrate without directly participating in the transfer of ubiquitin to the substrate. The RING motif of the RING containing E3 ligases is maintained by eight Cys and His residues and chelates two zinc ions whereas the ring in the U box is maintained by

intramolecular interactions other than zinc chelation. This family of ubiquitin ligases include the SCF, VBC-Cul2 and the anaphase promoting complex (APC) (reviewed in (Hatakeyama and Nakayama, 2003)).

HECT domain E3 ligases

These E3 ligases have been found to form thiol ester bonds with ubiquitin (a property originally assigned to E2 conjugases) by utilizing a C-terminal domain (referred to as the HECT domain for "homology to E6-AP carboxy terminus"), which is a highly conserved region with strong similarity to the UBC domain found within E2 ubiquitin conjugases. In addition, HECT domain E3 ligases contain an N-terminal extension which is involved in defining substate specificity. E6-AP was the first identified member of a family of novel HECT domain E3s which can form thiol esters with ubiquitin and acts as a donor in the target ubiquitination of the tumor suppressor p53 following its association with the E6 oncoprotein derived from the human papilloma virus (HPV16, (Huibregtse et al., 1991; Scheffner et al., 1993)). The association of E6 and p53 with E6-AP is mediated through the N-terminal extension in E6-AP(Huibregtse et al., 1993). Similarly, the N-terminal extension of Nedd4, an E3 ubiquitin ligase involved in the regulation of the epithelial sodium channel (ENAC) has been shown to contain a structural motif (referred to as the WW domain) that is important for this association ((Fotia et al., 2003), (Harvey et al., 1999) and (Abriel et al., 1999)). Similar proteins to E6-AP have been identified in yeast (RS5p/Npi1p and Pub1) and are involved in ubiquitination of the cell surface yeast uracil permease ((Hein et al., 1995) and cdc25, a mitotic activating tyrosine phosphatase respectively (Nefsky and Beach, 1996). Of interest the E6-AP E3 ubiquitin ligase is mutated in Angelman syndrome, an imprinted genetic disorder (Kishino et al., 1997).

E4 ubiquitin elongation factor

More recently a polyubiquitin chain elongation factor (E4 or otherwise known as UFD2 in yeast) has been identified whose putative role is to elongate the polyubiquitin chain (attach successive ubiquitin moieties to the previously attached ubiquitin (Koegl et al., 1999). Yeast cells lacking this factor do not exhibit a growth deficiency (Johnson et al., 1995) under normal metabolic conditions and as such the E4 factor is speculated to exhibit specificity for only a few selective substrates under conditions of cellular stress ((Koegl et al., 1999).

1.4 The degradation sequences

N-rule pathway of protein degradation

It is clear that the recognition of protein substrates by ubiquitin is dependent on the concerted action of enzymes constituting the conjugating system. Elegant work from Alexander Varshavsky, a pioneer in ubiquitin research, in 1986, demonstrated the existence of a strong correlation between the half-life of a protein substrate and the amino acid identity of its N-terminal residue (reviewed in (Varshavsky, 1997)). This observation gave rise to the N-end rule model of protein degradation whereby one could generally predict the lifespan of a protein based on the amino acid sequence of its N-terminal residue. Whereas proteins with N-terminal amino acids consisting of Val, Ser, Cys, Met, Ala, Thr and Gly were found to have the longest half-lives (over 20 hours), proteins with amino-terminal Leu, Phe, Trp, Asp, Asn and Arg exhibit the shortest half life (2 or 3 minutes). Proteins with amino-terminal Ile, Glu, Tyr, Gln and His were found to have variable half-lives ranging from 10 to 30 minutes. The requirement of the presence of one or more lysine residues in close proximity of the N-terminus for efficient ubiquitination has suggested the signal be bipartite (Bachmair and Varshavsky, 1989). One part of the signal may be

regarded as the primary determinant that is directly recognized by respective E2 or E3's (rad6/N-recognin in yeast and rad6/E3α in higher eukaryotes) whereas the other would serve as an attachment site where ubiquitin can be covalently appended. The mechanism that couples recognition of the N-terminal amino acid and the protein's half-life remains poorly defined but it is interesting to note that the N-end rule applies to bacteria even though they do not contain ubiquitin (Tobias et al., 1991).

The destruction box

The most characterized degradation signal is the destruction box (D box) originally identified in mitotic cyclins. It consists of a 9 amino acid sequence (RXALGXIXN) with a highly conserved Arg and Leu found in all cyclins with the exception of cyclin B3 in which Leu is replaced by Phe. Mutations affecting the D-box have been shown to stabilize cyclins by reducing their ability to be efficiently recognized and ubiquitinated by the APC complex (an E3 ubiquitin ligase complex involved in the degradation of cyclins, (King et al., 1996), (Sudakin et al., 1995), (Kobayashi et al., 1992);(Lorca et al., 1992), (Glotzer et al., 1991)). The cyclin B destruction box is a portable element and the addition of its D box to irrelevant proteins has been shown to result in their rapid degradation ((Glotzer et al., 1991), (Amon et al., 1994), (Brandeis and Hunt, 1996), (Yamano et al., 1996)). Recent studies have demonstrated that a stretch of 27 amino acid residues as well as the presence of at least one lysine residue (no particular one was essential or preferred) in the protein is required in the efficient proteolysis of cyclin B (King et al., 1996).

Other signals

Unlike B cyclins, the D-box present in cyclin A does not behave as a transportable module ((Klotzbucher et al., 1996) and (King et al., 1996)). This highlights the stringent regulation of the UPP and the requirement of ancillary proteins or post-translational modifications to

result in efficient degradation. As such, intracellular protein substrates can be recognized by the UPP components following the unmasking of motifs within the protein; prior phosphorylation or dephosphorylation ((Ganoth et al., 2001), (Musti et al., 1997), (Chen et al., 1995), (Mitsui and Sharp, 1999)), acetylation/deacetylation ((Rausa et al., 2004), (Giandomenico et al., 2003; Butcher et al., 2004; Jin et al., 2004)) sumoylation ((Ghioni et al., 2005), (Floyd and Stephens, 2004)), hydroxylation (Jaakkola et al., 2001), glycosylation ((Yoshida et al., 2002) and (Shimura et al., 2001)) and oxidation (Pacifici et al., 1993) and recently reviewed in (Iwai, 2003)) have all been shown to reduce or increase the life of proteins that would otherwise be short or long-lived. A signal could be hidden as part of a protein-protein interaction and following the removal of the masking protein the substrate can efficiently be ubiquitinated. Conversely, the substrate may require prior association with an ancillary protein to entice components of the UPP. Other signals include partially unfolded or mutated proteins that are prone to ubiquitination and degradation (reviewed in (Goldberg, 2003)). Such reversible masking degradation motifs add a level of complexity to the UPP and appear to be involved in the regulation of the protein levels of many transcription factors.

The PEST sequence

In contrast to mitotic cyclins, G1 cyclins do not contain a destruction box but rather a region highly enriched in proline, glutamic acid, serine and threonine residues (reviewed in (Rechsteiner and Rogers, 1996)). This amino acid signature constitutes a motif referred to as a PEST sequence which has been identified in a variety of short-lived proteins (reviewed in (Rechsteiner and Rogers, 1996)). A PEST sequence has been identified in the transcription factor Gcn4p (reviewed in (Irniger and Braus, 2003)) involved in the activation of amino acid and purine biosynthesis genes in yeast and in the p105 precursor

of the p50 subunit of NF-κB ((Wulczyn et al., 1998) and (Lin et al., 1996)). The PEST sequence is not a portable module in the case of Gcn4p; PEST sequences derived from other proteins have however been successfully appended to mammalian proteins (example Luciferase and EGFP, (Li et al., 1998)) which serve to enhance their proteolysis (Rechsteiner and Rogers, 1996). This is highly indicative of the contextual significance of the PEST domain.

The Ubiquitin Fusion Degradation pathway

Unlike the poly-ubiquitinated conjugates assembled though Lys48 linkages, linear Ub derivatives are formed as translational products of natural or engineered Ub gene fusions (Bachmair *et al.*, 1986; Finley *et al.*, 1987, 1989). The processing of ubiquitin fusion proteins of the latter type is reliant on enzymes with ubiquitin cleaving activity which recognize and cleave at the Gly-Met boundary of adjacent ubiquitin moieties. Fusions of N-terminal ubiquitin moieties with mutations in their C-terminal G76 which preclude their processing are directed for proteolysis as a consequence of the ubiquitin fusion degradation pathway (UFD system). As such, linear fusions of ubiquitin with heterelogous proteins have been expressed in cultured mammalian cells to test the hypothesis that fusions that escape deubiquitination would have shorter half lives as a consequence of this pathway (Stack et al., 2000) or to study proteasomal function in living cells ((Dantuma et al., 2000) and (Luker et al., 2003)). Contrary to the conventional G76-K48 linkages, the UDF based system utilizes primarily K29 linked polyubiquitin chains as degradation units. By these measures, ubiquitin itself can serve as a degradation motif (degron) when appended at the N-terminus of heterologous proteins.

1.5 The complexity of the 26S proteasome

The 26S proteasome (depicted in Figure 4) is a ubiquitously expressed, ATP-dependent cytoplasmic and nuclear multi-subunit enzyme protease charged with the elimination of cellular protein substrates. Many short-lived and long-lived proteins are subject to complete proteolysis under normal metabolic conditions, whereas others are only partially processed (e.g. the transcription factor NF-κB) resulting in their activation. Among the key regulatory proteins degraded by the 26S proteasome are the transcription factor c-Fos (Tsurumi et al., 1995), c-jun ((Treier et al., 1994; Tsurumi et al., 1995), p300/CBP acetyltransferases ((Iwao et al., 1999) and (Miyake et al., 2000)), c-mos (Ishida et al., 1993), cyclins ((Glotzer et al., 1991) and (Sudakin et al., 1995)) and cyclin dependent kinase inhibitors ((Alessandrini et al., 1997) and (Tam et al., 1997)) as well as a variety of oncoproteins and tumor suppressors (Rb, (Wang et al., 2001), E2F-1, (Hateboer et al., 1996) and p53 (Ciechanover et al., 1994) just to name a few). The 26S proteasome has a molecular mass of approximately 2.5 MDa and can functionally be divided in a core (20S) particle and a cap (19S) which can further be subdivided into a base and a lid component (reviewed in (Pickart and Cohen, 2004)). Each of these self-compartmentalizing proteases is assembled from a barrel shaped 20*S* core particle in which the proteolytic activity is sequestered, and one or two 19*S* regulatory particles that cap the ends of the 20*S* particle. While most eubacteria lack a 20S or 26S proteasome, they are enriched in a primitive type of 20S proteasome consisting of two different subunits (*alpha* and *beta*). All eubacteria appear to contain Clp proteases which are analogous to the 26S proteasome and consist of a proteolytic core structure (two heptameric rings of subunit ClpP) and ATPase subunits (ClpA), which are part of the AAA superfamily of ATPases (Katayama et al., 1988), (Kessel et al., 1995).

The 20S core particle

The 20S proteasome complexes (~700 kDa) of eukaryotes contains up to 28 different subunits consisting of two copies of 7 distinct α and 7 distinct ß-type subunits (reviewed in (Hilt and Wolf, 1995)). The structural rearrangement of the eukaryotic proteasome is such that the α subunits constitute the two outer layers whereas the ß subunits form the 2 inner rings of the structure [(a1-a7)(b1-b7)(b1-7)(a1-a7)], ((Schauer et al., 1993) and (Kopp et al., 1993)). Three distinct proteolytic activities have been associated with the 20S proteasome; the trypsin-like, the chymotrypsin-like, and the post-glutamyl peptidyl hydrolytic (PGPH) activities which cleave after basic, large hydrophobic and acidic residues respectively (Orlowski et al., 1993). Two additional specificities induced by gamma interferon have been determined for mammalian proteasomes, BrAAP and SNAAP activities, which cleave branched-chain amino acids and between small neutral amino acids respectively (Orlowski et al., 1993). The proteins that constitute the activities of the 20S proteasome as well as the catalytic ß-type subunits are synthesized as inactive proenzymes which are processed to their mature forms upon assembly into the 20S proteasome core (reviewed in (Schmidt and Kloetzel, 1997)). The active sites are sequestered in the catalytic compartment of the 20S proteasome in which access is blocked by the presence of the 19S regulatory particle. The structural organization of the 20S core particle is the basis of how arbitrary degradation of cellular proteins is avoided and why the proteasome is inactive in the proteolysis of properly folded proteins. The assembly of the 19S regulatory complex at one or both ends of the 20S proteasome was shown to result in the formation of pores at the ends of the 20S proteasome particle through which the substrates can be fed into the active sites. The 20S proteasome has been shown to have proteolytic cleavage towards denatured or oxidized proteins (without requirement for ATP, (Shringarpure et al.,

2003)) while the efficient degradation of the 26S proteasome necessitates the hydrolysis of ATP (reviewed in (Pickart and Cohen, 2004)).

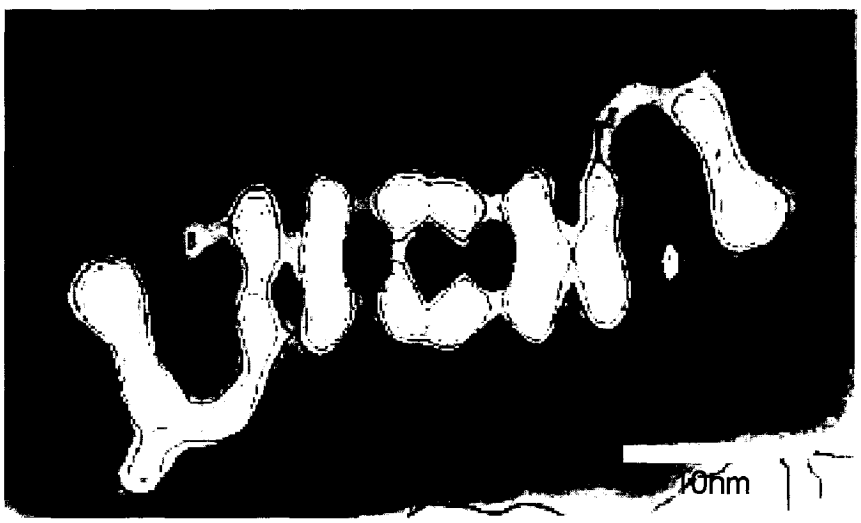
The 19S regulatory particle

The 19S complex (depicted in Figure 4) is composed of a lid and a base that caps the ends of the 20S proteasome; the base contains six ATPases and three non-ATPases (Rpn1,2 and 10) while the lid is composed of eight non-ATPase subunits (Rpn3,5,6,7,8,9,11 and 12) that serve in the recognition of ubiquitinated substrates (Glickman et al., 1998) and reviewed in (Hilt and Wolf, 1995)). The putative role of the base is one of a molecular chaperone involved in the unfolding and translocation of protein substrates into the catalytic chamber of the 20S particle in a reaction that requires ATP (Braun et al., 1999). The subunit Rpn10/S5a/Mcb1 has been shown to have affinity for polyubiquitin chains however it is dispensable in yeast as demonstrated by deletion experiments (van Nocker et al., 1996). This suggests that the recognition ability of the 26S proteasome is far more complex than the polyubiquitin signal. It has become evident that certain proteins can be degraded by the 26S proteasome in the absence of a polyubiquitin chain as typified by the degradation of ornithine decarboxylase whose degradation is antizyme-dependent and ubiquitin-independent (Li and Coffino, 1992; Murakami et al., 1992). Recently the 19S ATPase S6' (otherwise referred to as Rpt5) has been shown to contact the polyubiquitin chain in an ATP dependent fashion (Lam et al., 2002). Elegant work from Cecile Pickart has demonstrated that a threshold length of four ubiquitin moieties is the minimal targeting signal for proteolysis and that the ubiquitin moieties at position two and four contact the 19S particle in a reaction that is dependent on hydrophobic interactions (Thrower et al., 2000). Mutations in a hydrophobic patch of the globular domain ($Leu^{8}/Ile^{44}/Val^{70}$) of the ubiquitin monomer have been shown to interfere with the targeting of substrates

Figure 4. Structural organization of the eukaryotic 26S proteasome

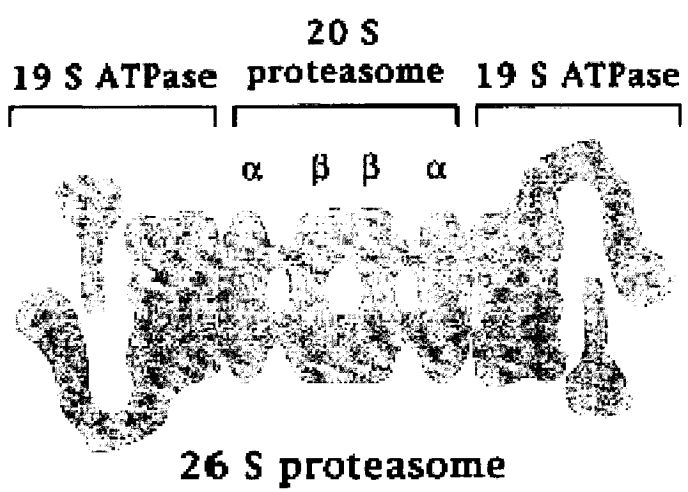
A) Electron micrograph of the 26S proteasome isolated from rat liver. B) Schematic representation of the 26S proteasome. The particle consists of a 20S core capped at either end by a 19S regulatory particle. The base and lid components that constitute the 19S regulatory element are indicated by horizontal bars.

A



Yoshimura et al, 1993

B



Kessel et al, 1995

to the 26S proteasome (Beal et al., 1996). The proteasome trims the substrates into peptides that are further degraded into single amino acids serving in the *de novo* synthesis of proteins.

The regulation of proteasome subunits by gamma interferon

Three additional ß-type subunits of mammalian proteasomes have been identified: LMP2, LMP7 and MECL1 which are inducible by gamma interferon (reviewed in (Groettrup et al., 2001)). The functional relevance of this induction pertains to the role of the proteasome (in this case the immuno-proteasome) in the intracellular processing of peptides to be presented on MHC class I molecules during an immune response. These inducible subunits replace three ß-subunits of the 20S core particle and serve to generate peptides of an appropriate length to be presented at the surface of the cell. Two proteasome interacting proteins, also induced by interferon (REGα and REGß) have been shown to assemble in a ring-shaped hexamer and constitute the 11S regulator (otherwise known as PA28, (Song et al., 1997)). The 11S regulator assembles with a 20S core particle in a manner that is reminiscent to the traditional rearrangement of the 26S proteasome (PA28-20S-PA28) and has been shown to digest peptides rather than ubiquitin-conjugated structures (Song et al., 1997). It has been speculated that this type of proteasomal re-arrangement acts downstream of the conventional 26S particle to further trim peptides released by the latter. The report of an asymmetrical, chimeric proteasome (19S-20S-PA28) has suggested that this process be coupled (Hendil et al., 1998).

1.6 Ubiquitin recycling enzymes

The efficient targeting and degradation of cellular protein structures relies on the presence of a targeting motif which is afforded by the presence of a threshold polyubiquitin chain

assembled though G76-K48 linkages. Although the degradation of wild-type ubiquitin has in some cases been reported (when it is lured into the proteasome by the substrate, (Hanna et al., 2003)), it generally escapes proteolysis and is recycled by the cell. Upon substrate degradation the polyubiquitin chain is disassembled to yield functional monomeric ubiquitin moieties and the concerted action of two types of enzymes mediate the release of the polyubiquitin signal. One is an inherent proteasome-bound deubiquitinating enzyme (Doa4p, (Papa et al., 1999)) that removes the attached remnant of the substrate from the C-terminus of ubiquitin. Yeast cells expressing dominant-negative Doa4 mutants accumulate polyubiquitin chains (with attached remnants), demonstrate impaired proteolysis and altered ubiquitin pools (Swaminathan et al., 1999). The removal of the remnant is an absolute requirement in the further processing/disassembly of the polyubiquitin chain; cytosolic enzymes can discriminate between un-anchored (no substrate) and chains modified or blocked by a substrate or a protein remnant (Wilkinson et al., 1995). The basis for this discrimination may be to preclude premature disassembly of the polyubiquitin signal. The most characterized enzyme with such catalytic specificity is isopeptidase T that acts on the distal end of the ubiquitin chain (the end which was previously attached to the target protein, ((Wilkinson et al., 1995) and (Hadari et al., 1992)). The deletion of isopeptidase T ((Amerik et al., 1997) or mutations affecting the G76 residue of ubiquitin (which prevent disassembly, (Hodgins et al., 1992)) have deleterious effects on ubiquitin metabolism; mainly a phenotype of ubiquitin deficiency as a consequence of assembly of poly-ubiquitin chains that are refractory to disassembly by isopeptidases ((Amerik et al., 1997) and (Wilkinson et al., 1995)). Threshold unanchored poly-Ub chains, via their affinity for proteasomes, have been proposed to exhibit a dominant-negative effect on

proteolysis and their expression results in the accumulation of cellular protein substrates. A mechanistically related phenomena has been proposed to contribute to the pathogenesis of Alzheimer's disease wherein transcriptional frameshifting of the ubiquitin B mRNA generates an aberrant C-terminal ubiquitin moiety (termed Ubb^{+1}) with an inherent ability to inhibit the 26S proteasome ((Lindsten et al., 2002), discussed in detail in a subsequent section).

The regulation of the UPP by the antagonistic effects of deubiquitinating enzymes

Ubiquitin metabolism is counteracted by ubiquitin catabolism by the action of enzymes that serve in the disassembly/processing of polyubiquitin chains and precursor proteins. These enzymes have important roles in ubiquitin processing and recycling and it is not surprising that they regulate important biological processes such as growth and oncogenesis, differentiation, development, disease and ubiquitin pools. They can functionally be divided into two sub-types: ubiquitin carboxyterminal hydrolases (UCHs) and ubiquitin specific proteases (UBPs) both of which were identified based on their ability to hydrolyze carboxyterminal esters, thiol esters and amides of ubiquitin (Pickart and Rose, 1985).

The UCH family of enzymes

Whereas the yeast genome encodes a single UCH enzyme designated UBLH or YUH1, (Tobias and Varshavsky, 1991), mammalian genomes encode multiple UCH enzymes having different patterns of tissue-specific expression (Wilkinson et al., 1992) and substrate specificity (Larsen et al., 1996). UCHL3, the most characterized member of the UCH family of enzymes has been reported to be most abundant in hematopoietic tissues (Wilkinson et al., 1992), although a more recent study has found it to be highly enriched in heart and skeletal muscle (Wada et al., 1998). The crystal structure of UCHL3 has been

elucidated revealing a catalytic triad (consisting of a Cys-His-Asp) with strong similarity to cathepsin B, a family of papain thiol proteases (Johnston et al., 1997). The expression of UCHL1 (or PGP 9.5, as it is also known) is largely restricted to neurons and neuroendocrine cells, where it is remarkably abundant (reportedly comprising 1–2% of total brain soluble protein, (Thompson et al., 1983)). The physiological roles of UCH enzymes are still poorly understood, but there are suggestions from biochemical data (Larsen et al., 1998) and from crystallography (Johnston et al., 1997) that the UCH enzymes may be sterically inhibited by large substrates. The natural substrates of the UCH enzymes might, therefore, include the precursor forms of ubiquitin. UCHL1 and UCHL3 differ in their ability to process these fusions (Larsen et al., 1998). UCHL3 may also be involved in the processing of the ubiquitin-like protein NEDD8 (Wada et al., 1998), an activity that has not been attributed to UCHL1. Recent reports have suggested that UCHL-1 may in addition, be capable of promiscuously altering the ubiquitination status of cellular proteins (Imai et al., 2000). A number of mutations have been identified and have been associated with disease; polymorphism in the gene encoding the neuronal specific UCHL-1 protein has been shown to increase the susceptibility to familiar Parkinson disease (Wang et al., 2002); (Choi et al., 2004) and deletion of the gene in mice results in sensory and motor ataxia (Kurihara et al., 2001). UCHL-1 over-expression is readily observed in many primary lung cancers (56%, (Hibi et al., 1998)), and almost all lung cancer cell lines (96%, (Hibi et al., 1998)).

The UBP family of enzymes

Ubiquitin-specific processing proteases (UBPs), the second sub-type of enzymes with ubiquitin-cleaving activity serve to recycle ubiquitin from larger substrates and in the disassembly of both unanchored polyubiquitin chains and in the regulation of protein

stability by trimming the polyubiquitin signal. There are at least 16 UBPs that have been identified in yeast and at least 80 identified in *C.elegans, Aplysia, Drosophila*, mouse and human. These enzymes contain a catalytic domain of about 450 amino acids flanked by catalytic Cys and His domains (reviewed in (Soboleva and Baker, 2004) and (Fischer, 2003)). UBPs are rather larger in size than UCHs with a molecular mass varying between 50 and 250kDA. Their substrate specificities are largely unknown but given the number of such enzymes in mammalian cells, one can speculate that their roles are as diversified as their number. Some enzymes appear to be involved in removing chains on specific substrates typified in yeast by FAF (the deubiquitinating enzyme with substrate specificity towards Liquid facet, (Chen et al., 2002) and humans by HAUSP (deubiquitinating enzyme of the p53 tumor suppressor, (Li et al., 2002a)) although a recent report has proposed that the substrate is MDM2 (Li et al., 2004), whereas the deletion of others (UBPY) results in global proteolysis defects as assessed by the accumulation of cellular protein substrates (Naviglio et al., 1998). Other roles involve the regulation of transcription by the deubiquitination of histones (mainly H2A and H2B, discussed in a subsequent section, reviewed in (Osley, 2004)) and the disassembly of multiubiquitin chains from peptides still bound to the proteasome (reviewed in (Amerik and Hochstrasser, 2004)). Of interest, mutation of Ubp14 in mice, the functional homologue of isopeptidase T, has been shown to result in an ataxic like phenotype reminiscent to the phenotype observed in the pathogenesis of SCA-1 (Wilson et al., 2002). The net effect of these enzymes is to counteract the ubiquitin conjugation system and to edit polyubiquitin chains (whether in the cytoplasmic compartment or at the level of the proteasome) thereby rescuing substrates from destruction.

1.7 Some roles of ubiquitin in proteolysis

Endoplasmic reticulum associated degradation pathway (ERAD)

Proteins unable to fold correctly in the ER are extracted from the ER, via an inter-membrane channel (Sec61) for degradation in proteasomes associated with the cytoplasmic aspect of the ER membrane (Kopito, 1997), a process that has been designated ERAD. Membrane-associated proteins otherwise destined for the plasma membrane may be degraded by this route (Kopito, 1999; Yu and Kopito, 1999), which depends on the assembly of K48-linked chains on these substrates by ER membrane-associated ubiquitinating enzymes (Lenk et al., 2002). The failure of the UPP to extract misfolded proteins found within the ER compartment induces the unfolded protein response (UPR) resulting in the "shut-down" of translation (Shen et al., 2004).

Peroxisomal assembly

Ubiquitin-mediated proteolysis has been implicated in the biogenesis of peroxisomes whereby the functioning of peroxisome biogenesis factors (Pex18p and Pex21p) is accompanied by their rapid and obligatory degradation by the 26S proteasome resulting in efficient peroxisomal assembly (Purdue and Lazarow, 2001). This is the first report of the UPP requirement in the functional assembly of an organelle.

Regulation of proliferation

It is well documented that the dysregulation of the cell cycle results in the progression of tumorigenesis. The eukaryotic cell cycle is driven by the fluctuation of cyclin-dependent kinase (Cdk) activities, which in turn are regulated by the episodic synthesis and degradation of cyclins (their regulatory subunits) in a process dependent on the ubiquitin proteasome pathway. The destruction of cyclins through ubiquitin-mediated proteolysis

serves to inactivate Cdk activities and mediate uni-directional transitions in the cell cycle. As an example, the cyclin-dependent kinase inhibitor p27 (a UPP substrate) negatively regulates the activity of its cognate CDK2/cyclinE and CDK2/cyclinA kinase complexes to prevent illicit progression from the G1 to S phase transition of the cell cycle. The dysregulation of the protein levels of p27 is often associated with the progression of colorectal, prostate and breast cancers (Ciaparrone et al., 1998) ; Sgambato et al., 1997), (Cordon-Cardo et al., 1998).

Regulation of cell death pathways

Not unexpectedly, ubiquitin-mediated proteolysis is omnipresent in cellular pathways that result in cell death as typified by the degradation of the inhibitor of apoptosis molecules (IAPs) in response to apoptotic stimuli (Holley et al., 2002). The degradation of IAPs results in commitment to apoptosis, a cascade of events mediated by a class of cysteine proteases otherwise referred to as caspases (Holley et al., 2002). IAPs exhibit E3 ubiquitin ligase activity and serve in the ubiquitination of cell death proteins thereby influencing the the death potential of cells (Hu and Yang, 2003; Olson et al., 2003). Other regulators of cell death include (but are not limited to) bcl-2 (an anti-apoptotic molecule), IκK (regulator of NFκB transcription factor implicated in both apoptosis and survival pathways) and p53 all of which have the ability to affect cell death pathways and their degradation by the proteasome are well documented ((Ciechanover et al., 1991; Dimmeler et al., 1999) and (Ciechanover et al., 1994)). These simplified examples serve to highlight the importance of the UPP in regulating proliferation, cancer and cell death and suggest that mutations in ubiquitin or its components have the potential to result in disease.

1.8 Roles of ubiquitin in non-proteolytic cellular processes

K63-linked mono- and poly-Ubiquitin linkages

The formation of non-canonical polyubiquitin chains assembled via lysine 63 of ubiquitin has been shown to have a function in processes other than proteolysis. Linear chains assembled through K63 linkages play non-proteolytic roles in DNA repair signaling, receptor mediated endocytocis (all recently reviewed in (Sun and Chen, 2004)) and ribosome assembly (Spence et al., 2000). The role of K63 linked chains in ribosomal activity and assembly are poorly defined but it has been speculated that ubiquitin may serve a chaperone-like function in directing proper assembly of ribosomal subunits (Spence et al., 1995). The disruption of K63 linked polyubiquitin chains in yeast has been shown to sensitize cells to radiation-induced damage and in defects in the error prone DNA repair pathway ((Arnason and Ellison, 1994) and (Spence et al., 1995)). Recent reports have implicated K63 polyubiquitin chains in signaling of the NFkb transcription factor resulting in the activation of downstream genes (Deng et al., 2000b).

The mono-ubiquitin signal

Mono-ubiquitination has long been appreciated in transcriptional control whereby the attachment of a single ubiquitin to histone moieties modulates gene expression (Sun and Allis, 2002; Kao et al., 2004). Mono-ubiquitination serves as a signal in vesicular sorting (Katzmann et al., 2001; Waterman and Yarden, 2001), as a sub-cellular localization motif (a function shared by ubiquitin like molecules, (Plafker et al., 2004)) as well as in the internalization of membrane bound receptors (Haglund et al., 2002; Monsonego-Ornan et al., 2002; Dupre et al., 2003). The internalization of cellular receptors by mono-ubiquitination has long been appreciated in the yeast model system but recent reports have implicated this post-translational modification in the recycling of higher eukaryotic cell

surfaces receptors (growth hormone (GHR) and epithelial growth factor receptor (EGFR) recently reviewed in (Dupre et al., 2004)). The internalization of receptors in yeast does not require the G76 residue of ubiquitin but rather the presence of two hydrophobic patches surrounding the F4 and I44 residues of ubiquitin (Sloper-Mould et al., 2001).

Viruses have also been shown to exploit the mono-ubiquitin signal which serves in virus assembly and budding presumably by covalent modification of the Gag protein by ubiquitin (Patnaik et al., 2000a). The modification of the Gag protein by ubiquitin has been suggested to allow the virus to exploit the endososomal machinery of the host and there is growing evidence that this process involves the E3 ubiquitin ligase Nedd4 (Kikonyogo et al., 2001; Vana et al., 2004) and variant E2-like protein TSG101 ((Demirov et al., 2002), (Garrus et al., 2001), (VerPlank et al., 2001)) acting through interactions (Pornillos et al., 2002) with late domain motifs (Freed, 2002) in viral Gag proteins to ligate ubiquitin. It remains unclear whether it is the mono-ubiquitination of the Gag protein or the effect on the cellular ubiquitin pools that is the determinant of retroviral budding. The addition of proteasome inhibitors has been shown to interfere with the budding of Rous sarcoma virus (Patnaik et al., 2000b) and human immunodeficiency virus (Schubert et al., 2000b), an effect that can be at least partially reversed by providing Gag as a linear fusion with ubiquitin (Patnaik et al., 2000b). A possible explanation for the sensitivity of viral assembly to proteasome inhibitors is that the induction by these agents of the accumulation of polyubiquitinated substrates depletes free pools of ubiquitin, pools that are therefore unavailable for membrane-associated events. This model is supported by data showing that the effects of proteasomal inhibition were suppressed by the addition of an exogenous source of ubiquitin (Patnaik et al., 2000b).

Modulation of the immune response

Recent reports have demonstrated that ubiquitin is present in the extracellular milieu (Taniguchi et al., 1989). It has been suggested that extracellular ubiquitin may be involved in the regulation of hematopoeisis as evidenced by the effects on the growth of human hematopoietic progenitor cells and a variety of leukemic cell lines (Daino et al., 1996) and to attenuate the production of TNF alpha in LPS-induced septic shock (Majetschak et al., 2003). The involvement of exogenous ubiquitin in the modulation of intracellular signaling cascades remains poorly defined; a recent report has suggested that the cell permeating ability of exogenously applied ubiquitin permits its entry into cells where it can induce cell death by modulating the protein levels of the stat3 transcription factor (Daino et al., 2000).

1.9 Ubiquitin and transcriptional regulation

The mechanistic relationship between ubiquitin and transcription has been limited to the mono-ubiquitination of histones (H2A and H2B) and to the degradation of transcription factors or components of the basal transcriptional machinery (RNA polymerase II) which serves in the modulation of gene expression. The most characterized signaling cascades involving ubiquitin pertain to the activation of the transcription factors nuclear Factor κb (NFκB), hypoxia-inducible factor 1 alpha (HIF1α) and β-catenin all of which are subject to ubiquitin mediated proteolysis in response to various stimuli.

Regulation of transcription by UPP components

The aforementioned examples serve to emphasize the direct role of ubiquitin in the activation of signaling pathways culminating in gene transcription by modulating their localization and abundance. There is accumulating evidence that ubiquitin and the proteasome are involved in the modulation of gene transcription through proteolytic and

non-proteolytic mechanisms to actively modulate the expression of genes. In addition to the well characterized role of ubiquitin in the DNA-damage induced degradation of RNA polymerase II (which permits the recruitment of DNA repair factors in transcription-coupled-repair, (Woudstra et al., 2002)), recent reports have demonstrated that components of the basal transcription machinery may exhibit or be associated with components that have dual functions in ubiquitin processes. TAFIID, which is a core component of the basal transcription factor complex (Chen et al., 1994) has been found to exhibit E3 ubiquitin ligase activity which serves in the ubiquitination of histone H1 (Pham and Sauer, 2000). The components of an E3 ubiquitin ligase complex (elongins B and C, (Brower et al., 2002)) have been shown to associate with the PolII holoenzyme and play a role in stimulating the elongation phase of RNA polymerase II (Aso et al., 1995). A mediator of the polII component complex (a complex formed by association of Paf1-Leo-1-Ctr9) has been shown to associate with the 26S proteasomes (Verma et al., 2000) and has a role in transcription elongation of RNA polymerase II (Mueller and Jaehning, 2002) whereas the CCR4-Not transcriptional repressor complex contains an E3 ubiquitin ligase activity (Albert et al., 2002). Conversely, components associated with the UPP have dual roles in ubiquitin and transcription; Rsp5, a ubiquitin-ligase enzyme (Huibregtse et al., 1995) is involved in the degradation of RNA Polymerase II (Beaudenon et al., 1999) and UBP8, a deubiquitinating enzyme, associates with TBP-associated factors (Sanders et al., 2002). The significance of these associations remains unclear. The most striking evidence linking the ubiquitin/proteasome pathway to transcription comes from recent reports implicating the proteasome as an active participant in transcriptional control. Sug1 (Rpt6) and Sug2 (Rpt4), two subunits of the 19S particle (reviewed in ((Voges et al., 1999)) have been

shown to be regulators of transcription presumably in the elongation phase of RNA polymerase II (Kim et al., 1994) and mutations in these components in yeast result in elongation defects (Ferdous et al., 2001). In addition to the SUG components, there is a report linking at 6 distinct ATPases of the 19S particle to actively transcribed genes in yeast (Gonzalez et al., 2002). The molecular basis for the presence of 19S subunits in these regions is presently unclear. Also remaining unclear is whether the 20S core is associated with the 19S regulatory particle. A recent report has suggested that the 19S particle acts independently of the 20S core proteasome and serves as a molecular chaperone in the conversion of polII to an elongation-competent polymerase by inactivating inhibitors or by stimulating chromatin remodeling events (Ferdous et al., 2001). All these observations led to a proposed model of gene regulation by ubiquitin and proteasomes recently described by William Tansey (reviewed in (Muratani and Tansey, 2003)). His model stipulates that E3 ubiquitin ligases are recruited to promoters of genes to regulate the activity of transcriptional components (including specific gene regulators or the basal transcriptional machinery, RNA polII and histones). Proteasomes, via their affinity for poly-ubiquitin chains, are recruited to the active sites and serve in the destruction of the gene activators to promote the elongation of transcription by RNA polII. The chaperone activity provided by the 19S subunits would serve to modify chromatin during transcription until its dissociation from the polymerase resulting in a cessation of transcription. The basis for the dissociation of the 19S particle from the polymerase is presently unclear. It has been speculated that the mechanism be the degradation of the polymerase or a conformational change due to its dephosphorylation (actively transcribing polII is phosphorylated).

1.10 Ubiquitin-like molecules

In recent years, a number of proteins of structural and functional diversity with an ubiquitin-like homology (UbH) have been identified (recently reviewed in (Schwartz and Hochstrasser, 2003)). A hallmark of ubiquitin like proteins is the presence of a protruding C-terminus defined by the presence of –LRGG suggesting the possibility of processing and conjugation. The signature motif has been identified in viral ubiquitin (vUb), UCPR (the gene product of the ISG15 gene), Fau/S30 (a gene encoding the S30 protein of the mammalian 40S ribosomal subunit), Sumo-1 (otherwise referred to as GMP-1, a covalent modifier of the GTPase activating protein RanGAP1 involved in nuclear import) and Nedd8. A common feature of modification of cellular proteins by ubiquitin like molecules seems to pertain to the conjugation of a single moiety (rather than the formation of conjugates with the exception of Sumo-2 and 3, (Tatham et al., 2001) and to roles other than proteolysis. Sumo-1 and Nedd8 are by far the two most characterized ubiquitin-like molecules possessing an enzymology similar but yet distinct from that of ubiquitin in modifying proteins. Nedd8 and Sumo can be activated by their cognate E1 like enzymes (Ual1/Uba3 and Sae1a/Sae1b respectively) and conjugated by dedicated E2 and E3s (Ubc12 and Ubc9 conjugases respectively and the PIAS E3 ligase for Sumo). The number of substrates subject to modification by Sumo is in the dozens whereas six proteins (family of cullins) are conjugated by Nedd8. The molecular association of ubiquitin with ubiquitin like molecules can be viewed as paralleled rather than distinct based on the observation that these two post-translational modifications can serve either in an antagonistic or convergent manner. As an example, Sumo-1 antagonizes ubiquitin conjugation to a lysine residue on IkBα and p53 thereby affecting their steady state levels. The net effect is abrogation of the transactivation potential of NFκB (Desterro et al., 1998) whereas Sumo-1 modification of

p53 results in an increase in its transcription ability (Gostissa et al., 1999). Included in the family of ubiquitin-like molecules are enzymes containing an ubiquitin like (UBL) domain but lacking the terminal glycines. Unlike the UBL domains of proteins containing the signature motif (LRGG) these latter proteins have not been shown to be processed. These include elongin B (transcription elongation factor and E3 ubiquitin ligase complex), RAD23 (yeast homologue of HHR23A and HHR23B with a role in nucleotide excision repair) and DSK2 (a yeast protein with a putative role in spindle body duplication). The recent identification of HUB1, a processed ubiquitin-like protein lacking terminal glycines (McNally et al., 2003) would indicate that possession of C-terminal glycines should not be considered a *sine qua non* for processing by enzymes with cleaving activity.

1.11 Ubiquitin, aging and neurodegeneration; a common link?

Age-related decline in UPP components

Among the more prevalent theories of aging are those involving the inability to repair accumulated DNA mutations and the loss of quality control systems culminating in the accumulation of mutated and/or misfolded proteins. Whether the source of these abnormal proteins is the surprisingly high "normal" background of translational errors reported in eukaryotic systems (Schubert et al., 2000a), elevated oxidation arising from progressive mitochondrial derangement (Hirai et al., 2001) or any number of environmental sources, there may ultimately be too much abnormal protein for a cell to deal with, and pathological consequences may ensue. This raises the question of whether there is a general age-related decline in the ability of the ubiquitin/proteasome pathway (UPP) to recognize and eliminate abnormal proteins, and whether such a decline would be reflected by changes in the abundance or activity of some or all components of the UPP. Such a consistent decline has been reported for the pepditylglutamyl peptide activity ((Shibatani et al., 1996), (Conconi

et al., 1996), (Bardag-Gorce et al., 1999), (Anselmi et al., 1998)) and the chymotrypsin-like activity associated in with 20S core particle of the proteasome (Keller et al., 2000). In an extensive study comparing the brains of 5-month-old versus 30-month-old mice, Lee *et al.* detected increased expression of inflammatory genes indicative of microglial activation and induction of the complement cascade (Lee et al., 2000). Also induced in the aged brains were genes that encode lysosomal proteases and genes involved in the stress response, including early response transcription factors and heat shock proteins. In this study, the level of ubiquitin mRNA was found to be elevated, whereas the level of mRNAs encoding other UPP components was decreased. This latter group of components included the ubiquitin ligase Nedd4, the proteasome β-2 subunit, and the deubiquitinating enzyme Usp4 (previously designated Unp). A recent microarray study of gene expression in the aging rat hippocampus also found evidence of increased gene expression consistent with inflammation and identified the proteasome β-2 subunit as being down-regulated at the RNA level (Blalock et al., 2003), but changes in other UPP transcripts were not reported in this study. For the most part, the UPP targets revealed by the microarray approach await validation by Northern and Western blotting or proteomic methodologies, and it would be premature to draw conclusions based on the available evidence, but they do point to age-related dysregulation of the UPP at the transcriptional level, a feature shared by polyglutamine diseases. Also shared is the presence of proteinaceous inclusions that are immuno-reactive for ubiquitin and components of the UPP; ubiquitinated inclusion bodies are a hallmark of neurodegenerative disorders and have been observed in the brains of elderly individuals as typified by the Marinesco body, an ubiquitinated intra-nuclear inclusion predominantly affecting nigral neurons of the substantia nigra (Dickson et al.,

1990). The molecular mechanism defining the role of the UPP in inclusion formation during disease progression is not fully elucidated. One theory stipulates that the failure of the UPP may contribute to the accumulation of aberrant protein structures with an increased propensity to aggregate in distinct inclusions (the inclusion is by definition the causative agent) while the other suggests that inclusions are cyto-protective and that ubiquitin (due to its stability), serves in their isolation preventing further interactions with cellular components (the coating ability of ubiquitin, (Gray, 2001)). It is conceivable that these theories are not mutually exclusive; a failure of the UPP results in the accumulation of inclusions that are coated by ubiquitin to preclude further interactions.

Lysosomal-proteasomal theory of aging

The mechanistic association of ubiquitin, the lysosomal pathway and neurodegenerative disease has long been appreciated (reviewed in (Mayer et al., 1992)), and the association of these two pathways and the role of ubiquitin in directing traffic within the lysosomal pathway has only recently become the focus of considerable research (reviewed in (Bonifacino and Traub, 2003) and (Pelham, 2004)). It is emerging that this relationship exists in the form of a complex feedback loop whereby the physiological or pharmacological inhibition of one results in an inducible and transient compensatory enhancement of the proteolytic activity (presumably by induction of proteasomal genes) of the other eventually resulting in the failure of both systems. The association of these two pathways has predominantly been characterized in the physiological response to aging as it occurs in mammals (a decline in lysosomal and proteasomal degradation during aging has been reported) and has recently been extrapolated to in vitro models of cultured mammalian cells. It is expected that a failure of either one or both of these systems may exacerbate the process; a failure in the UPP results in the accumulation of cellular proteins

with increased propensity to aggregate in distinct proteinaceous inclusions readily detected in the brains of the elderly and in patients with neuronal pathologies (tauopathies and polyglutamine induced pathogenesis) whereas the inhibition of the lysosomal pathway results in the accumulation of lipofuscin, a toxic aging biomarker (composed mainly of partially digested mitochondria) whose abundance increases with age (recently reviewed in (Gray et al., 2003)). The opposing model would implicate the failure of the lysosomal pathway (due to an increase in damaged mitochondria) as an instigating event resulting in the accumulation of lipofuscin further impeding upon the degradation ability of the organelle. Free radicals produced by abnormal mitochondria would result in the accumulation of damaged and misfolded proteins with potential to overwhelm the UPP. In both cases pathological consequences may ensue.

The peculiar case of Ubb^{+1}

The mechanistic association between ubiquitin, aging and neurodegeneration can most relevantly be portrayed by the peculiar case of Ubb+1 based on its ability to perturb ubiquitin homeostasis. It has been suggested that molecular misreading by RNA polymerase II of a dinucleotide repeat in the ubiquitin-B gene (*UBB*, which encodes a stress-activated in-frame fusion of three tandem ubiquitin proteins) results in an aberrant ubiquitin C terminus that is incapable of participating in conjugation (van Leeuwen et al., 1998). The neoepitope created by the frameshift event was detected by immunohistochemistry in the brains of Alzheimer's and Down syndrome patients (van Leeuwen et al., 1998) and has also been detected in the brains of non- demented elderly individuals (van Leeuwen et al., 1998). Ubb^{+1} has been suggested to exert its effect by serving as a substrate for the formation of ubiquitin chains that are destined to remain unanchored (Ubb^{+1} acting as a ubiquitin sink). Through their affinity for subunits within

the 19S proteasome cap structures, such unanchored chains have been suggested to exert a dominant negative effect on proteasome-mediated degradation of cellular substrates (Lam et al., 2000). An alternative model has been suggested wherein Ubb^{+1} may be recognized as an ubiquitin fusion degradation substrate (Lindsten et al., 2002) with an inherent ability to block the proteasome and induce neurotoxicity (the proteasome inhibitor model). The underlying theme linking the neurodegenerative phenotype is the depletion of ubiquitin pools eventually culminating in the accumulation of protein substrates. Recent reports have challenged these models (the substrate and the inhibitor) and have suggested that Ubb^{+1} is merely a sensor of proteasomal dysfunction; Ubb^{+1} protein only accumulates in diseased regions despite the ubiquitous expression of its mRNA (Fischer et al., 2003).

Ubiquitin and Trinucleotide-induced neurodegeneration

The family of polyglutamine-induced diseases encompasses at least 9 different disorders including Dentatorubropallidoluysian Atrophy (DRPLA), Huntington's disease (HD), Spinobulbar muscular dystrophy and six spinocerebellar ataxias (SCA-1, SCA-2, SCA-3, SCA-6, SCA-7 and SCA-14, reviewed in (Everett and Wood, 2004)). They are prevalent dominantly inherited progressive diseases typically featuring in the third or fourth decade eventually resulting in death. They are characterized by the presence of an expanded polyglutamine repeat (CAG) in their respective gene products of poorly defined function (with the exception of the gene product of SCA-6 identified as the α1A voltage-dependent calcium channel (Zhuchenko et al., 1997) and SCA-14 gene which encodes the γ isoform of PCK, (Chen et al., 2003). The number of repeats correlates with onset and severity of the disease; longer tracts are associated with an earlier onset and with a more severe phenotype (reviewed in (Orr, 2000)). At the ultrastuctural level they are defined by the presence of heat shock protein containing nuclear ubiquitinated inclusions that have

accumulated the abnormal protein. With the exception of SCA-2 and SCA-6 whose toxicity is predominantly cytoplasmic, (Huynh et al., 2000) the nuclear localization of the expanded protein is a pre-requisite for its toxicity; the expression of an expanded polyQ with a deleted the NLS sequence does not result in inclusion formation in cell culture or in pathology in transgenic mice (Klement et al., 1998). The two most characterized polyglutamine-induced diseases are SCA-1 and Huntington's due to the existence of numerous animal models which fully recapitulate the features of human disease. Spinocerebellar ataxia type 1 (SCA1) is an incurable neurodegenerative disorder characterized by an abnormally long tract of CAG codons within the gene encoding ataxin-1, a widely expressed gene product whose function is poorly understood (reviewed in (Orr, 2000)). Purkinje-specific expression of human ataxin-1 with an expanded CAG tract encoding 82 glutamines was found to recapitulate the key clinical features of SCA1 in a transgenic mouse model, including loss of Purkinje cells and progressive ataxia as measured by performance on rotating rod (rotarod). Transgenic mice expressing a similar construct but with a 30Q (line strain referred to as A02) polyglutamine tract do not display ataxia (reviewed in (Orr, 2000)).

The transcriptional dysregulation model of polyQ-induced pathology

The molecular mechanisms implicated in the onset/progression are poorly defined although evidence suggests an early dysregulation of transcription may be the basis of the phenotype. Modifiers of polyglutamine induced pathology of the SCA-1 phenotype in fly include genes whose products are involved in cytoplasmic/nuclear transport and RNA binding (Fernandez-Funez et al., 2000). These studies confirm work performed in transgenic mice and are consistent with ataxin-1 having RNA-binding activity ((Yue et al., 2001) and (Irwin et al., 2005)). Micro-array results have demonstrated a decline in

transcription at a time point before pathology ensues in both SCA (Lin et al., 2000) and Huntington's ((Luthi-Carter et al., 2002) and (Sipione et al., 2002)) mouse models of disease. The molecular basis for the decline of transcription is presently unclear however recent findings suggest that sequestration of transcriptional factors or co-factors might be the mechanism of toxicity. It has been demonstrated that the CAG expansion in atrophin-1 and the huntingtin proteins interact and sequester transcription factors/co-factors important in neuronal homeostasis (p300/CBP, Sp1 etc) resulting in the loss of expression of important neuronal genes (Nucifora et al., 2001). Whether this represents a unified mechanism by which expanded polyglutamine tracts adversely affect neuronal metabolism remains to be ascertained.

The altered conformation model of polyglutamine-induced pathology

It is likely that expanded polyglutamine (polyQ) proteins pose a serious problem for the cell, being at once potentially toxic and difficult to dispose of through the ubiquitin/proteasome pathway (UPP) although a dissenting view suggests efficient destruction of expanded polyQ proteins by the 26S proteasome (Michalik and Van Broeckhoven, 2004). If the cell is unable to properly fold the polyQ protein through the concerted action of chaperones, it may attempt to degrade the polyQ protein via the proteasome, and if all else fails sequester it in an inclusion body. There is accumulating evidence from experiments with cultured cells that this system of triage is operational. Enhanced expression of chaperones reduced the formation of protein aggregates (reviewed in (Kobayashi and Sobue, 2001)), whereas proteasome inhibition promoted their formation ((Ardley et al., 2003), (Harada et al., 2003), (Waelter et al., 2001)) although a dissenting view has been recently proposed wherein treatment of post-mitotic neurons with proteasome inhibitors suppressed polyglutamine induced inclusion formation (Kim et al.,

2004). Whereas small protein aggregates may be toxic, the large inclusion bodies appear protective, and it is the ubiquitin system that is pivotal in orchestrating their formation (reviewed in (Gray, 2001)). Interference with ubiquitin-mediated inclusion formation has been shown to result in fewer inclusions, but more severe pathology (Cummings et al., 1999). Evidence for the folding/degradation/inclusion triage also has emerged from an unbiased genetic screen in *Drosophila*, where expression of the 82Q ataxin-1 protein in retinal cells promoted their degeneration, an effect that could be modified by genes encoding chaperone proteins or components of the UPP (Fernandez-Funez et al., 2000).

1.12 The aims of the research

Much of what is known about the structure/function of ubiquitin and the phenotypes of site-directed mutation have been deduced from experiments performed in the yeast model system and in an *in vitro* reconstituted environment. From the high level of conservation between yeast and mammalian ubiquitin one would expect similar phenotypic consequences from the expression of dominant-negative ubiquitin mutants in higher eukaryotic cells. To test this hypothesis we generated expression constructs of wild type or mutant isoforms of ubiquitin that were introduced in mammalian cells and utilized in the generation of novel transgenic mice. The objective of this strategy was to investigate the constraints on processing and conjugation at the carboxy-terminus of ubiquitin and the phenotypic consequences of expression of mutant ubiquitin in higher eukaryotic cells and in the *in vivo* setting of novel ubiquitin transgenic mice. Contrary to the initial prediction and the findings in yeast, C-terminal ubiquitin mutants (that were found to impair proteolytic activity in yeast) were found to be efficiently recognized and processed by enzymes with ubiquitin cleaving activity and were found, in some cases, to conjugate to cellular proteins. In mice, it was found that the expression of a chain-terminating variant of

ubiquitin (K48R) conferred protective effects and alleviated the phenotype in a mouse model of spinocerebellar ataxia-1. The data generated suggest that mammalian cells are more tolerant of mutations in ubiquitin and suggest novel roles for this molecule in the regulation of cellular processes.

Chapter 2
Materials and methods

2.1 Plasmid construct

Eukaryotic expression constructs

A PCR strategy was used to append the residues MRGSHHHHHH to the amino terminus of human ubiquitin. The forward oligonucleotide, 5'-AAAAAGCTTAAAATGAGAGGCAGCCACCACCATCACCATCACATGCAGATCTTCGTG-3' and the reverse primer 5'-ACCACCTCTCAGACGCAGG-3 were used as primers in a reaction with a plasmid template containing the human UbB cDNA (a gift from Dr. Rohan Baker, Canberra). In a second reaction, an overlapping green fluorescent protein (EGFP) fragment was generated using the plasmid template pEGFP (Clontech Laboratories Palo Alto, California, USA), the forward primer, 5'-GCGTCTGAGAGGTGGTATGGTGAGCAAGGGCG-3', and the reverse primer, 5'-AAACTCGAGTTACTTGTACAGCTCG-3'. The products of the ubiquitin and EGFP reactions were mixed, and the forward and reverse primers for ubiquitin and EGFP, respectively, were used to amplify a product encoding the ubiquitin-EGFP fusion protein. The product of this reaction was digested with the restriction enzymes HindIII and XhoI and was subcloned into LITMUS28 (New England Biolabs, Ltd., Mississauga, Ontario, Canada) for the verification of the DNA sequence. The fusion cDNA was the excised using the same two enzymes and used to replace the junB cDNA in the plasmid Ubi-junB containing the human UbC promoter and the simian virus 40 (SV40) polyadenylation signal (a gift from Dr. Peter Angel, Heidelberg). The resulting plasmid was designated wt Ub-EGFP. The G76Y Ub-EGFP, insertion mutants IIGG Ub-EGFP, IGG Ub-EGFP and GGII Ub-EGFP were constructed in a similar manner as the wt Ub-EGFP in a PCR

reaction where wt Ub-EGFP plasmid served as a template. The forward ubiquitin and the reverse EGFP primers were the same as the ones used to generate the wt Ub-EGFP plasmid. The reverse ubiquitin primer as well as the forward EGFP primer used in these reactions was as follows: G76Y reverse ubiquitin oligonucleotide 5'-ATAACCTCTCAGACGCAGGACCAGGTGCAGGGTAGACTC- 3' and forward EGFP oligonucleotide 5'-CTGGTCCTGCGTCTGAGAGGTTATATGGTGAGCAAGGGCGAGGAG-3'. The primers utilized to generate the insertion mutants were as follows: IIGG ubiquitin reverse oligonucleotide 5'-ACCACCAATAATTCTCAGACGCAGGAC-3' and forward EGFP oligonucleotide 5'-CTGAGAATTATTGGTGGTATGGTGAGCAAGGGCG-3'; IGG ubiquitin reverse oligonucleotide 5'-ACCACCAATTCTCAGACGCAGGAC-3' and forward EGFP oligonucleotide 5'- CTGAGAATTGGTGGTATGGTGAGCAAGGGCG-3'; GGII ubiquitin reverse oligonucleotide 5'- AATAATACCACCTCTCAGACG-3' and forward EGFP oligonucleotide 5'-CTGAGAGGTGGTATTATTATGGTGAGCAAGGGCG-3'. The two templates were mixed together in a final reaction where the ubiquitin forward and EGFP reverse primers were utilized to amplify the Ub-EGFP fusion. The plasmid containing the K48R mutant version of ubiquitin was generated by a polymerase chain reaction-based site-directed mutagenesis strategy in which wt Ub-EGFP served as the template. The entire plasmid was amplified using primers overlapping at a position corresponding to Lys^{48}: upper strand primer, 5'-AAAGCCGGCAGCTGGAAGATGGCCGTACTC-3'; and lower strand primer, 5'-AAAGCCGGCCTCAAAGATGAGCCTCTGC-3'. These primers served to replace the lysine residue at position 48 with arginine and created a novel NgoMIV site in the plasmid.

The resulting plasmid was sequenced to confirm that the site-directed mutation was present and that no other sequence alterations had occurred during polymerase chain reaction amplification. The K63R Ub-EGFP plasmid was a gift from Dr. Brad Wouters, University of Maastricht. 'Stick' Ub-EGFP was generated using a PCR strategy in which the EGFP plasmid served as a template. Two successive PCR reactions were used to append the MRGS-6xhis and the sequences of the stick (LRLRGG) to the N-terminus of EGFP. In the initial reaction, the following forward EGFP oligonucleotide was used: 5'-CACCATCACCTGCGTCTGAGAGGTGGTATGGTGAGCAAGGGC-3' with the same EGFP reverse oligonucleotide that was described previously. The PCR product generated in the first reaction was used as a template in the second PCR reaction with the following forward oligonucleotide 5'-AAAAAGCTTATGAGAGGCAGCCACCACCATCACCATCACCTGCGT-3' and the reverse EGFP oligonucleotide that was described previously. The remaining G76 Ub-EGFP variants as well as the substitution mutants R72A Ub-EGFP, R74A Ub-EGFP, R72S Ub-EGFP, L67D Ub-EGFP, L69D Ub-EGFP and L73D Ub-EGFP were generated using QuickChange® site-directed mutagenesis (Stratagene, La Jolla, CA) in a reaction where the wt Ub-EGFP served as a template. The primers used in these reactions were as follows: G76A Ub sense strand primer 5'-CCTGCGTCTGAGAGGTGCGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCGCACCTCTCAGACGCAGG-3'; G76C Ub sense primer 5'-CCTGCGTCTGAGAGCTTGCATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATGCAACCTCTCAGACGCAGG-3'; G76D Ub sense primer 5'-CCTGCGTCTGAGAGGTGACATGGTGAGCAAGGGC-3'and anti sense primer 5'-

GCCCTTGCTCACCATGTCACCTCTCAGACGCAGG-3'; G76E Ub sense primer 5'-CCTGCGTCTGAGAGGTGAAATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATTTCACCTCTCAGACGCAGG-3'; G76F Ub sense primer 5'-CCTGCGTCTGAGAGGTTTCATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATGAAACCTCTCAGACGCAGG-3'; G76H Ub sense primer 5'-CCTGCGTCTGAGAGGTCACATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATGTGACCTCTCAGACGCAGG-3'; G76I Ub sense primer 5'-CCTGCGTCTGAGAGGTAAAATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATTATACCTCTCAGACGCAGG-3' ; G76K Ub sense primer 5'-CCTGCGTCTGAGAGGTAAAATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATTTTACCTCTCAGACGCAGG-3'; G76L Ub sense primer 5'-CCTGCGTCTGAGAGGTCTGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCATACCTCTCAGACGCAGG-3'; G76M Ub sense primer 5'-CCTGCGTCTGAGAGGTATGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCATACCACTCAGACGCAGG-3'; G76N Ub sense primer 5'-CCTGCGTCTGAGAGGTAACATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATGTTACCTCTCAGACGCAGG-3'; G76P Ub sense primer 5'-CCTGCGTCTGAGAGGTCCGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCGGACCTCTCAGACGCAGG-3'; G76Q Ub sense primer 5'-CCTGCGTCTGAGAGGTCAGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCTGACCTCTCAGACGCAGG-3'; G76R Ub sense primer 5'-CCTGCGTCTGAGAGGTCGCATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATGCGACCTCTCAGACGCAGG-3'; G76S Ub sense primer 5'-

CCTGCGTCTGAGAGGTCCGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCGAACCTCTCAGACGCAGG-3'; G76T Ub sense primer 5'-CCTGCGTCTGAGAGGTACTATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATAGTACCTCTCAGACGCAGG-3' ; G76V Ub sense primer 5'-CCTGCGTCTGAGAGGTGTAATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATTACACCTCTCAGACGCAGG-3'; G76W Ub sense primer 5'-CCTGCGTCTGAGAGGTTGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCCAACCTCTCAGACGCAGG-3'; R72S sense primer 5'CCCTGCACCTGGTCCTGAGCCTGAGAGGTGGTATGG-3' and anti-sense primer 5'-CCATACCACCTCTCAGGCTCAGGACCAGGTGCAGGG-3'; R72A sense primer 5'-CCCTGCACCTGGTCCTGGCGCTGAGAGGTGGTATGG-3' and anti-sense primer 5'-CCATACCACCTCTCAGCGCCAGGACCAGGTGCAGGG-3'; R74A sense primer 5'-CCCTGCACCTGGTCCTGCGTCTGGCGGGTGGTATGG-3' and anti-sense primer 5'-CCATACCACCCGCCAGACGCAGGACCAGGTGCAGGG-3'; L67D sense primer 5'-CCAGAAAGAGTCGACCAACCACCTGGTCCTGCGTC-3' and anti-sense primer 5'-GACGCAGGACCAGGTGGTTGGTCGACTCTTTCTGG-3'; L69D sense primer 5'-GAGTCGACCCTGCACAACGTCCTGCGTCTGAGAGG-3' and anti-sense primer 5'-CCTCTCAGACGCAGGACGTTGTGCAGGGTCGACTC-3'; L73D sense primer 5'-GCACCTGGTCCTGCGTAACAGAGGTGGTATGGTG-3'and anti-sense primer 5'-CACCATACCACCTCTGTTACGCAGGACCAGGTGC-3'. Double mutants G75AG76A Ub-EGFP, G75AG76I Ub-EGFP G75IG76A Ub-EGFP were also generated using QuickChange® site-directed mutagenesis (Stratagene, La Jolla, CA) in a reaction where either G76A Ub-EGFP or G75A Ub-EGFP served as a template. The primers used for

these reactions were as follows: G75AG76A sense primer 5'-CCTGCGTCTGAGAGCGGCGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCGCCGCTCTCAGACGCAGG-3', G75AG76I sense primer 5'-CCTGCGTCTGAGAGCGGCGATGGTGAGCAAGGGC-3' and anti sense primer 5'-GCCCTTGCTCACCATCGCCGCTCTCAGACGCAGG-3', G76IG76A sense primer 5'-CCTGCGTCTGAGAATAGCGATGGTGAGCAAGGGC-3' and anti-sense primer 5'-GCCCTTGCTCACCATCGCTATTCTCAGACGCAGG-3'. The resulting plasmids were sequenced to confirm that the site-directed mutation was present and that no other sequence alterations had occurred during the PCR reaction. The sequence of the CL1 degron (ACKNWFSSLSHFVIHL) that has been previously used to generate a destabilized version of EGFP (Bence et al., 2001) was appended to the C-terminus of ubiquitin using a sequential PCR strategy in a reaction where the wt Ub-EGFP plasmid served as a template. The primers used in the first PCR reaction were as follows: forward ubiquitin oligonucleotide 5'-AAAAAGCTTAAAATGAGAGGCAGCCACCACCATCACCATCACATGCAGATC-3' and ubiquitin reverse oligonucleotide 5'-GCTTAGGCTGCTGAACCAGTTCTTACATGCACCACCTCTCAG-3'. The PCR product of the first reaction was gel purified and used in the second PCR reaction with the same forward oligonucleotide and the following reverse oligonucleotide 5'-ACCTCGAGTCACAGGTGAATCACGAAGTGGCTTAGGCTGCT-3'. The product of this reaction was digested with restriction enzymes HindIII and XhoI and subcloned into the wt Ub-EGFP expression vector and designated wt Ub-CL1. The G76Y Ub CL1 expression construct was generated using QuickChange® site-directed mutagenesis

(Stratagene, La Jolla, CA) in a PCR reaction where the wild type Ub-CL1 plasmid served as a template. The primers used in this reaction were as follows: sense primer 5'-CCTGCGTCTGAGAGGTTACGCATGTAAGAACTGG-3' and anti-sense primer 5'-CCAGTTCTTACATGCGTAACCTCTCAGACGCAGG-3'. The sequence of the $^{+1}$ peptide (van Leeuwen et al., 1998) was appended at the C-terminus of ubiquitin by a sequential PCR strategy in a reaction where the wt Ub-EGFP plasmid served as a template. The following primers were used: ubiquitin forward primer 5'-AAAAAGCTTAAAATGAGAGGCAGCCACCACCATCACCATCAC-3' and ubiquitin reverse primer 5'-ACTTCCAGGGTCTCCACGAGGATCTGCATAACCTCTCAGACG-3'. The product of the first reaction was used in the subsequent PCR reaction with the following PCR primers: ubiquitin reverse primer 5'-TTTCTCGAGTTTTCACTGTTCTCCACTTCCAGGGTC-3'and the same ubiquitin forward primer as described previously. The plasmid was designated 6xhis Ubb^{+1}. The MRGS hexahistidine epitope was also appended at the C-terminus of ubiquitin following the $^{+1}$ peptide in a reaction where the 6xhis Ubb^{+1} served as a template. The primers used in these reactions were as follows: forward ubiquitin oligonucleotide 5'-AAAAAGCTTAAAATGCAGATCTTCGTG-3' reverse oligonucleotide 5'-TTTCTCGAGTTTCTAGTGCTGGTGATGGTGGTGGCTGCCTCTTCACTGGGCTCC-3'. The EGFP-Ubb^{+1} expression construct was generated with a PCR strategy in which an EGFP-Ub plasmid served as the template. The primers used to amplify the EGFP-Ub fusion were as follows: forward ubiquitin oligonucleotide 5'-AAAAAGCTTAAAATGGTGAGC AAGGGCGAGGAGC-3' and ubiquitin reverse oligonucleotide 5'-

ACTTCCAGGGTCTCCACGAGGATCTGCATAACCTCTCAGACG-3'. The product of the first reaction was used in the subsequent PCR reaction with the following PCR primers: ubiquitin reverse primer 5'-TTTCTCGAGTTTTCACTGTTCTCCACTTCCAGGGTC-3'and the same ubiquitin forward primer as described previously. The EGFP^{+1} expression vector was generated by appending the $^{+1}$ peptide at the C-terminus of EGFP in a PCR reaction using the EGFP plasmid as a template. The primers used in these reactions were as follows: forward EGFP primer 5'-AAAAAGCTTAAAATGGTGAGCAAGGGCGAGGAGC-3' and EGFP reverse primer 5'- CAGTCCAGGGTCTCCACGAGGATCTGCCTTGTACAGCTGGTC-3'. The PCR fragment was used as the template in the second PCR reaction with the same ubiquitin forward and the following EGFP reverse primer: 5'-TTTCTCGAGTTTTCACTGTTCTCCACTTCCAGGGTC-3'. All the products were sub-cloned in an expression vector driven by the UbC promoter using restriction enzymes HindIII and XhoI. The NoK Ub-EGFP expression vector was generated using QuickChange® site-directed mutagenesis (Stratagene, La Jolla, CA) in a PCR reaction in which K63 Ub-EGFP served as a template (all lysines except for lysine 63 had been replaced by Arg, a gift from Dr. Bradly Wouters, University of Maastricht). The primers used in these reactions were as follows: sense primer 5'-TTCTGACTACAACATCCAGCGGAAAGAGTGGACCCT-3' and anti-sense primer 5'-AGGGTCCACTCTTTCCGCTGGATGTTGTAGTCAGAA-3'.

2.2 Generation of Transgenic Mice

Wt Ub-EGFP, K48R Ub-EGFP and K63R Ub-EGFP ubiquitin transgenic lines were generated by Manon Dube in the laboratory of Dr.Barbara Vanderhyden, Ottawa Regional Cancer Center. NoK Ub-EGFP and EGFP-Ubb^{+1} transgenic mice were generated by Yves

Repentigny in the laboratory of Dr.Rashmi Kothary, Ottawa Hospital Research Institute. Briefly, sequences corresponding to the UbC promoter 6xHis-ubiquitin-EGFP and the SV40 polyadenylation signal were released from the plasmid backbone using a combination of NdeI and EcoR1. DNA containing this expression cassette was purified from an agarose slice by electroelution, followed by phenol/chloroform extraction and ethanol precipitation. The purified DNA was microinjected into the male pronuclei of either FVB/N oocytes (in the case of the wt Ub-EGFP, K48R Ub-EGFP and K63R Ub-EGFP transgenics) or C57BL16-C3H F1 oocytes (in the case of nok Ub-EGFP and EGFP-ubb^{+1} transgenics) which were cultured to the two-cell stage and implanted into pseudopregnant recipients. Progeny were examined for expression of the EGFP marker by illumination of tail biopsies (Fig. 5) with a Volpi Intralux 4000 light source fitted with a 470-nm excitation filter (Chroma Technology Corp., Brattleboro, Vermont, USA). Fluorescence was observed through a hand-held, 525-nm bandpass filter (Chroma Technology Corp., Brattleboro, Vermont, USA), which was also fitted to a 35-mm camera for photographs. Animals with intense tail fluorescence were chosen to serve as founders for the establishment of transgenic lines. The origin of the B05 transgenic line carrying an expanded polyglutamine in the Ataxin -1 allele with 82 CAG repeats and the A02 line with a CAG repeat of 30 codons was described in a paper from the laboratory of Dr. Harry Orr, Minnesota ((Burright et al., 1995)), from whom the lines were obtained.

2.3 Isolation and Culture of Embryos

Wild-type and heterozygous female mice, 1-5 months old, were super-ovulated by intra-peritoneal injection of 5 IU pregnant mares' serum gonadotropin (Folligon; Intervet Canada Ltd., Whitby, Ontario, Canada) followed 44h later by 5 IU human chorionic gonadotropin (hCG; Sigma, St. Louis, Missouri, USA). Immediately after the second

injection, the females were paired with either heterozygous or wild-type male mice, and mating was confirmed the following morning by the presence of vaginal plugs. Mated females were sacrificed 16-18h after hCG injection, the oviducts were excised, and the ampullae ruptured to release the ovulated eggs into CZ-B embryo culture medium supplemented with 875 mg/L taurine (CZ-B/T). The eggs were treated with hyaluronidase (Sigma, St. Louis, Missouri, USA) to remove the cumulus cells, and the cumulus-free eggs were washed twice through enzyme-free medium before transfer to 0.8 mL CZ-B/T in embryo culture dishes (Falcon; Becton Dickinson, Franklin Lakes, New Jersey, USA) for subsequent culture. After two days of culture, by which time embryos had cleaved to four cells, media were supplemented with 1 mg/mL glucose. Embryo development and EGFP expression were monitored at 12-h intervals for four days using a Leica MZFLIII fluorescence stereomicroscope.

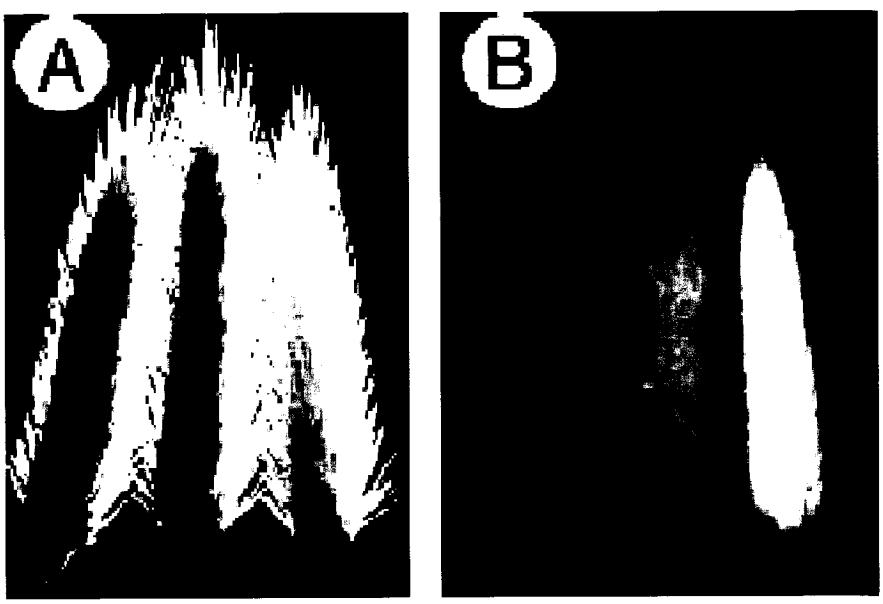
2.4 Cell culture and transfection experiments

HT4 murine neuroblastoma cell line

The parental HT4 cell line derived from a mouse neuroblastoma cell line (a gift form Dr. Maria Figueiredo-Pereira, City University of New York) and HT4 cells stably transfected with wt Ub -EGFP, K48R Ub-EGFP or K63R Ub-EGFP were maintained at 32°C in Dulbecco's modified Eagle's medium(D-MEM) containing 5% non-heat inactivated fetal calf serum in 5% CO_2. The HT4 cell line contains a recombinant temperature sensitive mutant of SV40 large T antigen and differentiate into neurons when grown at the nonpermissive temperature, synthesize and secrete NGF, express receptors for NGF (Whittemore et al., 1991) and glutamate (Lerma et al., 1993). For transient transfections, HT4 cells were plated at a density of $40x10^4$ cells/60-mm dish and maintained at 39°C for 3 days to induce differentiation into neurons. Following this incubation period, the cells

Figure 5. Genotyping by tail biopsy fluorescence

1 cm tail biopsies of nontransgenic mouse (left), heterozygote (middle) and homozygote (right) viewed under visible light (panel A) or ultraviolet light (panel B). The genotypes of the mice were confirmed by test matings to stock FVB/N animals.


A
B

were maintained at 37°C for at least 7 hours prior to transfection. Transfections were carried out at 37°C using Fugene6^{TM} (Roche Applied Science, Laval, Quebec, Canada) as per the supplier's protocol. Briefly, 15 µl of Fugene6^{Tm} transfection reagent was added to 285 µl of serum free medium and incubated at room temperature for 5 minutes. Subsequently 1.5 µg of plasmid DNA was added to the mixture and further incubated for 20 minutes at room temperature. The mixture containing the plasmid DNA, transfection reagent and serum free media was added to the cells. When cells were treated with 50 µM of proteasome inhibitor (Calbiochem, San Diego, California, USA) to reduce protein degradation and enhance the detection of ubiquitinated substrates, it was added in the culture medium 3 hours prior to lysis. To establish stable cell lines expressing wild-type and variants of epitope tagged ubiquitin, HT4 cells were co-transfected with a Pgk-puromycin expression vector (a gift from Dr. Michael McBurney, Ottawa Hospital Regional Cancer Centre) and either Wt, K48R or K63R Ub-EGFP plasmids (1:6 ratio). Following drug selection (2 µg/mL puromycin), clones were picked and expanded. Expression of the EGFP marker was monitored by fluorescence microscopy.

HEK-293T cells and NIH-3T3 cells

HEK-293T and NIH-3T3 cells were maintained in 5% CO_2 at 37°C in alpha-minimal essential medium (α-MEM) containing 10% FCS. For transient transfection experiments, the cells were plated in 6 well dishes at a density of $3x10^5$ cells/well and maintained at 37°C for 24 hours prior to transfection. Subsequent to this incubation period, cells were transfected using FuGENE6^{TM} (Roche Applied Science, Laval, QC) as per the supplier's protocol. 24 or 48 hours post-transfection, cells were collected and cell extracts were prepared for western blot analysis.

Cadmium and Canavanine-treated Cells

Untransfected HT4 cells and cells stably transfected with wt Ub-EGFP, K48R Ub-EGFP or K63R Ub-EGFP were plated at a density of $40x10^4$ cells/60 mm dish and maintained at 32°C in α-MEM containing 10% non heat inactivated FCS. The cells were either treated for 24h with 5μM cadmium sulfate (Sigma, St. Louis, Missouri, USA) alone or pretreated with 0.2 mM buthionine sulfoximine (Sigma, St. Louis, Missouri, USA) prior to a 24-h incubation period with 5 μM cadmium sulfate. Following the incubation period, cells were observed by light microscopy. For western blot analysis of extracts treated with cadmium, cells were treated with 25 μM cadmium sulfate for 0 to 8h prior to harvesting. When cells were treated with canavanine (Sigma, St. Louis, Missouri, USA) it was supplemented in the medium at a concentration of 20 mM for 30h prior to visualizing the cells by light microscopy. The toxicity of canavanine was measured by trypan blue exclusion in attached cells and in cells dislodged by 5 min of rotation at 100 rpm on a platform shaker. Three individual dishes from each cell line were counted and the experiments were performed in triplicates. The percentage of dead to live cells in floating *versus* adherent cells was determined independently. For Western blot analysis, untransfected HT4 cells and cell clones expressing wt Ub-EGFP or K48R Ub-EGFP were treated with 10 mM canavanine for 24h prior to harvesting.

2.5 Reporter assays

NIH-3T3 cells were plated in 6-well dishes and transiently transfected using Gene Juice™ as recommended by the manufacturer (Novagen, La Jolla, CA). Each well was transfected with a constant amount of plasmid DNA containing reporter plasmid 4xCRE-Luc (Stratagene, La Jolla, CA), *Renilla* expression vector (Promega, Madison, WI) and either with empty vector control (PCDNA3.1) or expression constructs encoding Ataxin-1 (Q82,

a gift from Dr. Huda Zoghbi, Baylor College of Medicine), CBP (a gift from Dr. Jim Dimitroulakos, Ottawa Hospital Regional Cancer Center), wt or K48R ubiquitin. Alternatively, cells were co-transfected with Ataxin-1 and either CBP or wild-type or mutant ubiquitin constructs. Forty-eight hours post-transfection, cells were treated with 20 μM forskolin (Sigma, St. Louis, Missouri, USA) for 12 hours, harvested in passive lysis buffer and 20 μl of cell extract was assayed for luciferase activity using a Dual-Luciferase ™ reporter assay system following the manufacturer's protocol (Promega, Madison, WI). Analysis was carried out in Prism (Graph pad software Inc, San Diego CA).

2.6 Fluorogenic assays

The protocol for these assays was described in Hope *et al* (Hope et al., 2003). Briefly, cells were cultured in 6-well dishes prior to transfection with wild-type or mutant ubiquitin expression vectors. 48 hours later, the cells were scraped with 250 μl of Lysis and Assay (L&A) buffer (20 mM Tris-HCl, pH 7.0, 1 mM EDTA and 3 mM sodium azide) and lysis was achieved by the hypotonic buffer action and three freeze thaw cycles using dry ice and room temperature. Extracts were spun at 5000g for 5 minutes to pellet cellular debris and protein quantification was carried out using the protein assay from Bio-Rad Laboratories Mississauga On. 60 μg of cell extract was diluted in a total volume of 300 μl L&A buffer. The fluorogenic substrates II and III that assay for the peptidylglutamyl and chymotrypsin-like activities of the proteasome (Calbiochem, San Diego, California, USA) were also diluted in L&A buffer at a final concentration of 10 μM/ml. An equal volume (300 μl) of buffer containing the substrate was added to the extract and each sample was dispensed into six 100 μl samples in a 96-well microtitre dish. A control sample consisted of a mock transfected cell lysate incubated in the presence of Proteasome Inhibitor I (a specific inhibitor of the chymotrypsin-like activity of the proteasome at a final concentration of 50

μM/mL) or epoxomycin (a more general inhibitor of the proteasome at a final concentration of 5 μM/mL, Calbiochem). Fluorescence (λem: 445 nm) was measured at various time points for up to 16 hours using a fluorimeter (Fluoroskan Ascent FL, Thermo Laboratory Systems). The measurement of proteasome activity in tissues was performed in a similar manner with the exception of gentle homogenization of the tissues prior to lysis by hypotonic freeze/thaw cycles.

2.7 Preparation of cells extracts and Western blot analysis

Cultured cells

The cells were washed 3 times with PBS and trypsinized for 5 minutes at 37°C. Cells were then collected in culture medium, centrifuged at 3000g for 5 minutes and resuspended in protein lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40 (Sigma, St. Louis, Missouri, USA), 0.5 mM EDTA, 20% glycerol) containing the following protease and phosphates inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin (Sigma, St. Louis, Missouri, USA), 2 μg/mL aprotinin (Sigma, St. Louis, Missouri, USA), 200 μM sodium Fluoride (NaF) and 200 μM sodium pyrophosphate (NaPPi). The cells were incubated on ice for 30 minutes and then centrifuged at 14 000rpm for 20 minutes at 4°C to pellet cellular debris. The soluble fractions were recovered and the protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories (Canada) Inc., Mississauga, Ontario, Canada). 20 or 30 μg of cytoplasmic extracts were then resolved on a two-phase SDS-polyacrylamide gel (15 and 8%) and electroblotted onto a hybond C nitrocellulose membrane (Amersham Pharmacia Biotech, Baie D'Urfé, Québec, Canada). The membranes were stained with Ponceau S (Sigma, St. Louis, Missouri, USA) prior to western blotting with the appropriate antibody to ensure the complete transfer of the proteins. Membranes containing tissue extracts were

autoclaved for 30 minutes on the liquid cycle prior to immunoblotting to enhance the detection of ubiquitinated conjugates ((Mimnaugh et al., 1999)). Unless indicated otherwise, the primary and secondary antibodies were diluted in 5% skim milk in TBST (10 mM Tris-HCl (pH7.6), 150 mM NaCl and 1%Tween-20) for 1 hour at room temperature. The membranes were washed 3 times with TBST prior to incubation with the appropriate secondary antibody. The proteins were visualized by a horseradish peroxidase method using the ECL kit from Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA.

Animal tissues

Animals were anaestitized using CO_2 and sacrificed by cervical dislocation. Whole brains or cerebella of non-transgenic and transgenic animals were excised and homogenized in PBS (whole brain) or protein lysis buffer (cerebella) containing 1% NP-40, and the protease and phosphatase inhibitors previously described. The brains of no-k Ub-EGFP were prepared in a similar manner except in Figure 44C when SDS was added to the lysis buffer. The homogenates were then sonicated on ice (3x10 sec). The extracts were centrifuged for 30 minutes at 4°C to pellet cellular debris. Protein quantification and western analysis was carried out as previously described.

2.8 Nickel purification strategies

Batch purification

Cells were plated in 100 mm dishes as previously described. The cells were harvested in lysis buffer (50 mM NaH_2P0_4, 300 mM NaCl, 0.05% Tween-20 pH 8.0) containing 50mM imidazole and quantified as previously described. 1 mg of cell extract was incubated with 30 µl of pre-washed nickel beads (Qiagen Inc. Canada, Mississauga, Ontario, Canada) o/n at 4°C. The beads were centrifuged for 1 minute and washed 4 times in lysis buffer

containing increasing amounts of imidazole (2x50 mM, 1x100 mM and 1x250 mM imidazole, Sigma). The purified proteins were then eluted in lysis buffer containing 500 mM imidazole. The eluates were analyzed by western analysis with the 6xHis specific antibody.

FPLC

Cells were lysed in buffer as before and then analyzed by fast liquid chromatography by Sylvie Lavictoire Brett in the laboratory of Dr. Ian Lorimer, Ottawa Hospital Research Institute.

2.9 Immunohistochemistry

Cerebella or whole brains were excised and fixed in 10% phosphate-buffered formalin o/n at room temperature. Tissues were paraffin-embedded and sectioned using a microtome at a thickness of 5μm. Deparaffinized sections were heated in a solution of 10 mM sodium citrate (pH 6.0) in 700W microwave for 10 minutes. Endogenous peroxidase activity was blocked by incubating in methanol containing 3% hydrogen peroxide for 20 minutes. Sections were washed with 0.1 M PBS (pH 7.4) and incubated for 30 minutes with 1.5% normal goat serum (Santa Cruz Biotechnologies, SC CA) to block nonspecific binding. Sections were then incubated overnight at 4°C with the appropriate primary antibody. The reaction product was visualized by the Envision^{+} system (DAKO Diagnostics Canada Inc.).

2.10 Behavioral analysis

Six mice of each genotype were tested for motor performance on the rotating rod apparatus. Mice were placed on the rotating rod for 3 trials per day for 4 consecutive days. The duration of the individual trials was approximately 6 minutes during which time the rod underwent linear acceleration in increments of 0.1 rpm/sec. Animals were scored for their latency to fall (in seconds) for each trial. Animals were allowed to rest between every

individual trial to avoid fatigue. A period of several seconds before each trial without any acceleration of the rod was allocated to acclimatize the animals to the apparatus. Standard errors for the data were calculated using Prism software (GraphPad Software Inc. San Diego CA).

2.11 Immunoprecipitations

293T cells were plated in 100mm dishes at a density of $1x10^6$ cells and maintained at 37°C for 24 hours prior to co-transfection with a plasmid encoding E2F-1 and either wt Ub-EGFP or mutant ubiquitin constructs. Three hours prior to harvesting the cells, 50 μg/mL of PI was added to the culture medium to inhibit protein degradation and enhance the detection of ubiquitinated substrates. Cell extracts were incubated with an anti-E2F-1 mouse monoclonal antibody in the presence of G-sepharose gamma bind beads (Amersham, Pharmacia Biotechnologies, Baie D'Urfe, QC) o/n at 4°C. Immunoprecipitates and total cell extracts were analyzed by western blot analysis.

2.12 Northern blot analysis

RNA was extracted from the cerebella of 3 month old aged-matched wt Ub-EGFP, K48R Ub-EGFP, BO5 and BO5 compound transgenic mice with the RNAse easy kit from Qiagen as per the supplier's protocol. 15 μg of total RNA was denatured by heating at 65°C for 30 minutes and was separated on a 1% agarose-5% formaldehyde gel. The migration and integrity of the RNA was confirmed by staining the gel with ethidium bromide for 15 minutes at room temperature prior to transferring o/n onto a Hybond N+ membrane (Amersham Pharmacia Biotechnologies, Baie D'Urfe, QC). The membrane was incubated at 42°C in pre-hybridization solution (50% formamide, 20xSSPE, 50X Denhardt solution, 10% SDS and 250μg/ml denatured sperm DNA) for 3 hours prior to the addition of the radiolabelled probe. A 2.7 kb cDNA fragment encoding the non-expanded ataxin-1 protein

(a gift from Huda Zoghbi, Baylor College of Medicine) was excised from the PGEMII backbone with EcoR1 restriction endonuclease and labeled with [α -32P] dCTP using the Megaprime DNA labeling kit from Amersham. The membrane was washed three times (two 15 minute washes at room temperature with 2xSSC/0.1%SDS and one 15 minute wash at 65° with 0.2xSSC/0.1%SDS), exposed on a phosphor screen (Amersham) and visualized with a phosphoimager (Amersham). The membrane was re-probed with a 1.1 kb human β-actin cDNA fragment excised from the Bluescript SK$^-$ backbone with EcoR1 restriction endonuclease (purchased from American Type Culture Collection, Manassas VA).

2.13 Antibodies

Western blot analysis was performed using a mouse monoclonal antibody raised against the RGS-His epitope (1/2000 Qiagen Inc.). The rabbit polyclonal antibody recognizing ubiquitin (1/600) was purchased from DAKO. EGFP (1/2000), p35 (1/500) and E2F-1(1/3000) were detected with rabbit polyclonal antibodies purchased from Santa Cruz Biotechnologies (Santa Cruz, CA. The mouse monoclonal E2F-1 (1/500) and the goat polyclonal HSP40 (1/200) antibodies were obtained from Santa Cruz (1/500). The mouse monoclonal actin antibody (1/10 000) and the rabbit polyclonal anti-PKCγ antibody (1/5000) was purchased from Sigma-Aldrich. Rabbit polyclonal antibodies used for the detection of Ac-H4 (K8), H4, PCAF and Ubb^{+1} were from Upstate Biotechnologies, Chicago IL. P300/CBP protein levels were assessed using a mouse monoclonal antibody (Upstate Biotechnologies). Anti-Calbindin rabbit polyclonal antibody was purchased from Chemicon International, Temecula CA. HSP 70 and Bip polyclonal antibodies were purchased from Stressgen (San Diego, CA). The anti-tubulin (1/50) was a gift from Dr.Michael McBurney (Ottawa Hospital Regional Cancer Centre). The horseradish

peroxidase conjugated mouse (1/5000) and rabbit secondary antibodies (1/5000) were purchased from Bio-Rad and Pierce respectively.

Chapter 3
Results

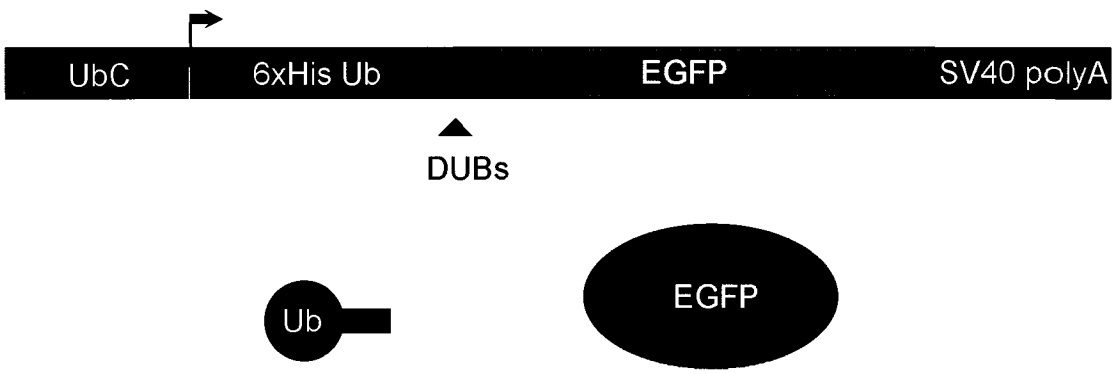
3.1 Analysis of ubiquitin variants in mammalian cells

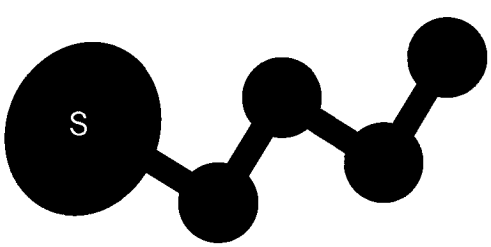
3.3.1 Expression of 6xHis-wild-type and mutant ubiquitin-EGFP constructs in cultured mammalian cells

Under natural conditions, ubiquitin is never expressed as a monomer, but as a linear fusion, either in the form of ubiquitin polymers (Finley et al., 1987) that comprise tandemly repeated ubiquitin coding sequences (Ozkaynak et al., 1984) or as a linear fusion between ubiquitin and ribosomal subunits (Ozkaynak et al., 1987; Hochstrasser, 1996). In both cases, it is thought that the translated fusions are processed to provide monomeric ubiquitin by enzymes with ubiquitin-cleaving activity, either the ubiquitin-specific proteases (Baker et al., 1992) or the ubiquitin carboxyterminal hydrolases (Larsen et al., 1998). This property of ubiquitin processing was exploited to generate in frame fusion of RGS-6xHis epitope tagged ubiquitin moieties with EGFP thus replacing the ribosomal subunit with an easily detectable marker that would serve as a proxy for detection of the tagged ubiquitin (schematically depicted in Fig 6). The objective of this strategy was to incorporate an epitope tag at the amino terminus of human ubiquitin that, when expressed in both cultured mammalian cells and transgenic animals, would facilitate the *in vivo* detection and recovery of ubiquitinated substrates. Prior to the creation of transgenic mice, the ubiquitin-EGFP expression constructs consisting of wt Ub-EGFP, K48R Ub-EGFP and K63R Ub-EGFP were tested by transient transfection into cultured murine HT4 cells. The observation of transfected cells under fluorescence revealed that most if not all cells were uniformly fluorescent and stable clones of fluorescent HT4 cells were selected for further analysis. These stable clones were found to be uniformly fluorescent after several

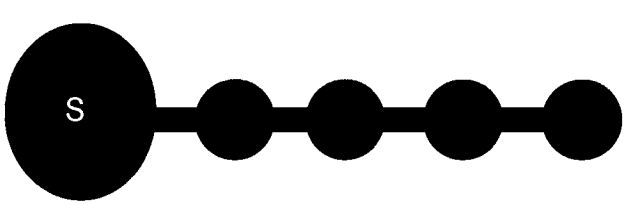
Figure 6. General design of ubiquitin expression constructs

The human ubiquitin C promoter (UbC) was used to drive the expression of hexahistidine-tagged human ubiquitin (Ub) fused in frame to enhanced Green Fluorescent protein (EGFP). The simian virus 40 polyadenylation signal (pA) was placed downstream. The arrow shows the Ub-EGFP cleavage site. The EGFP marker serves as a proxy for detection of the transgene. The epitope tagged ubiquitin moiety serves in the assembly of polyubiquitin chains assembled through Lys residues of ubiquitin (K48, K63 and others) or in the mono-ubiquitination of cellular substrates. Although ubiquitin chains are indicated by one color, the chains assembled via Lys residues are composed by a mixture of endogenous and transgene-derived ubiquitin. DUBs: Deubiquitinating enzymes. S: protein substrate.

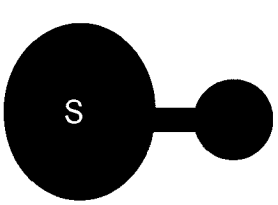




K48 linked chains



K63 linked chains



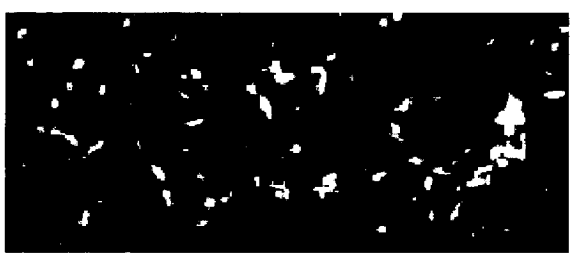
Mono-ubiquitin signal

passages, indicating that expression of epitope-tagged ubiquitin was not incompatible with cell growth and division (Fig 7A). Lysates of transfected cells were used to analyze the processing of the fusion protein and the availability of the tagged ubiquitin for conjugation. An antibody that was specific for EGFP detected a single protein species whose mass was consistent with processed EGFP and not of the ubiquitin-EGFP fusion protein (Fig 8, boxed panel). As expected, the detection of this protein species suggested that the fusion protein was recognized and efficiently processed by cellular enzymes in a manner that was similar to the endogenous ubiquitin fusions. The processing of the fusion protein was further confirmed by western analysis using an antibody directed against the 6xHis epitope-tag which detected a protein species whose mass was consistent with the 6xHis ubiquitin monomer as well as higher order ubiquitinated species (Fig 8, anti-His). The ubiquitin monomer and higher order conjugates were absent from extracts of un-transfected control cells. The presence of endogenous ubiquitin was confirmed by probing cellular extracts of transfected cells with an antibody directed against ubiquitin (Fig 8, anti-Ub). The antibody detected higher order ubiquitinated species as well as the ubiquitin monomer. The incorporation of epitope tagged ubiquitin in higher order ubiquitinated species was confirmed using co-immunoprecipitation experiments by the transient co-transfection of E2F-1, a well-characterized substrate of the ubiquitin/proteasome pathway with wild type ubiquitin (Hateboer et al., 1996; Hofmann et al., 1996; Campanero and Flemington, 1997). As expected, higher order ubiquitinated forms of E2F-1 were detected by western blot analysis using an antibody raised against the 6xHis epitope in cell extracts co-transfected with wt Ub-EGFP and E2F-1 plasmids (Fig 9A). These species were absent in cells co-transfected with the E2F-1 vector and a control EGFP plasmid.

Figure 7. Expression of 6xHis epitope tagged ubiquitin-EGFP in transfected cells

A) Murine HT4 neuroblastoma cells stably transfected with the expression plasmid encoding the wt Ub-EGFP showed intense green fluorescence when viewed under ultraviolet light. B) Comparison of the same field viewed under visible light revealed that most if not all cells were fluorescent, although there was variability in the intensity of the fluorescent signal.

A



B

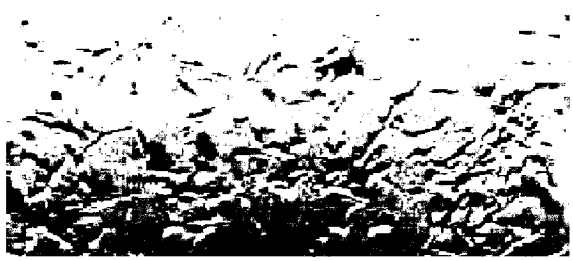
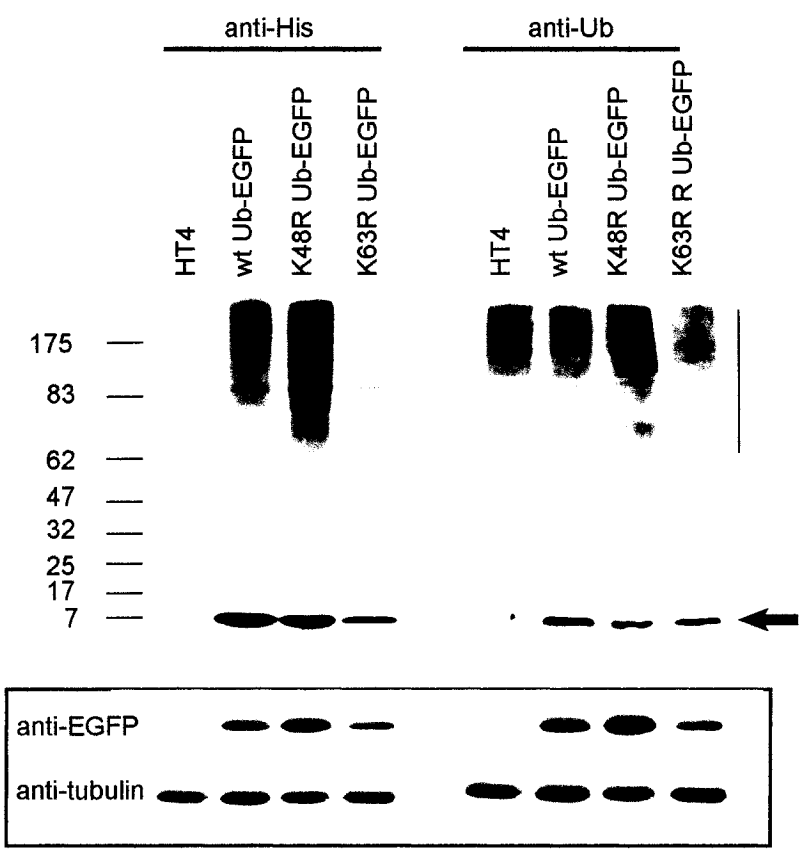


Figure 8. Detection of epitope-tagged ubiquitin variants

Western blot analysis of cell extracts from cells transfected with either wt Ub-EGFP, K48R Ub-EGFP or K63R Ub-EGFP with the 6xHis epitope-tag antibody (anti-His). The antibody detected the presence of high molecular weight ubiquitin conjugates (vertical line) as well as the epitope-tagged ubiquitin monomer (arrow) in lysates from cells stably transfected with the wt Ub-EGFP, K48R Ub-EGFP and K63R Ub-EGFP; these species were not detected in lysates from untransfected HT4 control cells. Monomeric ubiquitin (arrow) could be detected in all lysates using an anti-ubiquitin antibody (anti-Ub; which detected both endogenous and transgene-derived ubiquitin). The migration of markers is indicated on the left, with molecular mass in kilodaltons. The membrane was stripped and probed simultaneously for EGFP and tubulin expression (boxed panel). The EGFP-specific antibody detected the processed EGFP marker protein (anti-EGFP) and was indicative of the levels of transgene expression. Tubulin served as a loading control.


anti-His
anti-Ub
HT4
wt Ub-EGFP
K48R Ub-EGFP
K63R Ub-EGFP
HT4
wt Ub-EGFP
K48R Ub-EGFP
K63R R Ub-EGFP
175
83
62
47
32
25
17
7
anti-EGFP
anti-tubulin

The presence of E2F-1 in conjugates was confirmed by re-probing the membrane with an antibody directed against the E2F-1 protein (Fig 9B). Having established that the fusion proteins were efficiently processed and that the ubiquitin moieties generated therein were incorporated into higher order conjugates, it was determined if protein substrates could be recovered by standard nickel affinity chromatography. Stably transfected wt Ub-EGFP expressing cells treated with 50 μM of proteasome inhibitor for 3 hours (to block protein degradation and enhance the detection of ubiquitinated proteins) were harvested in protein lysis buffer and the soluble component of the cellular extract was purified by fast protein liquid chromatography (FPLC). Analysis of FPLC purified fractions by western blot analysis with the 6xHis antibody revealed the presence of monomeric ubiquitin as well as high molecular weigh conjugates (Fig 10A). These species were absent in fractions of untransfected control cells (Fig 10B). These data indicated that the expression vector was functional and that the purification strategy was feasible.

3.2 Sensitivity of mammalian cells expressing mutant ubiquitin to DNA and protein damaging agents.

Oxidative damage has been associated with aging as well as with the onset or progression of a variety of neuropathological states (reviewed in (Alves-Rodrigues et al., 1998)). It has been proposed that oxidation of cellular proteins may induce the formation of the proteinaceous inclusions that have long been associated with neuropathological states through the generation of aberrant protein structures with a propensity to aggregate. Proteins that misfold as a consequence of genetic mutations may suffer a similar fate. There is convincing evidence from studies in yeast that a functional ubiquitin/proteasome pathway is required to degrade misfolded or oxidatively damaged proteins (including

Figure 9. Conjugation of tagged wild-type ubiquitin to a cellular substrate

293T cells were co-transfected with an expression vector encoding E2F-1 and either an EGFP control or the wt Ub-EGFP plasmids. A) The lysates were immunoprecipitated with anti-E2F-1 antibody and analyzed by western blot analysis with the 6xHis antibody. The antibody detected high molecular weigh conjugates (vertical line) in immunoprecipitates from cells expressing wt Ub-EGFP that were absent in immunoprecipitates from cells expressing an EGFP control plasmid. B) Same membrane as in A) reprobed with an antibody raised against E2F-1 confirming the presence of the E2F-1 protein and respective conjugates in immunoprecipitates and total cell extracts. The asterisks denote the heavy and light chains respectively.

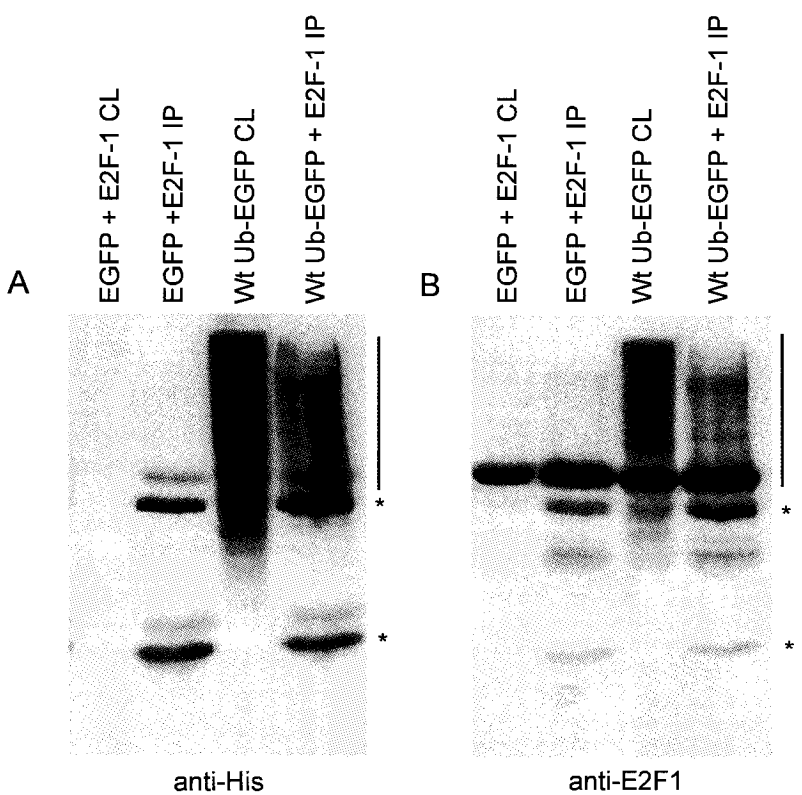
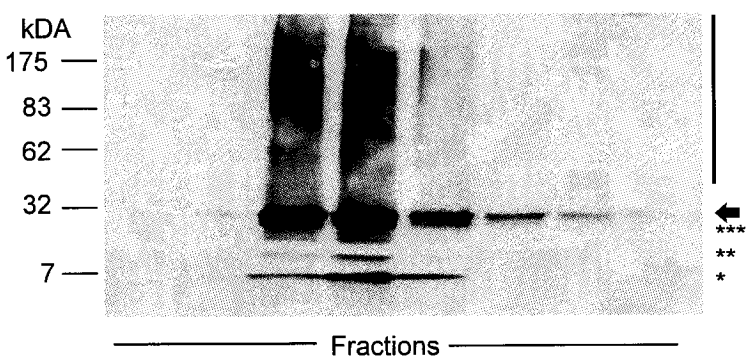

A
EGFP + E2F-1 CL
EGFP +E2F-1 IP
Wt Ub-EGFP CL
Wt Ub-EGFP + E2F-1 IP
*
*
anti-His
B
EGFP + E2F-1 CL
EGFP +E2F-1 IP
Wt Ub-EGFP CL
Wt Ub-EGFP + E2F-1 IP
*
*
anti-E2F1

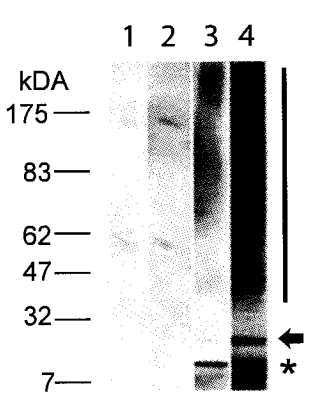
Figure 10. Recovery of 6xHis ubiquitin and ubiquitinated substrates by nickel matrix FPLC

A) A lysate from HT4 cells stably expressing wt Ub-EGFP was subjected to FPLC, and eluted fractions were analyzed by western blot analysis using the 6xHis epitope-tag antibody. Eluted fractions contained transgene-derived monomeric ubiquitin (indicated by an *) and slower migrating species consistent with di-and tri-ubiquitins (indicated by ** and ***, respectively). The smear (vertical line) probably represents various protein substrates with ubiquitin chains of variable length. The arrow indicates an ubiquitinated protein whose abundance and mass is consistent with monoubiquitinated histone H2A (uH2A). B) FPLC controls. Lysates from untransfected HT4 cells (Lane 1 and 2) and from cells stably transfected with wt Ub-EGFP (lane 3 and 4) were subject to FPLC as before. Eluted fraction 25 from both preparations was analyzed by western blotting using the 6xHis antibody (lane 2 and 4) or an anti-ubiquitin antibody (lane 1 and 3). Nothing was retained by FPLC from untransfected HT4 cells (lane 1 and 2) that was detected with either antibody, whereas the high molecular weight smear from the transfected cells (lane 3 and 4) was immunoreactive for both the epitope tag (lane 3) and for ubiquitin (lane 4). Symbols are as in panel A.

A



B



canavanyl proteins, (Finley et al., 1994; Chen and Piper, 1995; Lambertson et al., 1999) and the failure of the pathway to do so may account for the accumulation of protein aggregates that can compromise neuronal viability. A dominant-negative strategy was employed to test the hypothesis that a deficiency in the ubiquitin-mediated proteolytic pathway would sensitize mammalian cells to agents that induce oxidative damage to cellular components.

3.2.1 Accumulation of proteasome substrates in cells expressing a chain-terminating version of ubiquitin.

The expression of dominant-negative K48R mutant ubiquitin that interferes with the assembly of proteasomal targeting signals on cellular proteins was found to increase the half-life of protein substrates in yeast (Finley et al., 1994). To determine whether mammalian cells expressing K48R mutant ubiquitin present a general deficit in proteolysis, even in the absence of any exogenous stressors, the abundance of two unrelated substrates of the ubiquitin/proteasome pathway were examined by transient co-transfection. One was the natural substrate, E2F-1, whose degradation via ubiquitin proteolysis is well documented (Hateboer et al., 1996; Hofmann et al., 1996; Marti et al., 1999) and the other was GFP^u, a synthetic proteasome substrate generated through the addition of a destabilizing peptide sequence to EGFP. GFP^u has been shown to accumulate in cells presented with an overwhelming burden of misfolded protein or in cells treated with proteasome inhibitor (Bence et al., 2001). Western blot analysis with an E2F-1 specific antibody of cell extracts from wt Ub-EGFP and K48R Ub-EGFP expressing cells that were transiently transfected with E2F-1 revealed that E2F-1 was roughly twice as abundant in K48R Ub-EGFP expressing cells compared to cells expressing wt Ub-EGFP and endogenous ubiquitin (Fig 11). An even greater effect was observed for GFP^u. Western

blot analysis with an antibody raised against EGFP revealed a > 5 fold increase in GFP^u protein levels in cells stably expressing K48R Ub-EGFP compared with cells expressing the wild type ubiquitin transgene (Fig 11). As expected, this data suggested that protein substrates accumulate in mammalian cells expressing a chain-terminating version (K48R Ub-EGFP) of ubiquitin.

3.2.2 Sensitivity of transfected cells to Cadmium

The murine HT4 neuroblastoma cell line represents a well characterized system for the analysis of oxidative stress whereby treatment of cells with cadmium has been previously shown to result in both a decrease in intracellular glutathione levels and an increase in mixed protein disulfides (Figueiredo-Pereira et al., 1997, 1998). Most importantly, cadmium exposure has been shown to induce the profound ubiquitination of protein substrates, resulting in the accumulation of high molecular weight ubiquitinated conjugates (Figueiredo-Pereira et al., 1997, 1998; Figueiredo-Pereira and Cohen, 1999). In order to determine if expression of mutant forms of ubiquitin would sensitize mammalian cells to cadmium, a promiscuous agent previously reported to induce damage to cellular components, HT4 cells and pools of cells stably transfected with either wt Ub-EGFP, K48R Ub-EGFP or K63R Ub-EGFP were treated with 25μm Cd^{2+} for up to 8h. Western blot analysis with the 6xhis antibody of cell extracts from the stably transfected cells revealed that cadmium induced extensive ubiquitination of cellular proteins in all cell types in a time-dependent manner (Fig 12A). The accumulation of endogenous ubiquitinated proteins was detected by re-probing the membrane with an antibody directed against ubiquitin (Fig 12B). The results of the western analysis did not reveal any gross differences in the levels of ubiquitination among the different cell lines at any given point

Figure 11. Accumulation of proteasome substrates in cells expressing K48R mutant ubiquitin

A) Western blot analysis of HT4 extracts probed simultaneously for E2F-1 and tubulin. The ratios of E2F-1 intensities to tubulin intensities are presented in the boxed area below the blot. B) HT4 cell lysates probed simultaneously for EGFP and tubulin. Cells expressing transgene-derived ubiquitin also had detectable EGFP (asterisk), derived from processing of the ubiquitin-EGFP fusion protein. The GFP^{U} protein (arrow) migrated more slowly than EGFP.

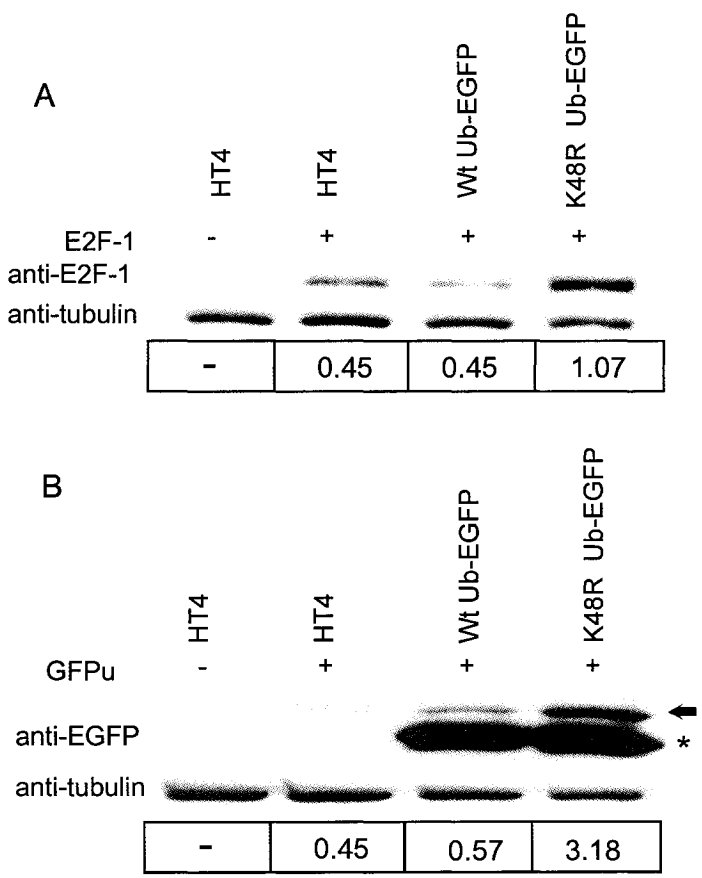
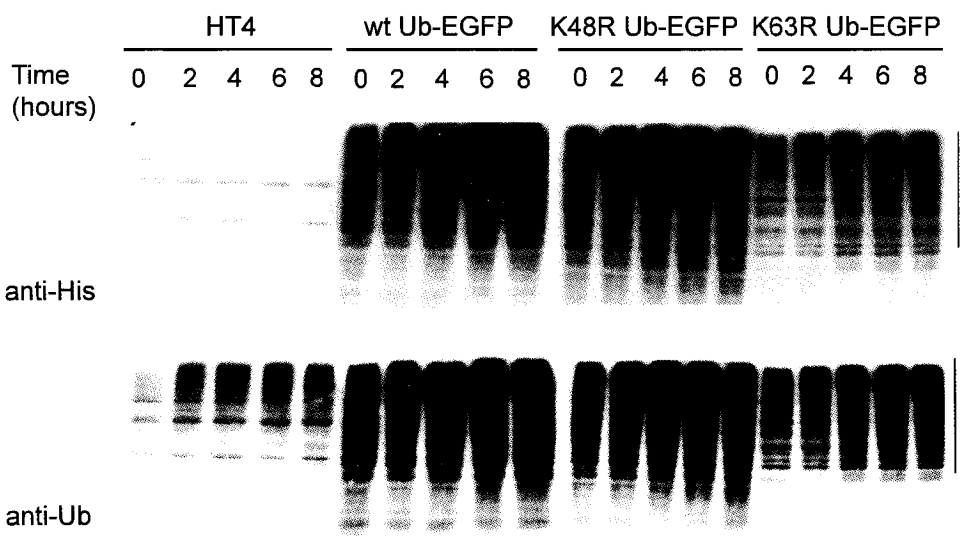

A
HT4
HT4
Wt Ub-EGFP
K48R Ub-EGFP
E2F-1
-
+
+
+
anti-E2F-1
anti-tubulin
-
0.45
0.45
1.07
B
HT4
HT4
Wt Ub-EGFP
K48R Ub-EGFP
GFPu
-
+
+
+
anti-EGFP
*
anti-tubulin
-
0.45
0.57
3.18

Figure 12. Time-dependent accumulation of ubiquitinated proteins following cadmium treatment

Cells were incubated with 25 μM cadmium sulfate for 0 (control) to 8 h prior to harvesting. The results shown are from Western blot analysis of extracts from cadmium sulfate-treated HT4 cells and pooled populations expressing wild-type ubiquitin, K48R mutant ubiquitin, or K63R mutant ubiquitin. Upper panel, Western blot probed with the 6xHis epitope-tag antibody. B) The same blot reprobed with an anti-ubiquitin antibody (anti-Ub). Conjugates were not detected in HT4 control cells using the 6xHis epitope-tag antibody, but could be detected when the blot was reprobed with an anti-ubiquitin antibody. High molecular weight conjugates are indicated by vertical bars. Cadmium sulfate was found to induce the accumulation of conjugates incorporating tagged ubiquitin in transfected HT4 lines.


HT4
wt Ub-EGFP
K48R Ub-EGFP
K63R Ub-EGFP
Time (hours)
0 2 4 6 8 0 2 4 6 8 0 2 4 6 8 0 2 4 6 8
anti-His
anti-Ub

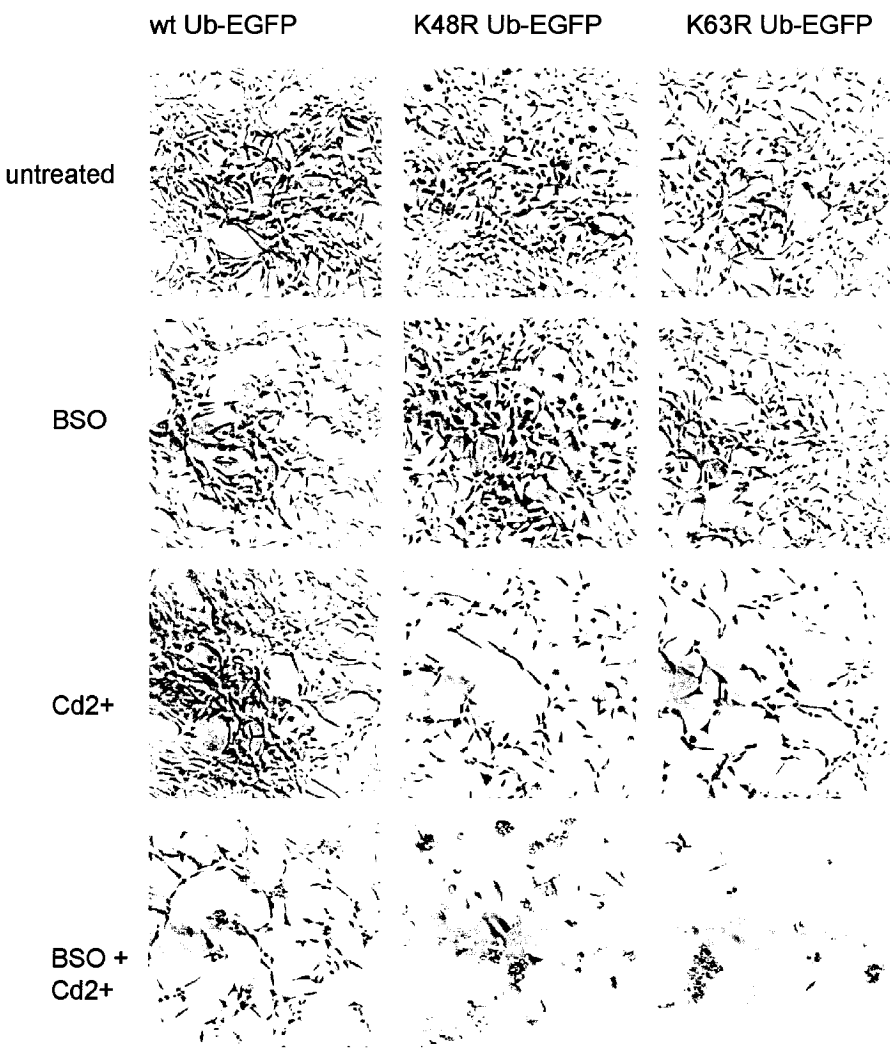
(Fig 12). However, when the effects of cadmium were assessed by light microscopy it was found that the cell morphology was altered in all cell types in a time and dose dependent manner mostly resulting in cell rounding and loss of adhesion (Fig 13). There was no profound difference in the percentage of dead to live cells among cells treated with 5 μm cadmium for 24h; however, there were notable differences in the appearance of cells, with K48R and K63R mutant ubiquitin-expressing cells being more severely affected than untransfected cells or cells expressing exogenous wild-type ubiquitin (Fig 13). It appears that under these conditions, many cells detached, but were still able to exclude the trypan blue dye. When glutathione was depleted by pretreatment with buthionine sulfoximine (BSO), thereby eliminating the predominant cellular defense against oxidation, the sensitivity to cadmium (as assessed by loss of adherence) was found to be increased in K48R pools, but was most pronounced in cells expressing K63R mutant ubiquitin (Fig 13), where very few attached cells remained. This alteration in morphology was not observed when cells were treated with BSO alone.

3.2.3 Sensitivity of transfected cells to canavanine

Unlike cadmium, which may directly or indirectly generate damage to nascent protein, canavanine is a naturally occurring amino acid analog that substitutes for arginine during translation, resulting in protein misfolding. In yeast, ubiquitin-mediated proteolysis is required to eliminate canavanyl proteins that would otherwise accumulate with deleterious effects (Chen and Piper, 1995; Lambertson et al., 1999). To determine whether mammalian cells expressing mutant forms of ubiquitin would be sensitive to a burden of misfolded protein, 20 mM canavanine was added to the culture medium for 30h and its toxicity to the various cell pools was assessed by phase-contrast microscopy of cells. It

Figure 13. Morphologic response to cadmium sulfate exposure

HT4 control cells and pooled populations expressing wild-type ubiquitin, K48R mutant ubiquitin or K63R mutant ubiquitin were treated with 5 μM cadmium sulfate for 24 h and visualized by light microscopy. In the lower panels, cells were pretreated with 10 mM buthionine sulfoximine (BSO) prior to 24-h incubation with 5 μM cadmium sulfate. Cells were photographed at low power (40 × magnification). These results are representative of four repeat experiments.


wt Ub-EGFP
K48R Ub-EGFP
K63R Ub-EGFP
untreated
BSO
Cd2+
BSO +
Cd2+

was found that K48R mutant ubiquitin expressing cells exhibited the highest sensitivity to canavanine exposure as indicated by the dramatically altered cell morphology (Fig 14). K63R Ub-EGFP expressing cells were found to be as sensitive to canavanine as the untransfected HT4 controls, whereas cells expressing wt Ub-EGFP showed increased resistance to canavanine as evidenced by their morphology (Fig 14). The response to canavanine was quantified by counting live and dead cells, both adherent and detached. The analysis revealed that among treated cells, there were significantly more detached cells in the pools of K48R Ub-EGFP expressing cells, and a higher proportion of these cells were dead compared with cells expressing other forms of ubiquitin (Fig 15). Western blot analysis of cell extracts from cells treated with 10 mM canavanine for 24h revealed that canavanine could induce the accumulation of high molecular weight conjugates in all three cell types, with the greatest effects noted in cells expressing K48R mutant ubiquitin (Fig 16A). The membrane was re-probed with an antibody directed against ubiquitin to confirm the presence of ubiquitin in the high molecular weight conjugates in extracts from the stably transfected cells as well as the ubiquitination and accumulation of endogenous substrates in the HT4 control cell extract (Fig 16B). Densitometric analysis revealed that there was an approximately 3 fold increase in the level of ubiquitin conjugates in K48R Ub-EGFP expressing cells (Fig 16C).

3.3 Functional constraints of the carboxyl terminus of ubiquitin

The covalent attachment of ubiquitin to the lysine residue of a protein substrate occurs through the carboxyl-terminal glycine of ubiquitin and alterations affecting the C- terminus have profound effects on ubiquitin metabolism in yeast (Ecker et al., 1987a; Hodgins et al., 1992). The expression of G76A variant of ubiquitin in yeast results in a diminished

Figure 14. Response of cells to canavanine

HT4 control cells and pooled populations stably expressing wild-type ubiquitin, K48R mutant ubiquitin, or K63R mutant ubiquitin were treated with 20 mM canavanine for 30 h and then visualized by light microscopy. Cells were photographed at low power (40 × magnification).

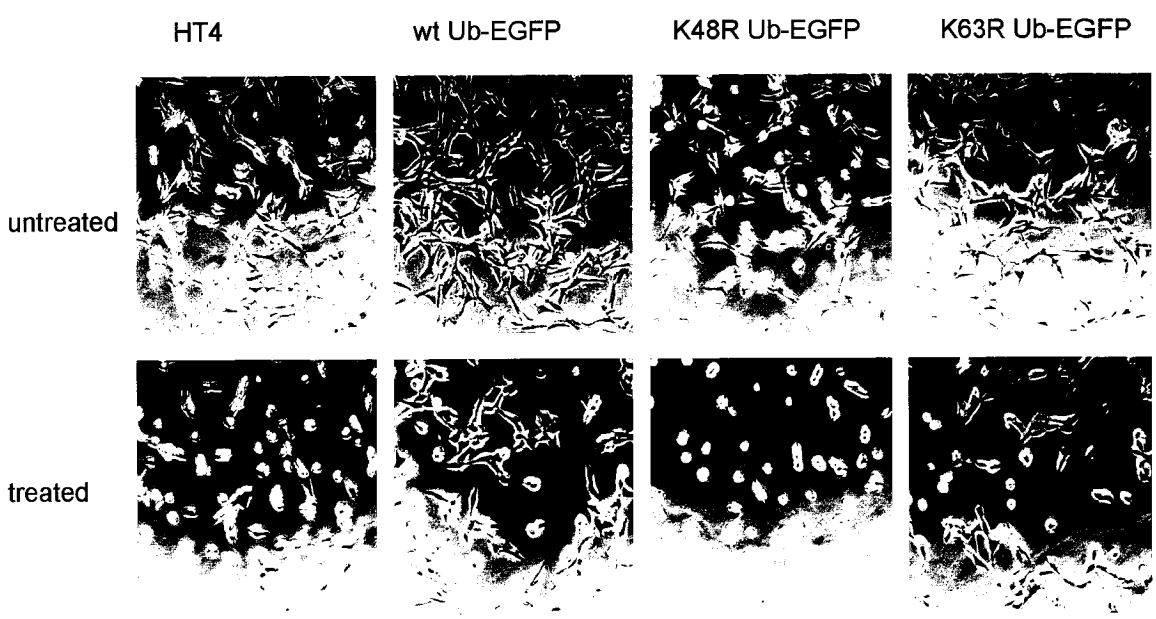

HT4
wt Ub-EGFP
K48R Ub-EGFP
K63R Ub-EGFP
untreated
treated

Figure 15. Quantitative analysis of both the attached and detached fractions

HT4 control cells and pooled populations stably expressing wild-type ubiquitin, K48R mutant ubiquitin, and K63R mutant ubiquitin were plated in triplicate and treated with 20 mM canavanine for 24 h prior to collecting the attached and detached fractions independently. Viability in the two fractions was measured by trypan blue exclusion. *wt Ub*, wild-type ubiquitin; *K48R Ub*, K48R mutant ubiquitin; *K63R Ub*, K63R mutant ubiquitin.

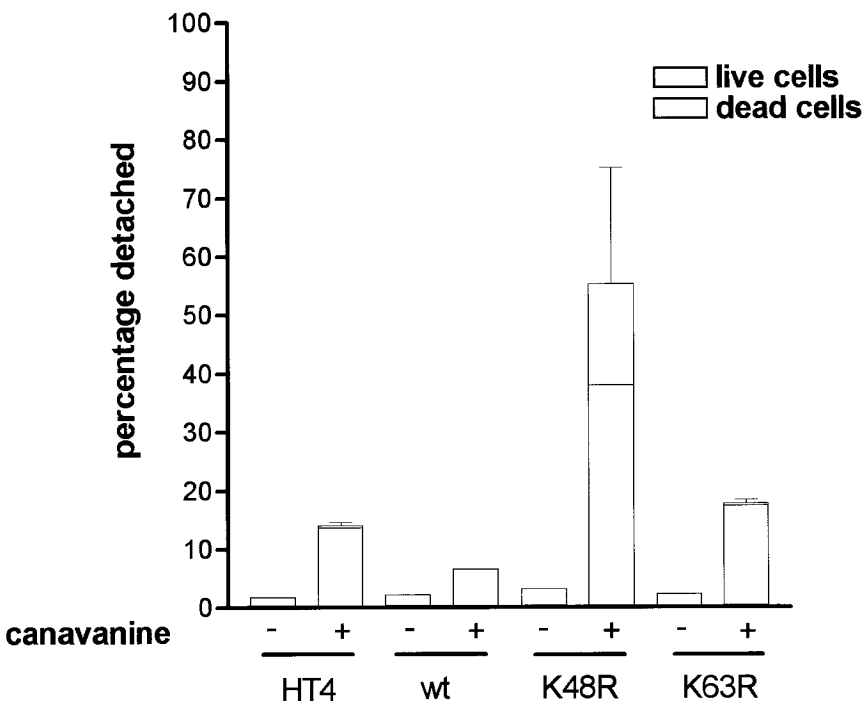
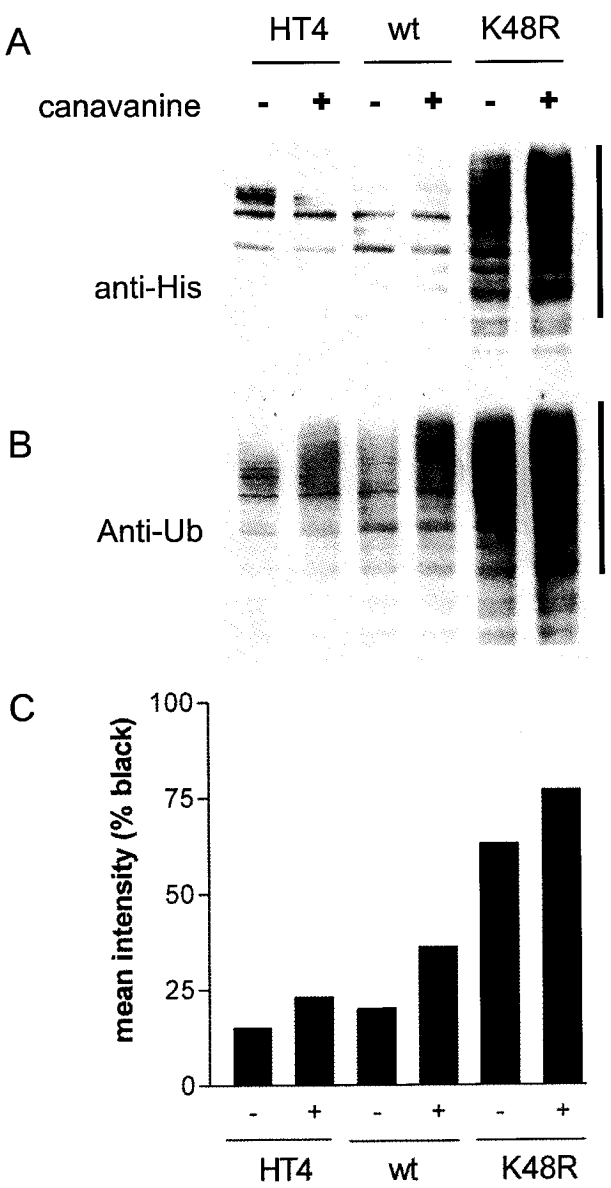

live cells
dead cells
percentage detached
100
90
80
70
60
50
40
30
20
10
0
canavanine
- + - + - + - +
HT4
wt
K48R
K63R

Figure 16. Accumulation of high molecular weight ubiquitin conjugates following incubation with 10 mM canavanine for 24 h

A) Western blot analysis of cell extracts from HT4 cells or stable clones expressing wild-type (wt) ubiquitin or K48R mutant ubiquitin probed with an antibody directed against the 6xHis epitope. B) The membrane from A) stripped and reprobed with an anti-ubiquitin antibody (Anti-Ub). Ubiquitin was detected in the conjugates (vertical bars) of untransfected HT4 control cells; in transfected cells, conjugates also incorporated the tagged ubiquitin. C) Quantification of the results presented in B. The values represent the mean intensity of pixels within entire lanes, expressed as the percentage of black (the raw scanned data were in the form of 256 shades of gray).


A
HT4
wt
K48R
canavanine
- + - + - +
anti-His
B
Anti-Ub
C
mean intensity (% black)
100
75
50
25
0
- + - + - +
HT4
wt
K48R

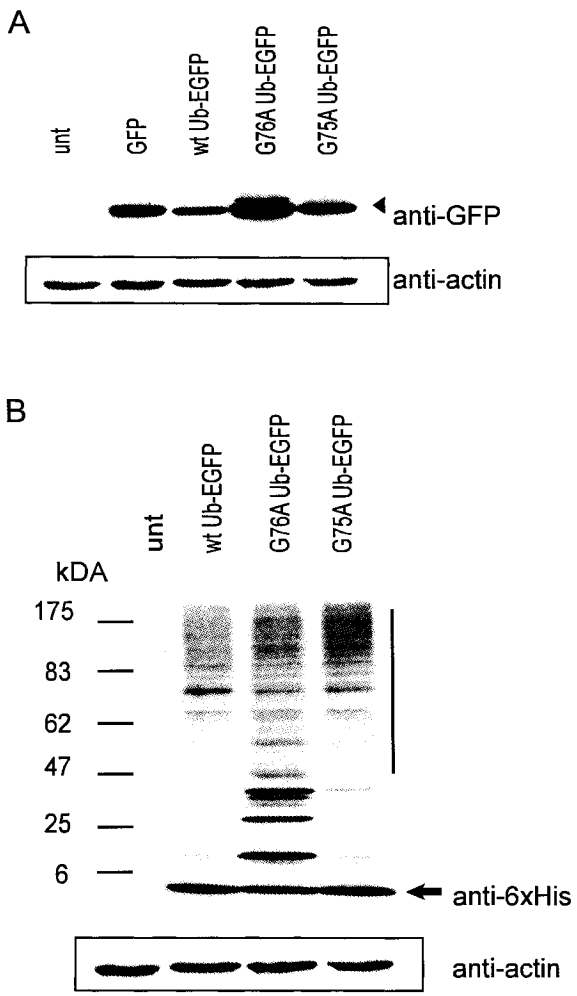
growth rate and to lethality when representing the sole source of ubiquitin presumably by interfering with the assembly of targeting signals on key cellular proteins (Hodgins et al., 1992). In order to determine the phenotype of cells expressing C-terminal ubiquitin variants an expression vector encoding a G76A Ub-EGFP was generated and transiently introduced in 293T cells.

3.3.1 The case of G76A mutant ubiquitin

Western blot analysis with the EGFP specific antibody of cell extracts from cells transiently transfected with G76A Ub-EGFP revealed the presence of a protein species at the expected size of the processed EGFP marker that co-migrated with EGFP from extracts transfected with an EGFP control plasmid (Fig 17A). Analysis of the same extracts with the 6xHis epitope-tag antibody revealed the presence of a protein species at a molecular weight that was consistent with tagged monomeric ubiquitin (Fig 17B). In addition to detecting this species, the antibody detected higher order ubiquitinated species that were similar to the higher order conjugates observed in extracts from cells transfected with the wt Ub-EGFP expression vector (Fig 17B). To assess whether the G76A Ub-EGFP fusion protein was differentially processed due to the presence of a highly conserved glycine at position 75, a G75A Ub-EGFP mutant was generated and its processing was investigated by transient transfection. Western blotting with the EGFP-specific antibody revealed that the G75A Ub-EGFP fusion protein was processed in the same manner as the wild type Ub-EGFP and G76A Ub-EGFP fusion proteins (Fig 17A). When membranes were probed with the antibody specific for the 6xHis epitope tag, HMW species as well as the ubiquitin monomer were detected by the antibody in extracts from G75A Ub-EGFP transfected cells (Fig 17B). In order to determine whether the HMW conjugates detected in G76A Ub-EGFP and G75A Ub-EGFP transfected cells were comprised of mutant ubiquitin attached

Figure 17. Detection of epitope tagged G75A and G76A mutant ubiquitin

A) Western blot analysis with an EGFP-specific antibody. The antibody detected the presence of the processed EGFP marker (arrowhead) in lysates from cells transiently transfected with wt Ub-EGFP, G76A Ub-EGFP and G75A Ub-EGFP; the EGFP marker was not detected in the lysates of untransfected control cells. B) Western blot analysis of the same extracts as in A) with the antibody raised against the 6xHis epitope-tag. High molecular weight ubiquitin conjugates (vertical line) were detected in the presumed conjugates as well as the epitope-tagged ubiquitin monomer (arrow). These species were absent from the untransfected control lysate. Actin antibody (boxed panels) served as a loading control.


A
unt
GFP
wt Ub-EGFP
G76A Ub-EGFP
G75A Ub-EGFP
anti-GFP
anti-actin
B
unt
wt Ub-EGFP
G76A Ub-EGFP
G75A Ub-EGFP
kDA
175
83
62
47
25
6
anti-6xHis
anti-actin

to cellular substrates or un-anchored (no substrate) poly-ubiquitin chains that had incorporated mutant ubiquitin, an expression construct encoding the transcription factor E2F-1 was co-transfected with mutant ubiquitin constructs. Western blot analysis of E2F-1 immunoprecipitates with the 6xHis antibody revealed the presence of higher order ubiquitinated E2F-1 species in immunoprecipitates of cells co-transfected with wt Ub-EGFP, G76A Ub-EGFP and G76A Ub-EGFP that were not detected in immunoprecipitates from cells transfected with a control EGFP expression vector (Fig 18A). Membranes were re-probed with an E2F-1 specific antibody to confirm the presence of the E2F-1 protein and E2F-1 conjugates in immunoprecipitates and total cell extracts (Figure 18B). These data suggested that contrary to what has been observed in yeast cells, G76A and G75A mutant ubiquitin were functional in mammalian cells.

3.3.2 The processing of mammalian ubiquitin fusions is influenced by both the G75 and G76 residues

The relative importance of these two residues in processing by deubiquitinating enzymes was assessed by the generation of variants of ubiquitin in which both glycines were substituted to alanines (G75AG76A), an alanine was followed by an isoleucine (G75AG76I) or an isoleucine was followed by an alanine (G75IG76A). Western analysis of lysates from these transfected cells using the EGFP or 6xHis epitope-tag specific antibodies revealed the presence of a protein species at the expected size of the Ub-EGFP fusion protein (Fig 19A and B). In addition to recognizing the ubiquitin fusion protein, the EGFP-specific antibody detected the processed EGFP marker in lysates from cells transfected with G75AG76A Ub-EGFP and to a lesser extend in lysates from cells transfected with G75IG76A Ub-EGFP and G75AG76I Ub-EGFP (Fig 19A). The 6xHis epitope tag-specific antibody detected monomeric ubiquitin in the cell extract from cells

Figure 18. G75A and G76A variants of ubiquitin can conjugate to a cellular protein

A) Western blot analysis with the 6xHis epitope-tag specific antibody of immunoprecipitates or total cell extracts from cells co-transfected with E2F-1 and either wt Ub-EGFP, G76A Ub-EGFP or G75A Ub-EGFP mutant isoforms of ubiquitin. The antibody detected higher order ubiquitinated forms of E2F-1 (vertical line) in immunoprecipitates from cells co-transfected with E2F-1 and either wt Ub-EGFP, G75A Ub-EGFP or G76A Ub-EGFP. These HMW species were absent in immunoprecipitates from cells transfected with an EGFP control expression vector. B) Same membrane as in A) re-probed with an E2F-1 specific antibody confirming the presence of E2F-1(arrow) and E2F-1 conjugates (vertical line) in cell extracts and in immunoprecipitates from wild-type and mutant ubiquitin transfected cells.

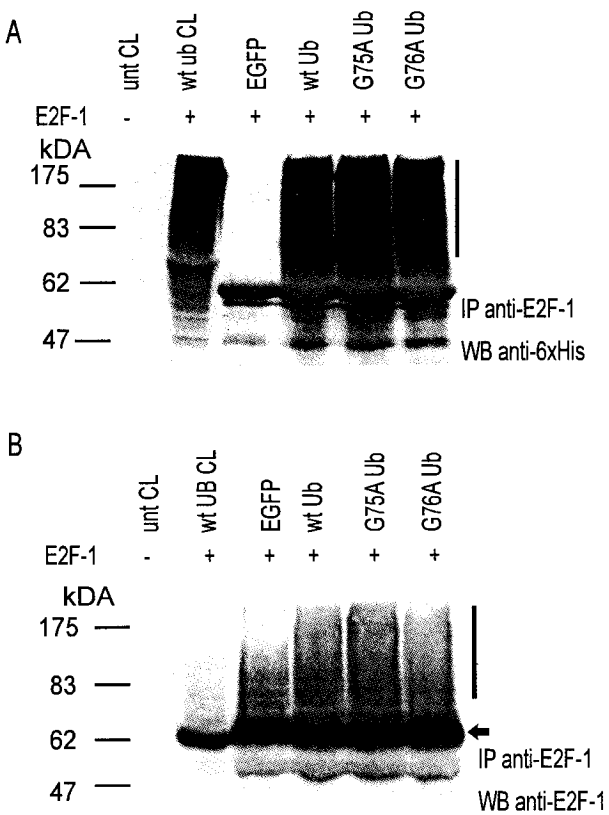
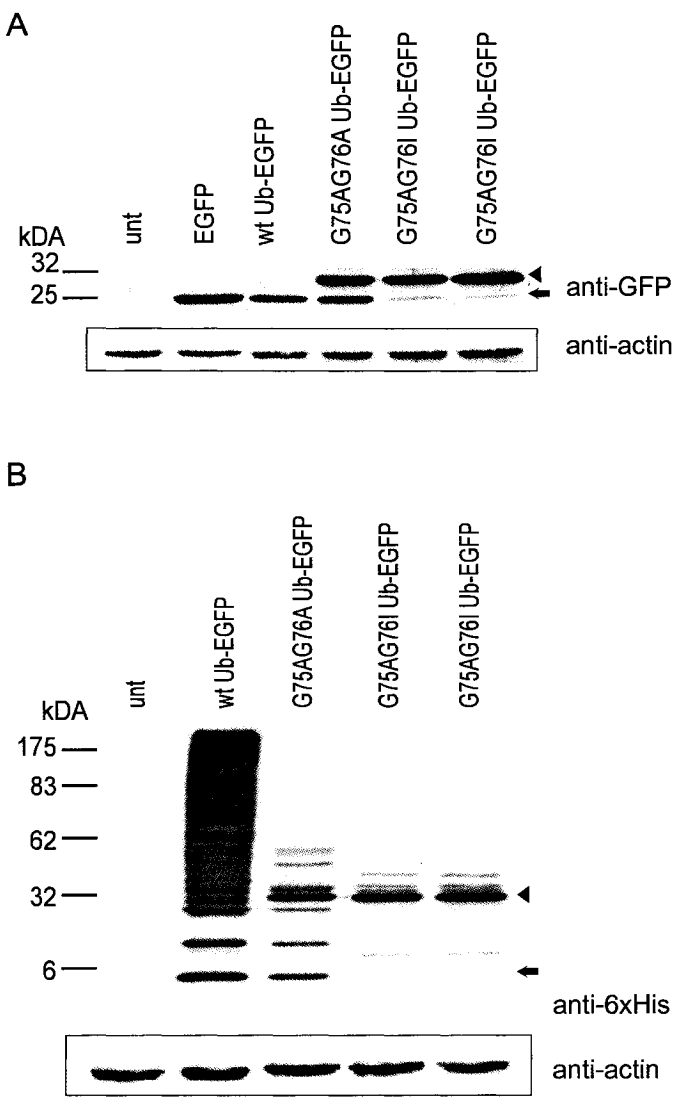

A
unt CL
wt ub CL
EGFP
wt Ub
G75A Ub
G76A Ub
E2F-1 - + + + + +
kDA
175
83
62
47
IP anti-E2F-1
WB anti-6xHis
B
unt CL
wt UB CL
EGFP
wt Ub
G75A Ub
G76A Ub
E2F-1 - + + + + +
kDA
175
83
62
47
IP anti-E2F-1
WB anti-E2F-1

Figure 19. Substitution of both G75 and G76 residues of ubiquitin impairs processing of the Ub-EGFP fusion protein

A) Western analysis of cell extracts from cells transfected with substitution mutants G75AG76A Ub-EGFP, G75AG76I Ub-EGFP and G75IG76A Ub-EGFP with an EGFP antibody detecting the Ub-EGFP fusion protein (arrowhead) as well as the processed EGFP marker (arrow). B) Western blot analysis of the same lysates as in A) using the 6xHis epitope-tag specific antibody. The antibody detected the Ub-EGFP fusion protein (arrowhead) in G75G76A Ub-EGFP, G75AG76I Ub-EGFP and G75IG76A Ub-EGFP transfected cells as well as transgene-derived epitope-tagged ubiquitin (arrow) in G75AG76A Ub-EGFP transfected cells. These species were absent in untransfected control lysates. Actin antibody (boxed panels) served as a loading control.


A
kDA
32
25
unt
EGFP
wt Ub-EGFP
G75AG76A Ub-EGFP
G75AG76I Ub-EGFP
G75AG76I Ub-EGFP
anti-GFP
anti-actin
B
kDA
175
83
62
32
6
unt
wt Ub-EGFP
G75AG76A Ub-EGFP
G75AG76I Ub-EGFP
G75AG76I Ub-EGFP
anti-6xHis
anti-actin

transfected with G75AG76A Ub-EGFP that was absent in cell extracts from cells transfected with the other mutant isoforms of ubiquitin (Fig 19B). HMW conjugates were absent in lysates from these transfected cells.

3.3.3 Constraints of the C-terminus of ubiquitin

The aforementioned findings suggested that the sequence constraints with regard to processing and conjugation of human ubiquitin were not as stringent as those previously reported in yeast. In order to investigate the constraints on the sequence identity of the G76 residue of human ubiquitin with regard to processing and conjugation to cellular substrates, a comprehensive study of the C-terminus of ubiquitin was undertaken wherein the G76 residue of ubiquitin was mutated to every possible amino acid residue and expressed as a linear fusion with EGFP. Western blot analysis of cell extracts with an antibody directed against EGFP revealed the presence of the Ub-EGFP fusion protein in extracts from mutant ubiquitin transfected cells with the exception of wt Ub-EGFP and G76A Ub-EGFP transfectants in which the processed EGFP marker was the only species detected (Fig 20A and B). In addition, the processed form of EGFP was detected in lysates from cells transfected with the majority of mutant ubiquitin variants with the exception of G76L Ub-EGFP, G76P Ub-EGFP, G76V Ub-EGFP and G76W Ub-EGFP in which the processed EGFP was not detected (Fig 20A and B). Re-probing the membranes with an antibody directed against the 6xHis epitope-tag antibody confirmed the presence of the Ub-EGFP fusion protein in lysates from mutant ubiquitin transfected cells and revealed the presence of transgene-derived monomeric ubiquitin in lysates from cells expressing the various mutant ubiquitin constructs. The epitope tagged ubiquitin monomer was not detected in cells expressing G76E Ub-EGFP, G76I Ub-EGFP, G76L Ub-EGFP, G76P Ub-

Figure 20. Processing of G76 variants of ubiquitin

A) and B) western blot analysis with an EGFP specific antibody of cell extracts from cells transfected with G76 variants. The Ub-EGFP fusion protein (arrow) and the processed EGFP marker (asterisk) were detected in mutant ubiquitin transfected cells. Actin (boxed panels) served as a loading control.

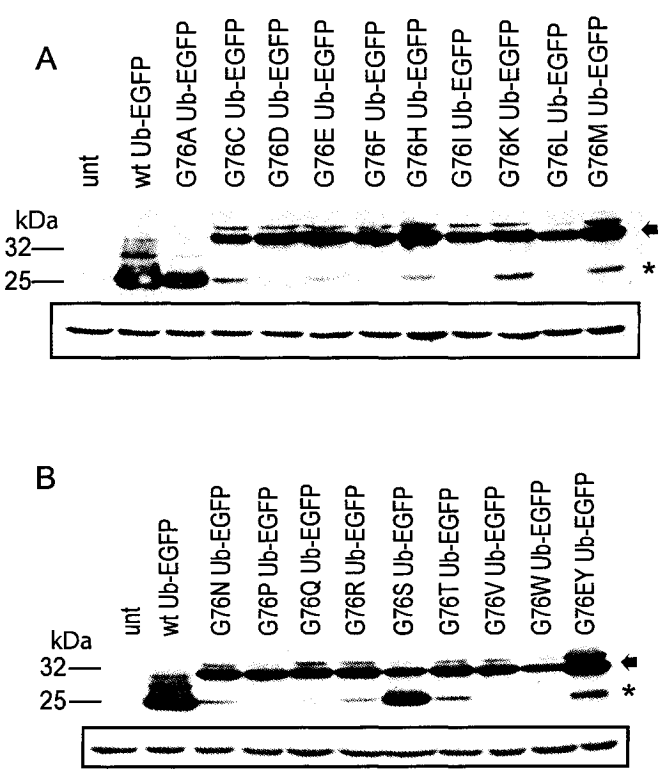
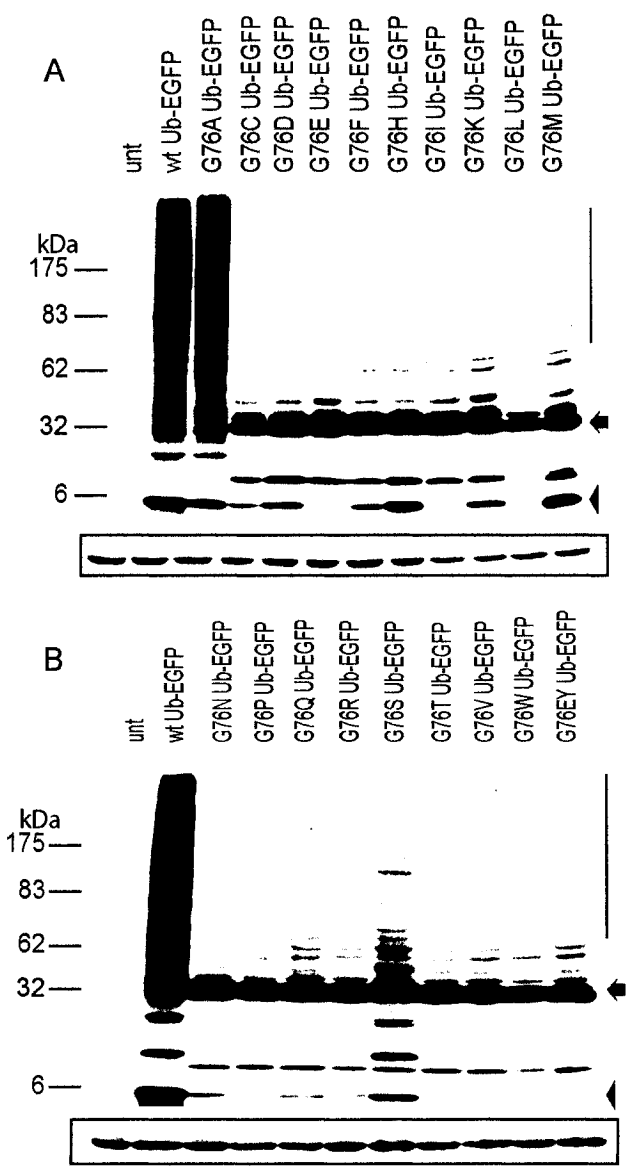

A
unt
wt Ub-EGFP
G76A Ub-EGFP
G76C Ub-EGFP
G76D Ub-EGFP
G76E Ub-EGFP
G76F Ub-EGFP
G76H Ub-EGFP
G76I Ub-EGFP
G76K Ub-EGFP
G76L Ub-EGFP
G76M Ub-EGFP
kDa
32
25
*
B
unt
wt Ub-EGFP
G76N Ub-EGFP
G76P Ub-EGFP
G76Q Ub-EGFP
G76R Ub-EGFP
G76S Ub-EGFP
G76T Ub-EGFP
G76V Ub-EGFP
G76W Ub-EGFP
G76EY Ub-EGFP
kDa
32
25
*

Figure 21. Detection of transgene-derived G76 mutated ubiquitin variants

A) and B western blot analysis with the antibody directed against the 6xHis epitope-tag. An arrow denotes the Ub-EGFP fusion protein. The epitope-tagged ubiquitin monomer derived from processing of the Ub-EGFP fusion protein is denoted by an arrowhead. HMW conjugates (vertical line) were detected in the extracts of cells expressing wt Ub-EGFP, G76A Ub-EGFP and to a lesser extend G76S Ub-EGFP and G76H Ub-EGFP.


A
unt
wt Ub-EGFP
G76A Ub-EGFP
G76C Ub-EGFP
G76D Ub-EGFP
G76E Ub-EGFP
G76F Ub-EGFP
G76H Ub-EGFP
G76I Ub-EGFP
G76K Ub-EGFP
G76L Ub-EGFP
G76M Ub-EGFP
kDa
175
83
62
32
6
B
unt
wt Ub-EGFP
G76N Ub-EGFP
G76P Ub-EGFP
G76Q Ub-EGFP
G76R Ub-EGFP
G76S Ub-EGFP
G76T Ub-EGFP
G76V Ub-EGFP
G76W Ub-EGFP
G76EY Ub-EGFP
kDa
175
83
62
32
6

EGFP and G76V Ub-EGFP (Fig 21A and B). High molecular weight (HMW) conjugates were detected in lysates from wt Ub-EGFP, G76A Ub-EGFP and to a lesser extend in G76S Ub-EGFP and G76Q Ub-EGFP expressing cells (Fig 21 and B). As expected, the HMW smear and ubiquitin monomer were absent in the untransfected control cell extract. Co- immunoprecipitation experiments were performed to determine whether the HMW conjugates detected in G76S Ub-EGFP and G76Q Ub-EGFP transfected cells were comprised of mutant ubiquitin attached to cellular substrates or un-anchored poly-ubiquitin chains that had incorporated mutant ubiquitin. As previously described, an expression construct encoding the transcription factor E2F-1 was co-transfected with either EGFP, wt Ub-EGFP or mutant ubiquitin constructs. Western blot analysis of E2F-1 immunoprecipitates with the 6xHis antibody revealed the presence of higher order ubiquitinated E2F-1 species in immunoprecipitates of cells co-transfected with wt Ub-EGFP, G76A Ub-EGFP and G76S Ub-EGFP that were not detected in immunoprecipitates from cells transfected with a control EGFP expression vector or other mutant ubiquitin constructs (Fig 22A). Membranes were re-probed with an E2F-1 specific antibody to confirm the presence of the E2F-1 protein and respective conjugates in immunoprecipitates and total cell extracts (Fig 22B). As might be expected, other variants were found to be conjugation-deficient (a summary of the mutants, their processing and conjugation is shown in Table 1).

3.3.4 The globular domain of ubiquitin and the length of the C-terminus influence the processing of ubiquitin fusions.

The tertiary structure of ubiquitin is frequently depicted in cartoon form as a lollipop or ball and stick, representing the compact globular domain and the extended C-terminus

Figure 22. G76S isoform of ubiquitin can conjugate to a cellular substrate

A) Western analysis with the 6xHis epitope-tag antibody of E2F-1 immunoprecipitates or total cell extracts. Higher order ubiquitinated species (vertical line) were detected in immunoprecipitates from cells co-transfected with an E2F-1 plasmid and either wt Ub-EGFP or G76S Ub-EGFP constructs that were absent in immunoprecipitates from cells transfected with an EGFP control plasmid or other ubiquitin variants. B) Same membrane as in A) re-probed with an E2F-1 specific antibody showing the E2F-1 protein (arrow) and respective conjugates (vertical line). The arrowhead denotes the endogenous immunoglobulin heavy chain.

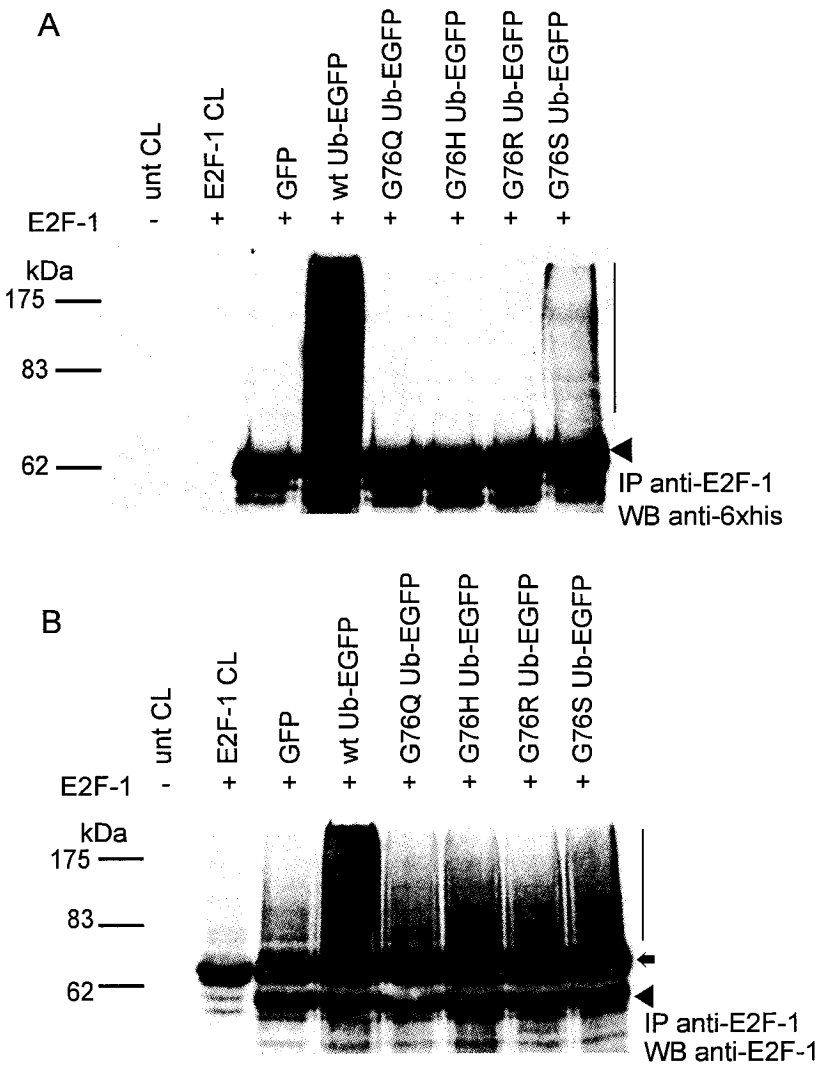

A
unt CL
E2F-1 CL
GFP
wt Ub-EGFP
G76Q Ub-EGFP
G76H Ub-EGFP
G76R Ub-EGFP
G76S Ub-EGFP
E2F-1
-
+
+
+
+
+
+
+
kDa
175
83
62
IP anti-E2F-1
WB anti-6xhis
B
unt CL
E2F-1 CL
GFP
wt Ub-EGFP
G76Q Ub-EGFP
G76H Ub-EGFP
G76R Ub-EGFP
G76S Ub-EGFP
E2F-1
-
+
+
+
+
+
+
+
kDa
175
83
62
IP anti-E2F-1
WB anti-E2F-1

Table 1. Processing and conjugation constraints at the G75 and G76 residues of mammalian ubiquitin

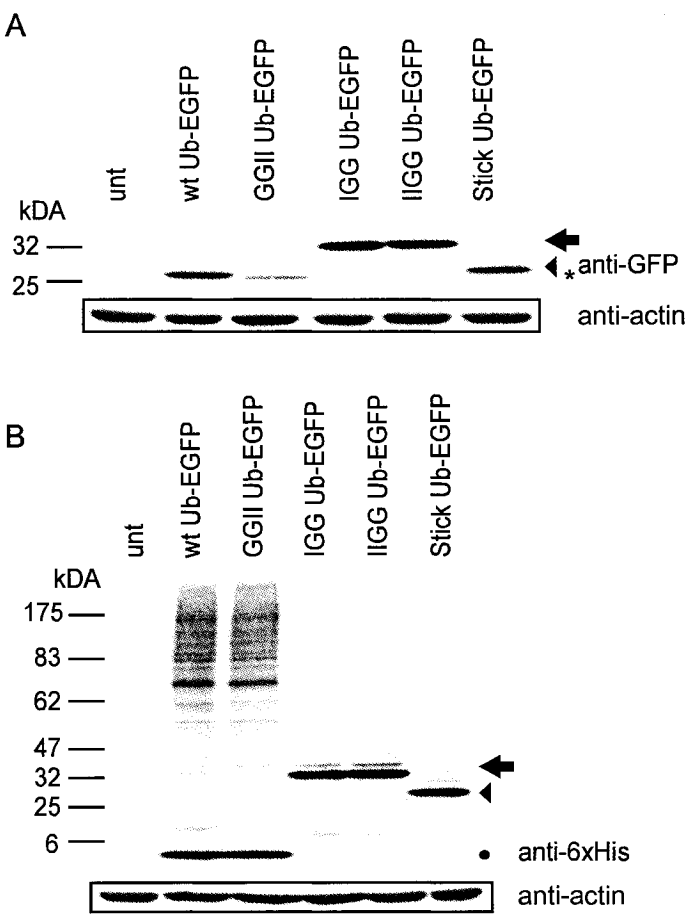
Summary of the constraints on processing and conjugation of ubiquitin following mutation in the G75 and/or G76 residues. A + sign indicates efficient processing and/or conjugation whereas partial processing is indicated by a +/-.

Mutation	Amino acids	Processing	Conjugation
Ala	75	+	+
Ala	76	+	+
Cys	76	+/-	-
Asp	76	+/-	-
Glu	76	+/-	-
Phe	76	+/-	-
His	76	+/-	-
Ile	76	+/-	-
Lys	76	+/-	-
Leu	76	-	-
Met	76	+/-	-
Asn	76	+/-	-
Pro	76	-	-
Gln	76	+/-	-
Arg	76	+/-	-
Ser	76	+/-	+
Thr	76	+/-	-
Val	76	-	-
Trp	76	-	-
Tyr	76	+/-	-
Ala-Ala	75-76	+/-	+
Ala-Ile	75-76	-	-
Ile-Ala	75-76	-	-

respectively. The processing of ubiquitin fusions is thought to be mediated through interactions of deubiquitinating enzymes with both the globular domain and the amino acid sequence within the C-terminal 'stick' domain (Johnsson and Varshavsky, 1994a; Johnsson and Varshavsky, 1994b). To confirm that the sequences of the globular region were necessary for efficient recognition and processing of ubiquitin fusions in mammalian cells an expression vector in which the sequence of the stick region (LRLRGG) was expressed as a linear fusion with EGFP was generated and expressed in 293T cells. This plasmid was designated stick Ub-EGFP. Western blot analysis with EGFP and 6xHis epitope-tag specific antibodies revealed that the amino acid sequences contained within the stick region were not sufficient to allow processing; both antibodies detected a protein species at the expected size of the stick Ub-EGFP fusion protein (Fig 23A and B). To further elucidate the mechanism involved in the recognition and in the processing of ubiquitin fusion proteins by cellular deubiquitinating enzymes expression constructs were generated in which the terminal glycine residues were displaced by one or two amino acid residues. These plasmids were generated via insertion of isoleucine residues between R74 and the terminal glycine pair and were designated IGG Ub-EGFP and IIGG Ub-EGFP. The positioning of the glycines was found to be critical for processing as assessed by western blot analysis of lysates from cells transfected with IGG Ub-EGFP and IIGG Ub-EGFP. Both the EGFP and 6xHis epitope tag-specific antibodies detected the presence of a protein species at the expected size of the Ub-EGFP fusion protein (Fig 23A and B). As might be expected a GGII Ub-EGFP was processed in the same manner as the wt Ub-EGFP fusion protein and became incorporated into higher order conjugates.

Figure 23. The efficient processing of the Ub-EGFP fusion proteins requires the globular domain and is dependent on the length of the C-terminus

A) Detection of the Ub-EGFP fusion proteins (arrow) in cell extracts from cells transfected with IGG Ub-EGFP, IIGG Ub-EGFP and stick Ub-EGFP by western analysis with an EGFP specific antibody. The processed form of EGFP (arrowhead) as well as stick-Ub-EGFP (*) was detected in cell extracts from GGII Ub-EGFP and stick-Ub expressing cells. B) Western analysis of the same extracts as in A) with an antibody directed against the 6xHis epitope tag. The antibody detected the presence of the Ub-EGFP fusion protein (arrow) in cell extracts from cells transfected with IGG Ub-EGFP, IIGG Ub-EGFP and stick Ub-EGFP (*) whereas HMW conjugates (vertical line) and the ubiquitin monomer (•) were detected in cell lysates from cells transfected with GGII Ub-EGFP. Actin (boxed panels) served as a loading control.


A
unt
wt Ub-EGFP
GGII Ub-EGFP
IGG Ub-EGFP
IIGG Ub-EGFP
Stick Ub-EGFP
kDA
32
25
anti-GFP
anti-actin
B
unt
wt Ub-EGFP
GGII Ub-EGFP
IGG Ub-EGFP
IIGG Ub-EGFP
Stick Ub-EGFP
kDA
175
83
62
47
32
25
6
anti-6xHis
anti-actin

3.3.5 Substitutions in the 'stick' region do not interfere with processing/conjugation of ubiquitin in mammalian cells.

In vitro activation and degradation assays performed with variants of yeast ubiquitin have revealed that site directed mutations/deletions in the stick region of ubiquitin (R72S, R72A, DL73) and in the region upstream of the tail (L67D, L69D) resulted in the loss of interaction with the E1 enzyme and the ability of mutant ubiquitin to participate in degradation of test substrates (Ecker et al., 1987b; Burch and Haas, 1994). To assess whether mutations within these regions would abolish the processing of ubiquitin fusion proteins or would disrupt the interaction of ubiquitin with the E1 enzyme in mammalian cells a site-directed mutagenesis approach was employed to generate and express constructs encoding R72A Ub-EGFP, R72S Ub-EGFP, R74A Ub-EGFP, L67D Ub-EGFP, L69D Ub-EGFP, and L73D Ub-EGFP in 293T cells. Western blotting with the anti-EGFP antibody revealed that (with the exception of L67D, which was only partially processed) all mutant ubiquitin fusions were efficiently recognized and processed by cellular deubiquitinating enzymes as evidenced by the presence of the processed EGFP in cell lysates (Fig 24A). Analysis of the same extracts with the 6xHis epitope-tag specific antibody revealed that R72A, R72S, R74A, L69D and L73D mutant ubiquitin was incorporated into HMW conjugates whereas L67D mutant ubiquitin was only partially incorporated (Fig 24B). Analysis of E2F-1 immunoprecipitates with the 6xHis antibody of extracts from cells co-transfected with E2F-1 and mutant ubiquitin isoforms revealed that all variants were conjugation-competent (Fig 25). These data demonstrate that in higher eukaryotes substitutions in the C-terminal extension of ubiquitin do not interfere with processing, nor do they disrupt the ability of ubiquitin to be conjugated to cellular protein substrates (see Table 2).

Figure 24. Point mutations in the C-terminal extension of ubiquitin do not interfere with processing or conjugation of ubiquitin to cellular substrates

A) Western blot analysis with an EGFP specific antibody. The processed EGFP marker (asterisk) was detected in cell extracts from R72A Ub-EGFP, R74A Ub-EGFP, R72S Ub-EGFP, L67D Ub-EGFP, L69D Ub-EGFP and L73D Ub-EGFP transfected cells. In L67D Ub-EGFP expressing cells the EGFP antibody also detected the Ub-EGFP fusion protein (arrowhead). B) Western analysis of the same extracts as in A) with the antibody directed against the 6xHis epitope-tag demonstrating the presence of high molecular weight species (vertical line) as well as the epitope-tagged ubiquitin monomer (arrow) in cell extracts from R72A Ub-EGFP, R74A Ub-EGFP, R72S Ub-EGFP, L69D Ub/GFP and L73D Ub-EGFP transfected cells. The HMW conjugates were less abundant in the lysate from cells transfected with L67D variant of ubiquitin and absent in the untransfected control cell lysate. Boxed panels, actin antibody control.

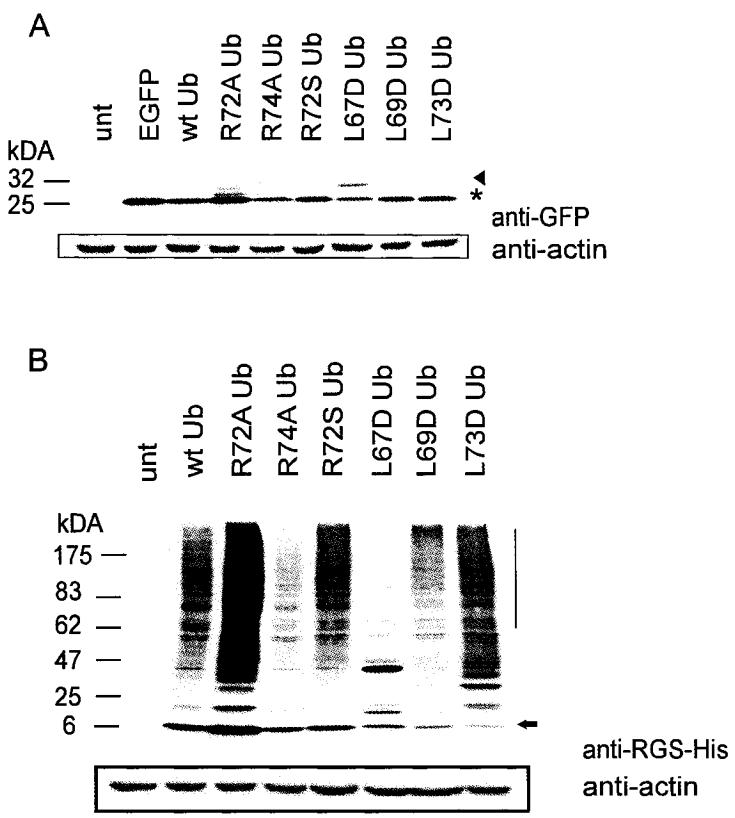

A
unt
EGFP
wt Ub
R72A Ub
R74A Ub
R72S Ub
L67D Ub
L69D Ub
L73D Ub
kDA
32
25
anti-GFP
anti-actin
B
unt
wt Ub
R72A Ub
R74A Ub
R72S Ub
L67D Ub
L69D Ub
L73D Ub
kDA
175
83
62
47
25
6
anti-RGS-His
anti-actin

Figure 25. Conjugation of C-terminal mutants to a cellular substrate

A) and B) Immunoprecipitates from cell extracts transfected with E2F-1 and either expression constructs encoding EGFP alone or variants of ubiquitin were analyzed by western blot with the 6xHis epitope-tag specific antibody. The antibody detected higher order ubiquitinated E2F-1 conjugates (vertical line) in immunoprecipitates from cells transfected with E2F-1 and all variants of ubiquitin. The HMW species were absent in immunoprecipitates from cells transfected with EGFP alone. C) and D) same membranes as in A) and B) re-probed with an antibody raised against E2F-1 confirming the presence of E2F-1 (arrow) and E2F-1 conjugates (vertical line) in immunoprecipitates and total cell extracts.

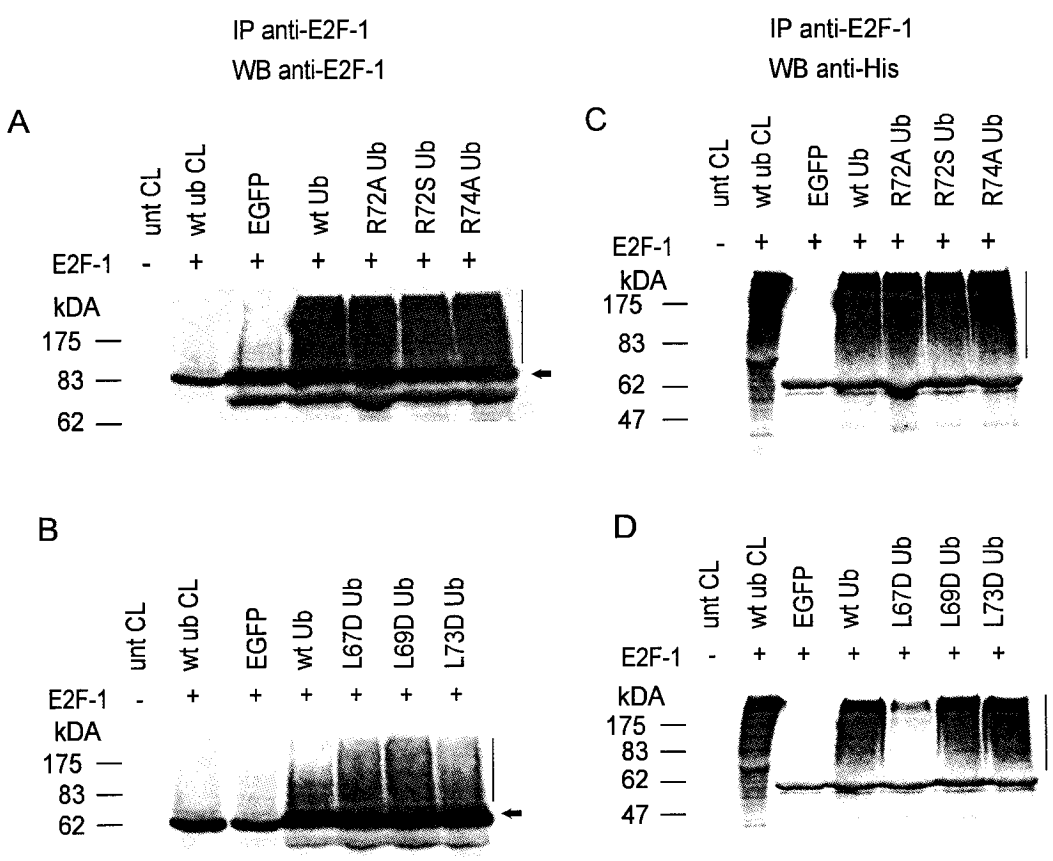

IP anti-E2F-1
WB anti-E2F-1
A
unt CL
wt ub CL
EGFP
wt Ub
R72A Ub
R72S Ub
R74A Ub
E2F-1 - + + + + + +
kDA
175
83
62
B
unt CL
wt ub CL
EGFP
wt Ub
L67D Ub
L69D Ub
L73D Ub
E2F-1 - + + + + + +
kDA
175
83
62
IP anti-E2F-1
WB anti-His
C
unt CL
wt ub CL
EGFP
wt Ub
R72A Ub
R72S Ub
R74A Ub
E2F-1 - + + + + + +
kDA
175
83
62
47
D
unt CL
wt ub CL
EGFP
wt Ub
L67D Ub
L69D Ub
L73D Ub
E2F-1 - + + + + + +
kDA
175
83
62
47

Table 2. Effects of mutations in the stick region of ubiquitin on processing, conjugation and proteolysis.

Removal of the globular domain of the ubiquitin moiety as well as the insertion of amino acids in the C-terminus of ubiquitin abolishes the processing of the Ub-EGFP fusion protein and the conjugation of ubiquitin to cellular proteins. C-terminal site-directed point mutations within the stick region were found to be processed and conjugated. The effects on proteasome activity are indicated. A + sign indicates efficient processing and/or conjugation whereas partial processing is indicated by +/-. ND, not determined.

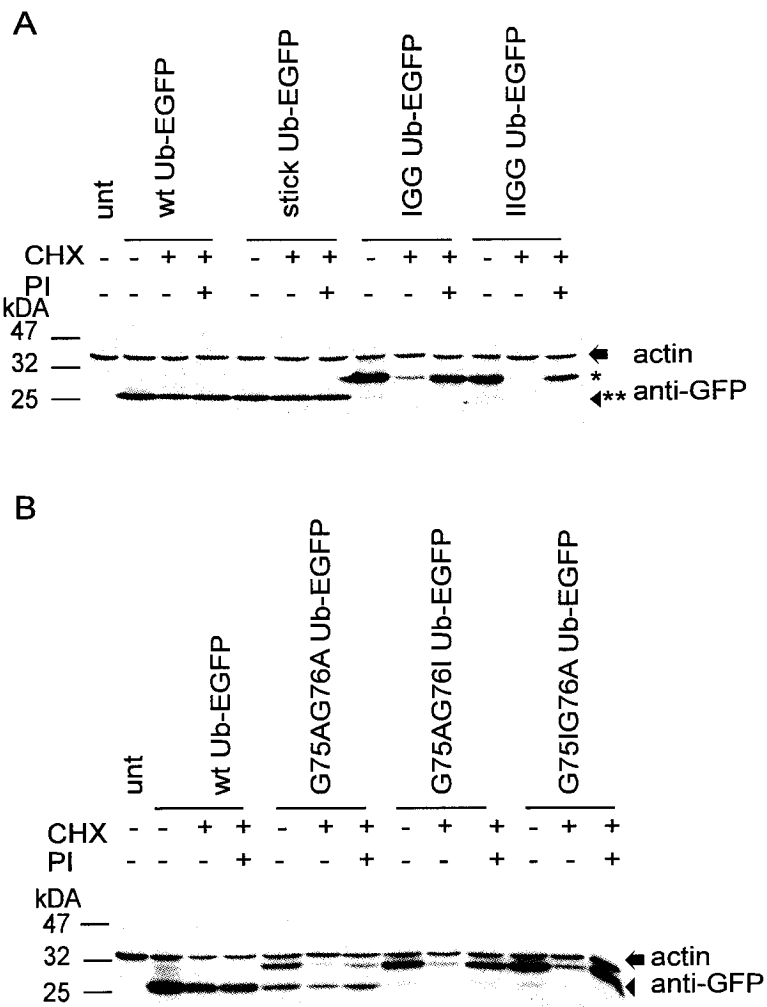
Mutant	Processing	Conjugation	Proteolysis
IGG	-	-	ND
IIGG	-	-	ND
GGII	+	ND	ND
Stick	-	-	ND
L67D	+/-	+	+
L69D	+	+	+
L73D	+	+	+
R72A	+	+	-
R72S	+	+	-
R74A	+	+	+

3.3.6 C-terminal G76 ubiquitin variants are targeted to the 26S proteasome as a consequence of the ubiquitin fusion degradation pathway.

It has been reported that fusions of ubiquitin with heterologous proteins that escape processing are recognized by enzymes of the ubiquitin fusion degradation pathway (UFD) and are degraded by the proteasome (both the reporter protein and the attached ubiquitin moiety, (Yao and Cohen, 2002)). The absence of monomeric ubiquitin in lysates from cells transfected with insertion mutants IGG Ub- EGFP and IIGG Ub-EGFP as well as double mutants G75AG76I Ub-EGFP and G75IG76A Ub-EGFP prompted the investigation of the half-lives of the fusion proteins and the fate of the ubiquitin moieties. Transiently transfected 293T cells were either pre-treated with cycloheximide (CHX, a translational inhibitor) or CHX in the presence of proteasome inhibitor 1 (PI) for a 12 hour period prior to protein extraction. Western blot analysis with the anti-EGFP antibody of lysates from cells transfected with IGG Ub-EGFP and IIGG Ub-EGFP revealed that treatment of cells with CHX alone resulted in an almost complete absence of the fusion protein. The fusion protein reappeared in lysates from cells that were cultured in CHX and PI (Fig 26A). Similar results were obtained with double mutants G75AG76A Ub-EGFP, G75AG76I Ub-EGFP and G75IG76A Ub-EGFP whereby treatment with CHX alone resulted in a substantial loss of the fusion protein that was restored upon treatment with PI (Fig 26B). As expected, cells expressing a processed ubiquitin isoform (wt Ub-EGFP) or an ubiquitin variant that is not recognized by UPP components (stick Ub-EGFP) were not affected by treatment with CHX or CHX+PI (Fig 26A). In order to determine whether fusions of G76 substituted ubiquitin with EGFP were uniformly susceptible to proteolysis, cells were transfected with the following expression constructs: G76D Ub-EGFP, G76Y Ub-EGFP, G76E Ub-EGFP, G76T Ub-EGFP, G76H Ub-EGFP, G76I Ub-EGFP, G76F

Figure 26. Fusion proteins that escape UBP processing are degraded by proteasomes

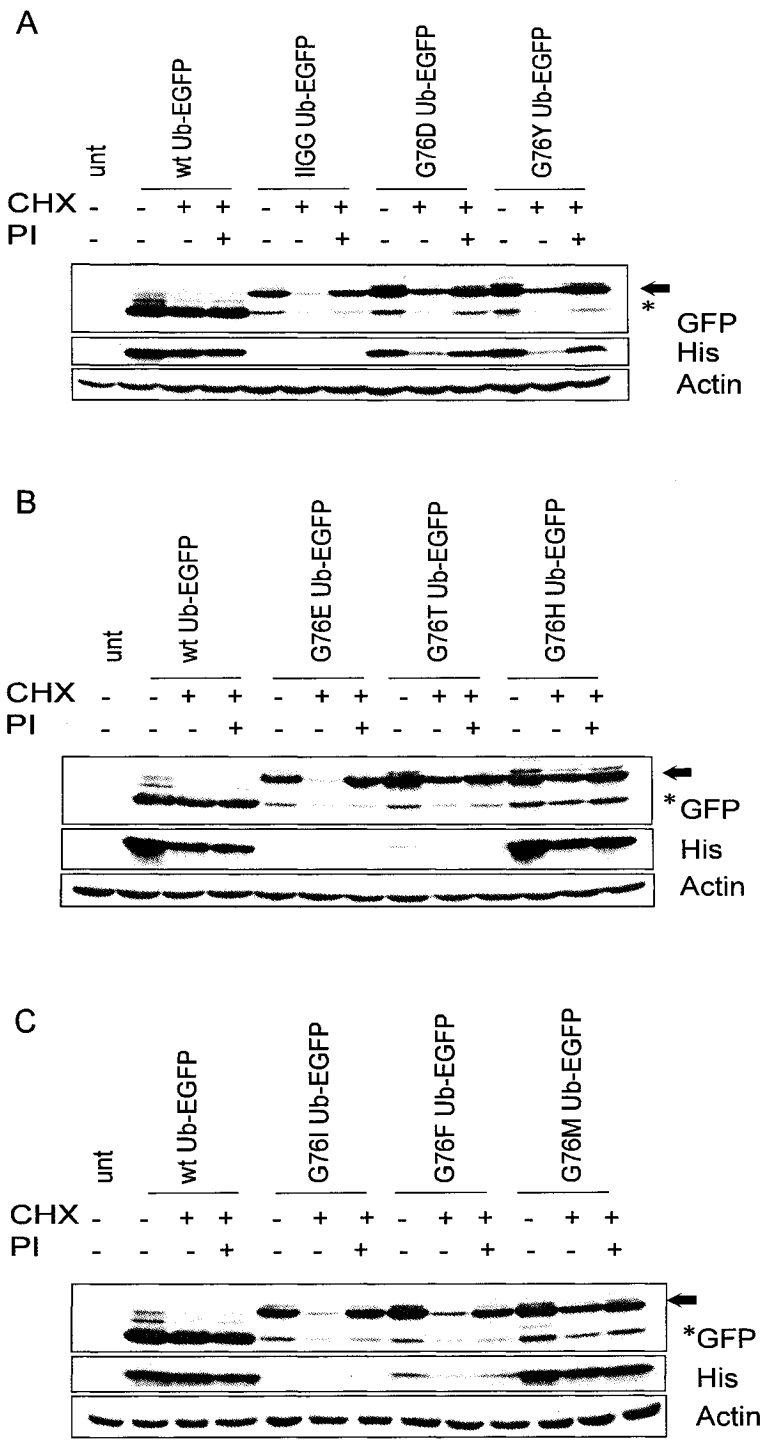
A) and B) Western analysis with an EGFP specific antibody of cell extracts from cells transfected with wt Ub-EGFP, stick Ub-EGFP, insertion mutants IGG Ub-EGFP and IIGG Ub-EGFP and substitution mutants G75AG76A Ub-EGFP, G75AG76I Ub-EGFP and G75IG76A Ub-EGFP that were either untreated or treated with cycloheximide (CHX) alone or cycloheximide (CHX) in the presence of proteasome inhibitor 1(PI). The antibody detected the presence of the Ub-EGFP fusion proteins (asterisk) in untreated lysates from cells transfected with double mutants and insertion mutants, a loss in detection of the fusion protein in the same lysates upon treatment with cycloheximide (CHX) that re-appeared with cycloheximide (CHX) treatment in the presence of proteasome inhibitor 1(PI). As expected, treatment of wt Ub-EGFP and stick Ub-EGFP expressing cells with cycloheximide (CHX) or cycloheximide (CHX) in the presence of PI had no effect. The EGFP marker derived from processing of the wt Ub-EGFP fusion protein and stick Ub-EGFP are denoted by an arrowhead and two asterisks (**) respectively). Blots were simultaneously probed for actin as a loading control.


A
unt
wt Ub-EGFP
stick Ub-EGFP
IGG Ub-EGFP
IIGG Ub-EGFP
CHX
PI
kDA
47
32
25
actin
*
** anti-GFP
B
unt
wt Ub-EGFP
G75AG76A Ub-EGFP
G75AG76I Ub-EGFP
G75IG76A Ub-EGFP
CHX
PI
kDA
47
32
25
actin
anti-GFP

Ub-EGFP and G76M Ub-EGFP and cycloheximide was used to assess their half-lives. As expected, western analysis with the EGFP specific antibody of extracts from mutant ubiquitin transfected cells revealed the presence of the Ub-EGFP fusion proteins in untreated culture (Fig 27). The treatment of transfected cells with CHX resulted in a mild loss in detection of the fusion protein in lysates from G76F Ub-EGFP, G76H Ub-EGFP and G76M Ub- EGFP transfected cells, which was more pronounced in cell extracts from cells transfected with G76D Ub-EGFP, G76Y Ub-EGFP, G76E Ub-EGFP, G76I Ub-EGFP and G76N Ub-EGFP mutant ubiquitin isoforms. The addition of proteasome inhibitor mildly restored the Ub-EGFP protein levels in G76F Ub-EGFP and G76H Ub-EGFP and G76M Ub-EGFP expressing cells but again the most pronounced effect was observed in G76D Ub-EGFP, G76Y Ub-EGFP, G76E Ub-EGFP, G76I Ub-EGFP and G76N Ub-EGFP transfectants. The processed EGFP marker was also more abundant in cells transfected with G76D Ub-EGFP, G76Y Ub-EGFP and to a lesser extent in G76M Ub-EGFP transfectants (Fig 26). No such accumulation was detected in lysates from the other mutant ubiquitin transfected cells. The analysis of the same extracts with the 6xHis epitope-tag antibody revealed that transgene-derived monomeric ubiquitin was accumulating in G76D Ub-EGFP, G76Y Ub-EGFP and to a lesser extent in G76N Ub-EGFP transfected cells treated with CHX+PI (Fig 27, boxed panel, anti-His). Cells expressing other mutant isoforms of ubiquitin did not accumulate transgene-derived monomeric ubiquitin. These data suggested that although fusions of ubiquitin with EGFP are targeted to the 26S proteasome as a consequence of the ubiquitin degradation pathway they are not all efficiently degraded by the 20S core proteasome.

Figure 27. G76 variants of ubiquitin are targeted to the 26S proteasome as a consequence of the ubiquitin fusion degradation pathway

Western analysis with the EGFP specific antibody of extracts from cells transfected with wt Ub-EGFP, IIGG Ub-EGFP, G76D Ub-GFP, G76Y Ub-GFP, G76E Ub-GFP, G76T Ub-GFP, G76H Ub-EGFP, G76I Ub-EGFP, G76F Ub-EGFP and G76M Ub-EGFP treated with cycloheximide alone or CHX in the presence of PI. The antibody detected the Ub-EGFP fusion protein (arrow) in untreated cell extracts, a loss in detection of the Ub-EGFP fusion protein upon treatment of cells with CHX that was restored in cells treated with CHX+PI. The processed EGFP marker (asterisk) also accumulated in extracts from cells expressing G76D Ub-EGFP, G76Y Ub-EGFP and to a lesser extend in G76M Ub-EGP transfectants. The same membranes re-probed with the hexahistidine antibody revealed an accumulation of the ubiquitin monomer (boxed panel) in G76D Ub-EGFP, G76Y Ub-EGFP and to a lesser a extend in G76F Ub-EGFP transfected cells. Actin served as a loading control.


A
unt
wt Ub-EGFP
IIGG Ub-EGFP
G76D Ub-EGFP
G76Y Ub-EGFP
CHX
PI
*
GFP
His
Actin
B
unt
wt Ub-EGFP
G76E Ub-EGFP
G76T Ub-EGFP
G76H Ub-EGFP
CHX
PI
*GFP
His
Actin
C
unt
wt Ub-EGFP
G76I Ub-EGFP
G76F Ub-EGFP
G76M Ub-EGFP
CHX
PI
*GFP
His
Actin

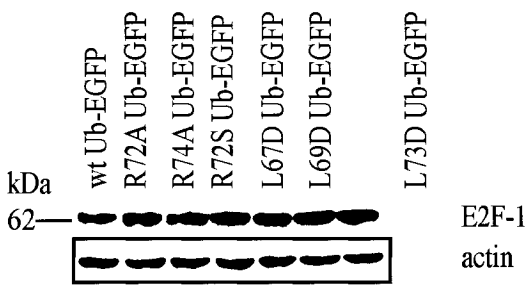
3.3.7 Expression of C-terminal ubiquitin variants do not result in the accumulation of UPP substrates

Mutations of residues on ubiquitin of importance for proteolytic targeting (and not in the C-terminus per se) have been shown to preclude the destruction of the substrate by inhibiting the association of ubiquitin with the 26S proteasome despite the assembly of a conformationally competent polyubiquitin chain (Beal et al., 1996). It was therefore conceivable that mutations in the C-terminus of ubiquitin would impede the targeting of substrates to the 26S proteasome. In order to investigate the targeting ability of mutant ubiquitin (rather than the conjugating ability) the accumulation of a UPP substrate was examined by transient co-transfection of E2F-1 with either wt Ub-EGFP or C-terminal mutant ubiquitin constructs. Western blot analysis with the E2F1-specific antibody of cell extracts from mutant ubiquitin transfected cells did not reveal a difference in abundance in the protein levels of E2F-1 (Fig 28A). This suggested that substrates are efficiently targeted to the 26S proteasome and that mutant ubiquitin is not irreversibly conjugated to cellular proteins which would otherwise be reflected by changes in the protein levels of E2F-1. However, when the chymotrypsin-like activity of the proteasome was tested for its ability to process a fluorogenic substrate in the presence of mutant ubiquitin, it was found to be differentially altered in transfected cells with Leu and Arg mutations exerting the most pronounced effect. Wild-type ubiquitin and ubiquitin variants which were previously found to be degraded by the 26S proteasome as part of the fusion protein had a minor adverse affect on proteolysis (Fig 28B).

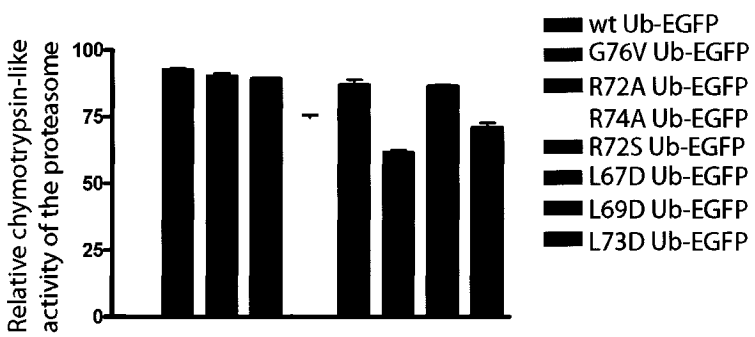
Figure 28. The expression of C-terminal ubiquitin mutants does not impair the targeting of substrates to the 26S proteasome

A) Western blot analysis with an E2F-1 antibody of cell extracts from cells co-transfected with E2F-1 and various ubiquitin expression vectors. No difference in E2F-1 levels were observed. B) Cells were transfected with either wt or R72S, R74A, R72A, L67D, L69D and L73D mutant ubiquitin plasmids and assayed for their ability to process a chymotrypsin-specific fluorogenic substrate. Cells were transfected, lysed and assayed in triplicates. The percentage of activity is represented at the 16 hour time point for each mutant. Addition of proteasome inhibitor to the lysate of the EGFP expressing cells served as a control for non-proteasomal degradation. Data was normalized to proteasome activity in lysates from cells transfected with an EGFP control vector.

A


wt Ub-EGFP
R72A Ub-EGFP
R74A Ub-EGFP
R72S Ub-EGFP
L67D Ub-EGFP
L69D Ub-EGFP
L73D Ub-EGFP
kDa
62
E2F-1
actin

B

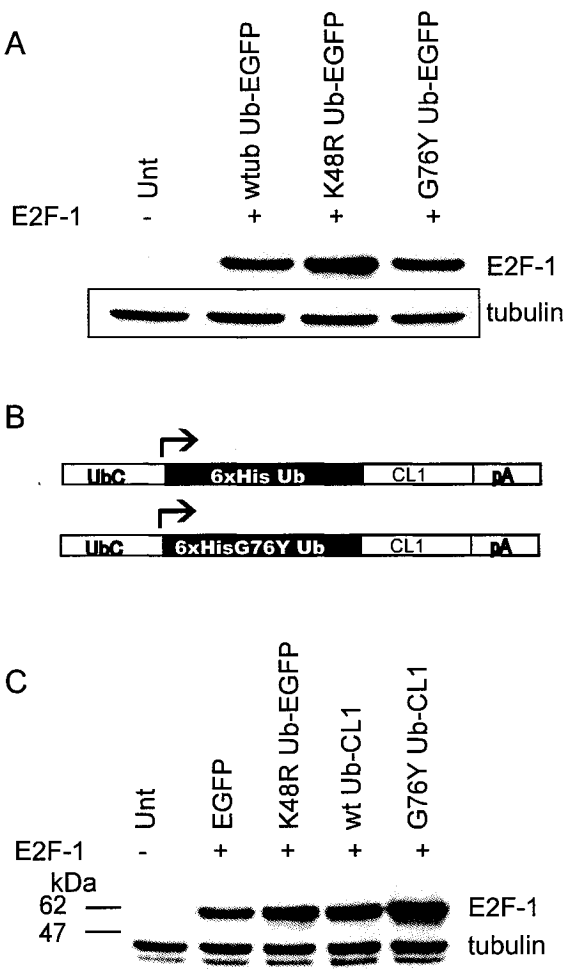

Relative chymotrypsin-like activity of the proteasome
100
75
50
25
0
wt Ub-EGFP
G76V Ub-EGFP
R72A Ub-EGFP
R74A Ub-EGFP
R72S Ub-EGFP
L67D Ub-EGFP
L69D Ub-EGFP
L73D Ub-EGFP

3.3.8 The expression of a G76Y Ub-EGFP fusion protein does not result in the accumulation of protein substrates.

It has been well documented in yeast and more recently in mammalian cells that expression of C-terminal ubiquitin variants resulted in a decrease in the rate of degradation by the 26S proteasome (Hodgins et al., 1992; Lindsten et al., 2002). To test the hypothesis that mutant ubiquitin decreases the rate of proteasomal degradation and results in the accumulation of protein substrates, an E2F-1 expression construct was co-transfected with G76Y Ub-EGFP. Western analysis of cell extracts with an E2F-1 antibody did not reveal a significant accumulation of E2F-1 protein levels in G76Y Ub-EGFP transfected cells whereas a robust accumulation of E2F-1 was observed in extracts from cells expressing a K48R chain-terminating version of ubiquitin (Fig 29). These data suggested that any depletion in ubiquitin pools and/or reduced rate of degradation by the 26S proteasome imposed by the expression of mutant ubiquitin was not sufficient to result in the accumulation of cellular substrates. It has been previously reported that the targeting of C-terminal ubiquitin isoforms to the 26S proteasome can be accelerated by the addition of degron signals (Lindsten et al., 2002). In an attempt to increase proteasomal burden and cause a more significant alteration in ubiquitin pools, a degron sequence was appended at the C-terminus of a G76Y mutant ubiquitin (schematically depicted in Fig 29B); such a construct would reveal potential consequences of increased trafficking of mutant ubiquitin to the proteasome. Co-transfection of destabilized mutant ubiquitin with E2F-1 resulted in an accumulation of E2F-1 protein levels beyond the one observed with chain-terminating K48R mutant ubiquitin (Figure 29C).

Figure 29. The expression of G76Y mutant ubiquitin does not result in accumulation of UPP substrates

A) Western blot analysis with an E2F-1 specific antibody of extracts from cells co-transfected with an E2F-1 expression vector and either wt Ub-EGFP, K48R Ub-EGFP or G76Y Ub-EGFP plasmids. The E2F-1 protein accumulated in cells expressing K48R Ub-EGFP. No difference in E2F-1 stability was observed in cells expressing wt Ub-EGFP or G76Y Ub-EGFP. B) Schematic representation of the CL1 expression constructs. C) Western analysis with an E2F-1 antibody of extracts from cells co-transfected with E2F-1 and either EGFP, K48R Ub-EGFP, wt Ub-CL1 or G76Y Ub-CL1. A robust accumulation in the protein levels of the E2F-1 transcription factor was detected in cell extracts from cells transfected with G76Y Ub-CL1 mutant ubiquitin. Tubulin served as a loading control.

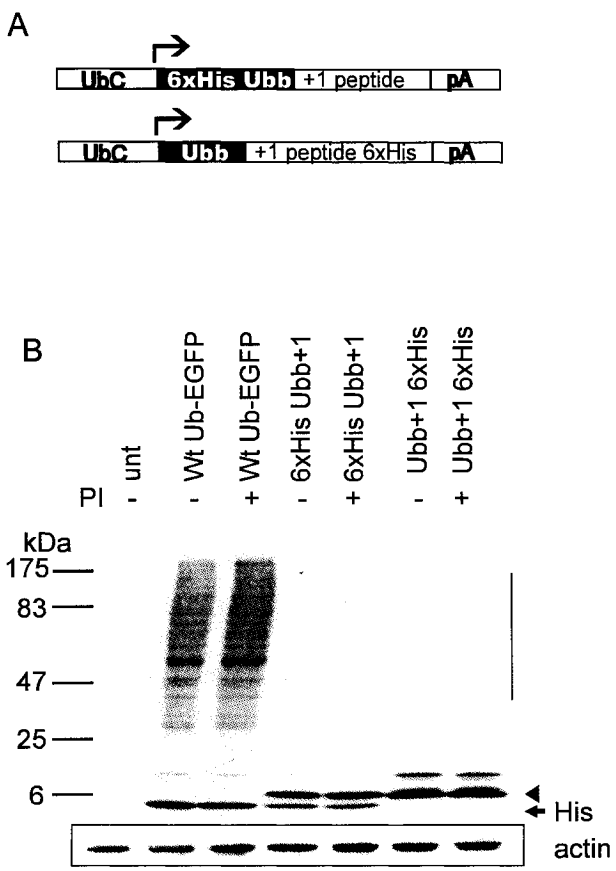

A
Unt
+ wtub Ub-EGFP
+ K48R Ub-EGFP
+ G76Y Ub-EGFP
E2F-1
-
E2F-1
tubulin
B
UbC
6xHis Ub
CL1
pA
UbC
6xHisG76Y Ub
CL1
pA
C
Unt
+ EGFP
+ K48R Ub-EGFP
+ wt Ub-CL1
+ G76Y Ub-CL1
E2F-1
-
kDa
62
47
E2F-1
tubulin

3.3.9 The case of Ubb+1, a C-terminal ubiquitin variant implicated in the pathogenesis of Alzheimer's disease.

Ubb^{+1}, arising from a frameshift mutation in transcription of the UBB gene, encodes an ubiquitin protein in which the C-terminal glycine residue is substituted to a Tyr and is extended by a 20 amino acid peptide sequence. Ubb^{+1} has been proposed to be an uncleavable ubiquitin variant (due to the G76 mutation) that is a substrate of the UFD pathway and a dominant-negative inhibitor of the proteasome through a mechanism involving the assembly of unanchored polyubiquitin chains that are refractory to disassembly by ubiquitin recycling enzymes (reviewed in (Layfield, 2001)). These properties of mutant ubiquitin have been proposed to be the mechanism by which this aberrant isoform contributes to the pathogenesis of AD. The data generated with the mutational analysis of mammalian ubiquitin suggested that the G76Y Ub-EGFP fusion protein was subject to partial processing by enzymes with ubiquitin cleaving activity and it has been previously documented that the stability of a G76V mutant ubiquitin in cells (in the absence of a C-terminal reporter protein) does not significantly differ from wild-type ubiquitin (Qian et al., 2002). In order to investigate the potential processing of Ubb^{+1} and its half-life, two plasmids (schematically depicted in Fig 30A) encoding a G76Y ubiquitin with an N-or C- terminal 6xHis epitope tag were generated; the peptide sequence of the Ubb^{+1} protein was appended at the C-terminus of these variants or on EGFP. Western analysis of extracts from cells transfected with the two isoforms of G76Y Ubb^{+1} with the 6xHis antibody revealed the presence of the full-length species in extracts from cells transfected with both ubiquitin isoforms (Fig 30B). In extracts from N-terminal Ubb^{+1} expressing cells, the antibody also detected a protein species that co- migrated with the epitope-tagged ubiquitin monomer from extracts of cells transfected with wt Ub-EGFP (Fig

Figure 30. G76 ubiquitin extension proteins are subject to processing

A) Schematic representation of the Ubb^{+1} expression vectors. B) Western analysis with the 6xHis antibody of extracts from cells expressing C- or N-terminal 6xHis-tagged ubb^{+1} expression constructs. A species at the molecular weight expected of the full length ubb^{+1} (arrowhead) was detected in extracts from cells transfected with both isoforms of Ubb^{+1}. Processed monomeric ubiquitin was detected in extracts from cells transfected with the N-terminal Ubb+1 isoform (arrow). Actin (boxed panel) served as a loading control.


A
UbC
6xHis Ubb
+1 peptide
pA
UbC
Ubb
+1 peptide 6xHis
pA
B
PI
-
unt
-
Wt Ub-EGFP
+
Wt Ub-EGFP
-
6xHis Ubb+1
+
6xHis Ubb+1
-
Ubb+1 6xHis
+
Ubb+1 6xHis
kDa
175
83
47
25
6
His
actin

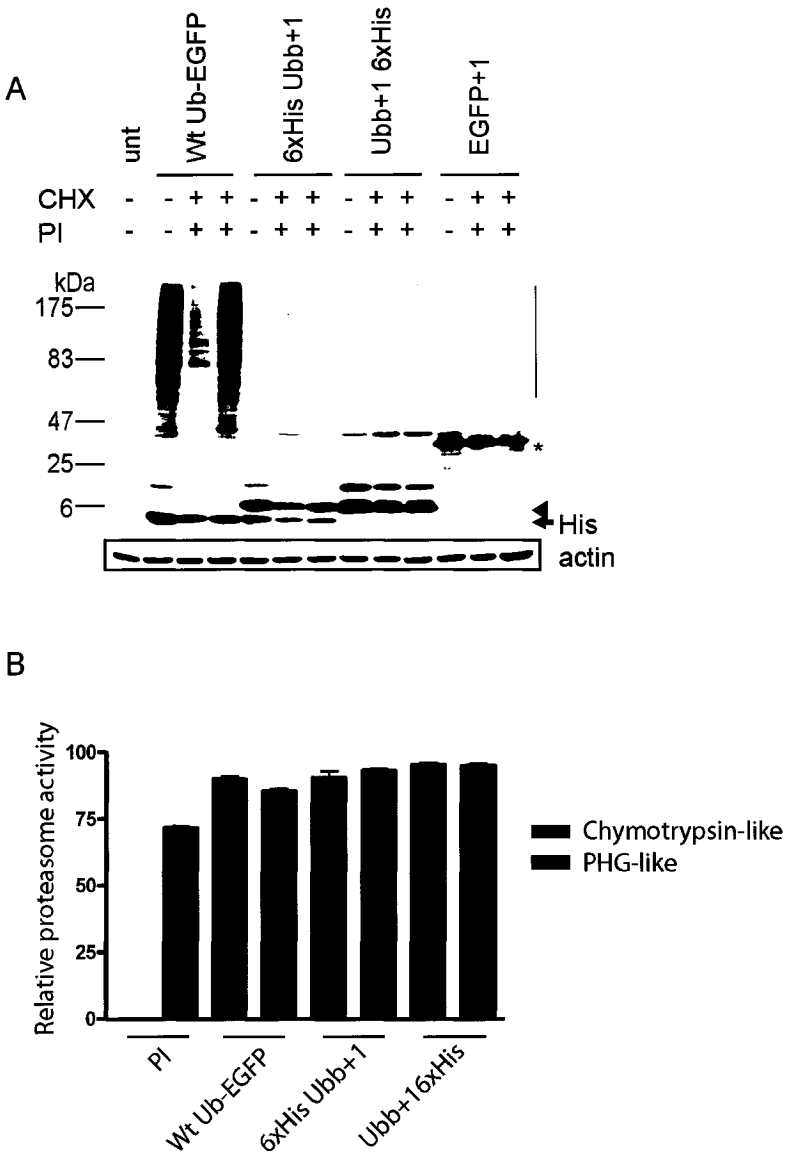
30B). The addition of proteasome inhibitor prior to harvesting the cells for western blot analysis had no effect on the G76Y Ubb+1 protein levels (Fig 30B). These data suggested that G76Y Ubb^{+1} proteins were not substrates of the proteasome. To further confirm this finding, transfected cells were treated with either CHX alone or CHX in the presence of PI. As expected, western analysis with the 6xHis antibody did not reveal a significant decrease in stability of Ubb^{+1} protein levels in CHX treated cells (Fig 31A). Furthermore, the addition of PI had no effect. Similar data were obtained for the $EGFP^{+1}$ protein; treatment with CHX or CHX +PI did not alter the protein levels. The expression of Ubb^{+1} variants did not result in an inhibition of the chymotrypsin-like or peptidylglutamyl-like activities of the proteasome as assessed by the cleavage of specific fluorogenic substrates. As expected, the treatment of cells with proteasome inhibitor I abolished the processing of the substrate (Fig 31B). These data suggest that transient expression of Ubb^{+1} does not result in overt proteolysis deficiencies and that the +1 peptide does not serve as a UFD motif.

3.4 Analysis of mutant ubiquitin in transgenic mice

The generation of novel ubiquitin transgenic mouse models would facilitate the analysis of ubiquitination events as they occur in vivo and make possible the identification of substrates of the ubiquitin pathway that feature in disease states. The primary objective in creating transgenic mice was to yield high levels of expression of transgene-derived ubiquitin in all tissues thus permitting the incorporation of exogenous ubiquitin in higher order ubiquitinated species. The UbC promoter has previously been shown to be ubiquitously active in adult transgenic mice and was selected to drive expression of the 6xHis ubiquitin expression vectors *in vivo* (Schorpp et al., 1996). The analysis of EGFP fluorescence in cultured embryos derived from the mating of heterozygous lines with non

Figure 31. G76 ubiquitin extension proteins are not proteasome substrates

A) Western analysis with the 6xHis antibody of extracts from cells transfected with either Ubb^{+1} expression constructs or an expression vector encoding an EGFP extension protein ($EGFP^{+1}$) treated with CHX alone or CHX +PI. No significant alteration in the protein levels was observed regardless of the treatment. D) Cells were transfected with the ub+1 expression constructs or wt Ub-EGFP and extracts were assayed for their ability to process chymotrypsin and peptidyl-glutamyl specific substrates. The cells were transfected in triplicates and the percentage of proteasome activity in the lysates is represented for the 24 hour time point for each mutant. The lysate from the mock transfected cells was incubated with PI and served as the control for proteasomal activity. Data was normalized to proteasome activity of lysates from cells transfected with a mock (PCDNA3) plasmid.


A
unt
Wt Ub-EGFP
6xHis Ubb+1
Ubb+1 6xHis
EGFP+1
CHX
PI
kDa
175
83
47
25
6
His
actin
B
Relative proteasome activity
100
75
50
25
0
Chymotrypsin-like
PHG-like
PI
Wt Ub-EGFP
6xHis Ubb+1
Ubb+16xHis

transgenic animals revealed that whereas all zygotes were negative for EGFP fluorescence, faint green fluorescence was detected in half the embryos at the early morula stage, increasing in intensity through the blastocyst stage (Fig 32A). Examination of midgestation embryos revealed that half of the progeny were fluorescent regardless of whether the male or female parent was transgenic. Fluorescence emanated throughout the embryos, although blood appeared to be negative or to quench the fluorescent signal as previously reported (Hadjantonakis et al., 1998). The most intense signals were detected in testes and brain (Fig 32B).

3.4.1 Epitope-Tagged ubiquitin in Transgenic Brains

Having visually confirmed the expression of EGFP in the brains of transgenic animals, cell extracts were prepared from transgenic and non-transgenic control brains to determine if the 6xHis-ubiquitin component of the transgene construct was as processed and available for conjugation as it was in transfected cells. Western blot analysis with both EGFP and 6xhis specific antibodies revealed that the fusion proteins were efficiently processed *in vivo* and that the transgene-derived ubiquitin moieties were becoming incorporated into higher order conjugates (Fig 33). Comparison of brain lysates from newborn pups and adult animals revealed differences in the pattern of conjugates present, an observation previously reported for endogenous ubiquitin in the brain (Fig 34). These data were indicating that the strategy employed was feasible in the *in vivo* setting of transgenic animal models. A batch purification method was employed to recover *in vivo* substrates of the ubiquitin/proteasome pathway from brains of the ubiquitin transgenic mice. Western analysis of eluates from wt Ub-EGFP and K48R Ub-EGFP expressing mouse brains with a 6xHis antibody revealed the presence of ubiquitinated substrates as well as the ubiquitin monomer in wild-type and K48R ubiquitin transgenic brains. These species were absent from the cell extract and the

Figure 32. In vivo expression of the EGFP marker in wt Ub-EGFP transgenic mice

A) Morula-stage embryo from a heterozygous X wild-type cross. In accordance with Mendelian genetics, approximately half the embryos visualized by phase contrast microscopy (upper panel) were found to be fluorescent when viewed under UV light (lower panel; the position of the embryos shifted slightly between exposures). B) Newborn littermates from a heterozygous X wild type cross. When viewed under UV light, the mouse on the right was found to be intensely fluorescent over its entire surface, although blood vessels could be seen in contrast. C) Dissected brains from a nontransgenic mouse (left) and its transgenic littermate (right). When viewed under fluorescent light (lower panel), the transgenic mouse brain was found to be intensely fluorescent.

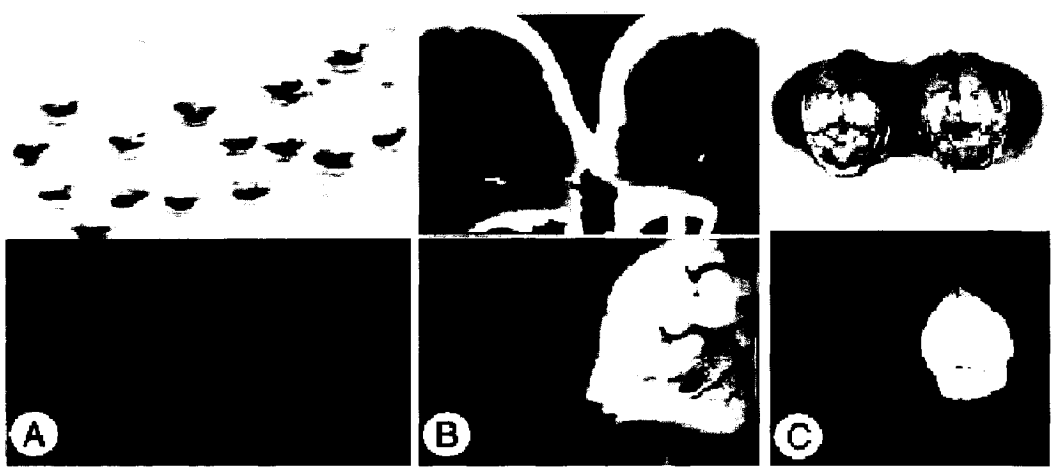

A
B
C

Figure 33. Processing and conjugation of transgene-derived ubiquitin in the brains of transgenic mice

A) Transgene-derived ubiquitin was detected by using an antibody specific for the 6xHis epitope tag. The presence of the low-molecular-weight band (arrow) confirmed that the Ub-enhanced EGFP fusion had been processed to provide free monomers, which were not detected in brain lysates from nontransgenic control mice (NT). The high-molecular-weight smear (particularly evident in brain lysates from K48R transgenic mice) corresponded to epitope-tagged ubiquitin conjugated to substrates. B) Analysis of total ubiquitin in brain lysates. The addition of transgene-derived ubiquitin was not found to greatly increase the overall levels of the ubiquitin monomer (arrow) or to grossly affect the levels of ubiquitin conjugates (smear). C) Comparison of transgene expression levels. With a GFP-specific antibody, only processed enhanced EGFP could be detected (asterisk), confirming that the ubiquitin moiety was rapidly released from the fusion. The levels of transgene expression appeared comparable in the K48R and K63R transgenic lines, whereas expression in the wild-type ubiquitin transgenic line may have been slightly lower. The simultaneous use of a ß-actin-specific antibody confirmed equal loading of lysates (arrowhead).

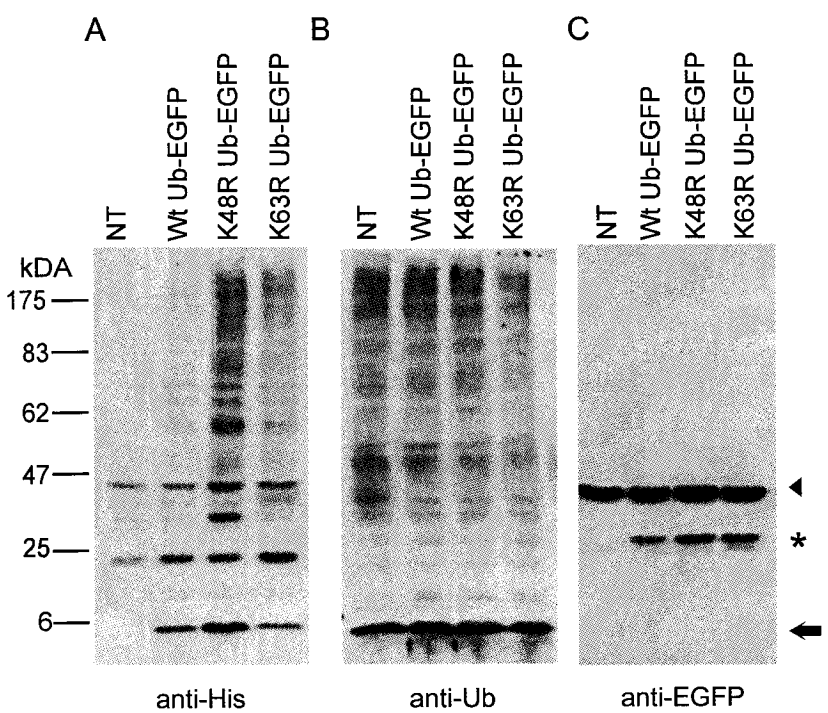
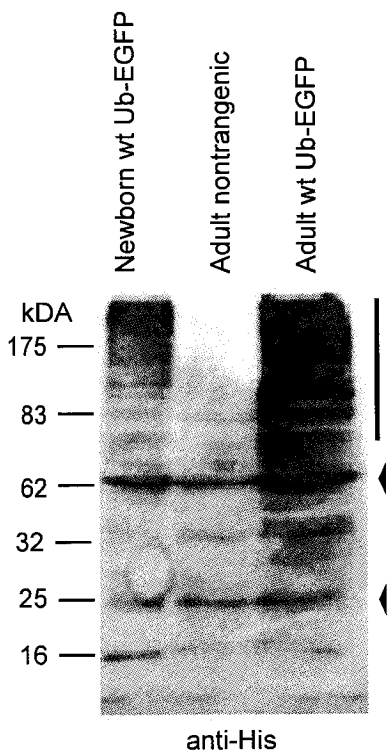

A
B
C
NT
Wt Ub-EGFP
K48R Ub-EGFP
K63R Ub-EGFP
kDA
175
83
62
47
25
6
anti-His
anti-Ub
anti-EGFP
*

Figure 34. Detection of 6xHis-Ub conjugates in transgenic mouse brains

Western blot analysis of brain lysates from a newborn transgenic mouse, adult nontransgenic and transgenic mouse, probed with an antibody to the 6xHis epitope tag. In addition to the nonspecific bands detected by this antibody in mouse tissues (adult nontransgenic), intense high molecular weight smears indicative of ubiquitin protein conjugates (vertical line) were detected in the lysates from transgenic mice. Arrowheads denote endogenous immunoglobulin heavy and light chains.


Newborn wt Ub-EGFP
Adult nontrangenic
Adult wt Ub-EGFP
kDA
175
83
62
32
25
16
anti-His

eluate of nontransgenic brains (Fig 35).

3.5 The in vivo expression of K48R mutant ubiquitin confers protective effects and alleviates the phenotype in a mouse model of SCA-1

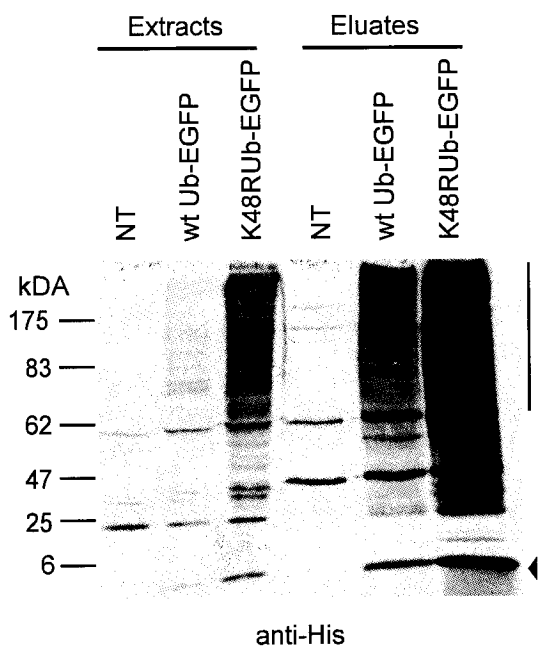
Neurodegenerative disorders are characterized by the presence of proteinaceous inclusions that stain positive for ubiquitin and components of the UPP (reviewed in (Berke and Paulson, 2003)). The presence of ubiquitin in these inclusions may reflect a failure of the UPP to deal with misfolded protein and it might be expected that mutations in ubiquitin would accelerate the onset or the severity of the disease. In order to test the hypothesis that exogenous expression of wild-type ubiquitin would delay the onset of a neuro-pathological state, compound transgenic mice were generated by crossing ubiquitin transgenics to the well characterized SCA-1 model (referred to as B05 with a pathological 83Q expansion in the gene product Ataxin-1). As a control, compound transgenics were generated by crossing ubiquitin mice with the A02 model encoding a non-pathological 30Q expansion.

3.5.1 Degeneration of Purkinje neurons is delayed in mice expressing K48R ubiquitin.

A feature of SCA-1 pathology is the morphological alterations that occur in the cerebellum of patients and transgenic mice typically represented by the degeneration of the Purkinje cell layer in the cerebellum (Clark et al., 1997). By this measure, the microscopical examination of the anatomical structure of cerebella serves as an indicator of the progression/severity of the disease. The cerebella of A02 (30Q) and B05 (82Q) heterozygous mice as well as compound heterozygous progeny of A02 or B05 crossed with each of the three ubiquitin transgenics (wild-type, K63R and K48R) were analyzed for Purkinje neuron alterations by immunohistochemistry with calbindin (a Purkinje-specific marker) for several time points up to eight months. A pronounced delay in the morphological deterioration of Purkinje neurons was evident in B05xK48R compound

Figure 35. Purification of 6xHis tagged proteins from brains of ubiquitin transgenics

Western blot analysis of total brain extracts and eluated fractions with the 6xHis antibody. HMW conjugates (vertical bar) and transgene-derived monomeric ubiquitin (arrowhead) was detected in extracts and eluates from brains expressing wt Ub-EGFP and K48R Ub-EGFP. These species were absent in the extract and eluate of NT control brains.


Extracts
Eluates
NT
wt Ub-EGFP
K48RUb-EGFP
NT
wt Ub-EGFP
K48RUb-EGFP
kDA
175
83
62
47
25
6
anti-His

transgenics at the 3 month time point, an age at which pronounced morphological and behavioral changes have been reported in the B05 strain (Burright et al., 1995; Clark et al., 1997). In B05 heterozygous mice or the B05x wt Ub-EGFP cross there was loss of Purkinje neurons as well as the appearance of many Purkinje neurons with aberrant dendritic morphology, unusual orientation, or positioning within inappropriate granular or molecular cell layers (Fig 36). The loss of calbindin immunoreactivity was in agreement with the levels of the calbindin protein in mouse cerebella as assessed by western blot analysis (Fig 37). By each of these measures K48R mutant ubiquitin reduced the magnitude of polyQ-induced pathology in the B05xK48R genetic cross. At the 8 month time point some Purkinje neurons with altered dendritic morphology and subcellular localization were still visible in B05xK48R compound transgenics whereas heterozygous B05 and wild-type ubiquitin crossed with B05 showed an almost complete loss of their Purkinje neurons (Fig 38).

3.5.2 Loss of coordination in SCA-1 mice is delayed by K48R mutant ubiquitin

The anatomical data suggested that the deterioration of the Purkinje cell layer was delayed in K48R crosses and in order to investigate whether that translated in an enhanced coordination, the performance of mice was examined by the rotating rod apparatus (an indicator of motor capability). The morphological data were in good agreement with behavioral analysis of the loss of coordination in simple and compound heterozygous mice at the 3 month time point. There was a pronounced difference in the performance of mice expressing K48R mutant ubiquitin as opposed to the Wt Ub isoform in the presence of the expanded polyQ protein (Fig 39A). As expected, no differences were observed in the performance of the A02 and respective crosses when compared to the nontransgenic

Figure 36. Representation of Purkinje cell morphology in mice at 3 months of age

Purkinje cells (reddish brown) were visualized by immunohistochemistry using an antibody specific for calbindin. Nuclei (purple). Sections were counterstained with hematoxylin. A) Nontransgenic FVB/N control cerebellum, showing orderly Purkinje layer with some dendritic processes visible. B) Heterozygous A02 cerebellum, similar in appearance to the nontransgenic control. C) Heterozygous B05 cerebellum showing pathological Purkinje cell morphology, including vesicle formation, aberrant orientation and localization of Purkinje neurons within the molecular layer, and some neuronal loss. D) and E) Cerebellum from B05xWt Ub and B05x K63R Ub cross with evidence of vesicles and some neuronal loss. F) Cerebellum from B05xK48R Ub cross, showing some evidence of vesiculation of Purkinje neurons and altered orientation, but less severe pathology than is evident in B05 heterozygous sections ©. Bar = 100μM.

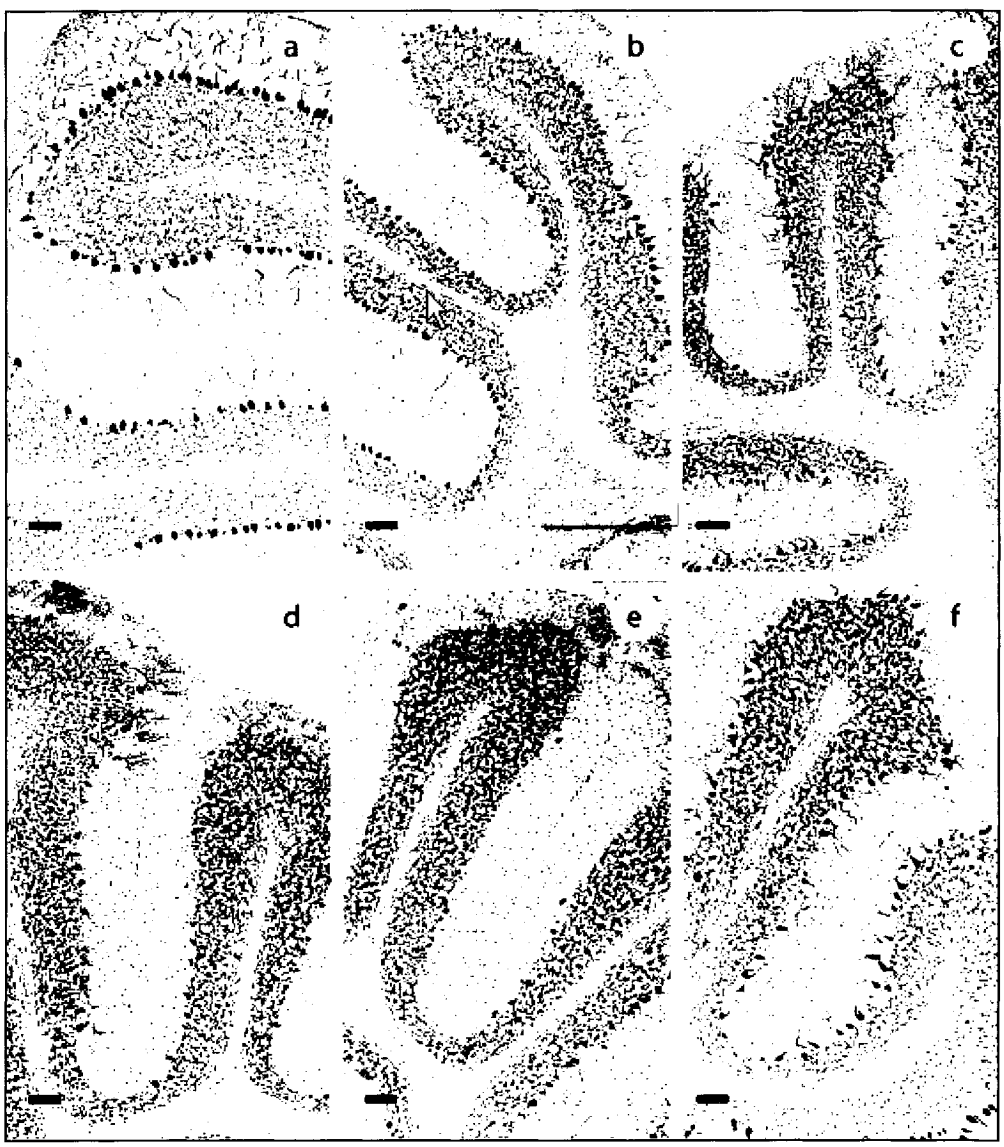

a
b
c
d
e
f

Figure 37. Western analysis of mouse cerebella with a calbindin-specific antibody

The levels of calbindin were not found to differ between ubiquitin transgenics, were drastically decreased in B05, partially restored in the B05 x wt compound transgenics with an even higher level detected in the B05xK48R. The levels Actin (boxed panel) served as a control.

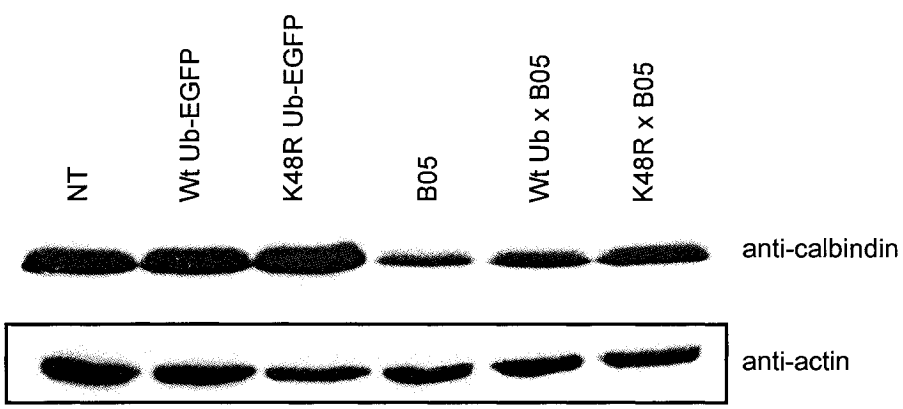
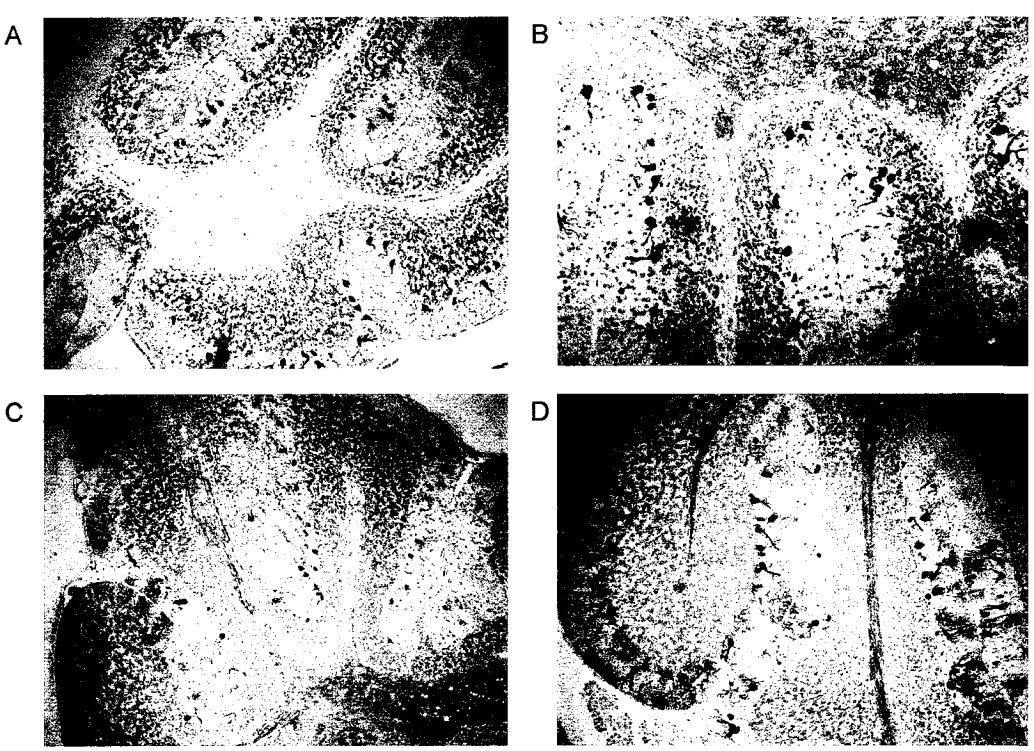

NT
Wt Ub-EGFP
K48R Ub-EGFP
B05
Wt Ub x B05
K48R x B05
anti-calbindin
anti-actin

Figure 38. Representation of Purkinje cell morphology in mice at 8 months of age

Purkinje cells (reddish brown) were visualized by immunohistochemistry using an antibody specific for calbindin. Sections were counterstained with hematoxylin. A) Nontransgenic FVB/N control cerebellum, showing Purkinje layer with some dendritic processes visible. B) Heterozygous A02 cerebellum, similar in appearance to the nontransgenic control. C) Heterozygous B05 and D) and E) B05x wt Ub and B05xK63R Ub cerebellums showing pathological Purkinje cell morphology with extensive neuronal loss. E) Cerebellum from B05xK48R Ub cross, showing some evidence of vesiculation of Purkinje neurons and altered orientation, but less severe neuronal loss than is evident in B05 heterozygous sections ©. Bar = 100μM.


A
B
C
D

control at 3 months of age (Fig 39B). The differences in performance observed in the presence of the expanded polyQ, while sizeable, were transient in nature, becoming less pronounced at later time points. At the 8 month time point, K48R mutant ubiquitin compound transgenics appeared to perform similarly to BO5 and B05 crossed with the wild-type ubiquitin isoform (Fig 40).

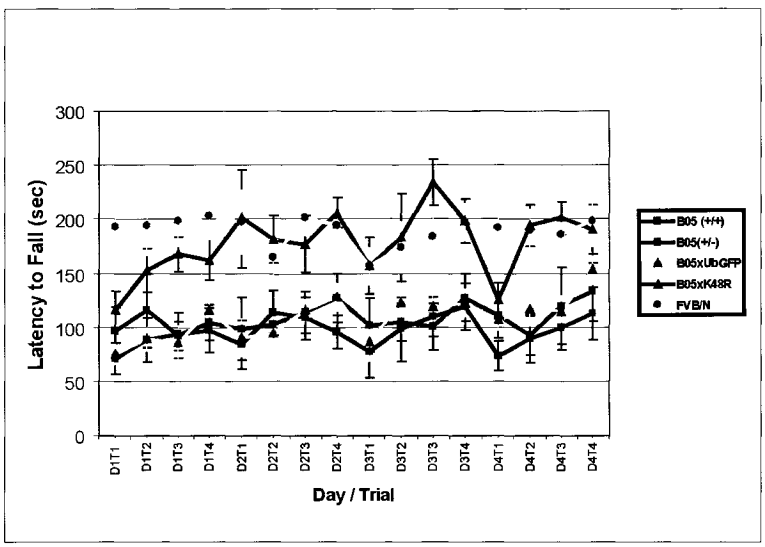
3.5.3 The delay in the phenotype is not attributed to a heat shock response, an alteration of the SCA-1 levels or differences in ubiquitin pools

Proteasomal inhibition has been shown to result in the accumulation of HMW ubiquitinated conjugates and to a concomitant up-regulation of HSP expression ((Grossin et al., 2004), (Goldbaum and Richter-Landsberg, 2004), (Stangl et al., 2002)). The exogenous expression of chaperone proteins have been shown to alleviate the phenotype of polyQ-induced disease in cell culture models as well as in transgenic mice ((Adachi et al., 2003), (Cummings et al., 1998), (Muchowski et al., 2000)). To rule out the possibility that elevated levels of heat-shock proteins in K48R ubiquitin mice is contributing to a delay in the phenotype (rather than the expression of the mutant isoform) the analysis of the protein levels of heat shock proteins were examined by immunoblot analysis. The protein levels of HSP70, HSP40 and Bip (ER chaperone with analogous function to HSP70) were not found to significantly differ in the various strains (Fig 41). In agreement with the data were the levels of HMW conjugates which were not found to differ significantly between the strains as assessed by western analysis with the 6xhis and ubiquitin-specific antibodies (Fig 42). In considering effects that would alleviate the phenotype is that of the levels of ataxin-1 whose fluctuation has recently been shown to affect the course of SCA-1 pathology (Zu et al., 2004). To formally exclude the contribution of reduced pathogenesis due to a decrease

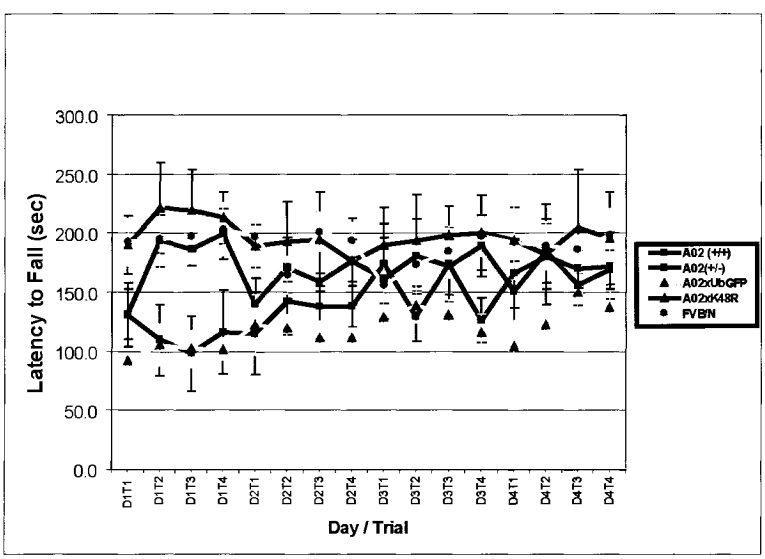
Figure 39. Assessment of motor skills using the rotating rod apparatus at 3 months of age

A) B05xK48R Ub compound heterozygous mice showed performance similar to that of nontransgenic FVB/N control animals, whereas other genetic crosses fared no better than B05 heterozygous mice. B) No difference in performance was noted in the A02 compound transgenics when compared to the control. Bars represent standard error of the mean.

A


300
250
200
150
100
50
0
Latency to Fall (sec)
D1T1
D1T2
D1T3
D1T4
D2T1
D2T2
D2T3
D2T4
D3T1
D3T2
D3T3
D3T4
D4T1
D4T2
D4T3
D4T4
Day / Trial
B05 (+/+)
B05(+/-)
B05xUbGFP
B05xK48R
FVB/N

B


300.0
250.0
200.0
150.0
100.0
50.0
0.0
Latency to Fall (sec)
D1T1
D1T2
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D1T4
D2T1
D2T2
D2T3
D2T4
D3T1
D3T2
D3T3
D3T4
D4T1
D4T2
D4T3
D4T4
Day / Trial
A02 (+/+)
A02(+/-)
A02xUbGFP
A02xK48R
FVB/N

Figure 40. Assessment of motor skills using the rotating rod apparatus at the 8 month time point

B05xK48R Ub compound heterozygous mice showed performance similar to that of B05 transgenic mice. Bars represent standard error of the mean.

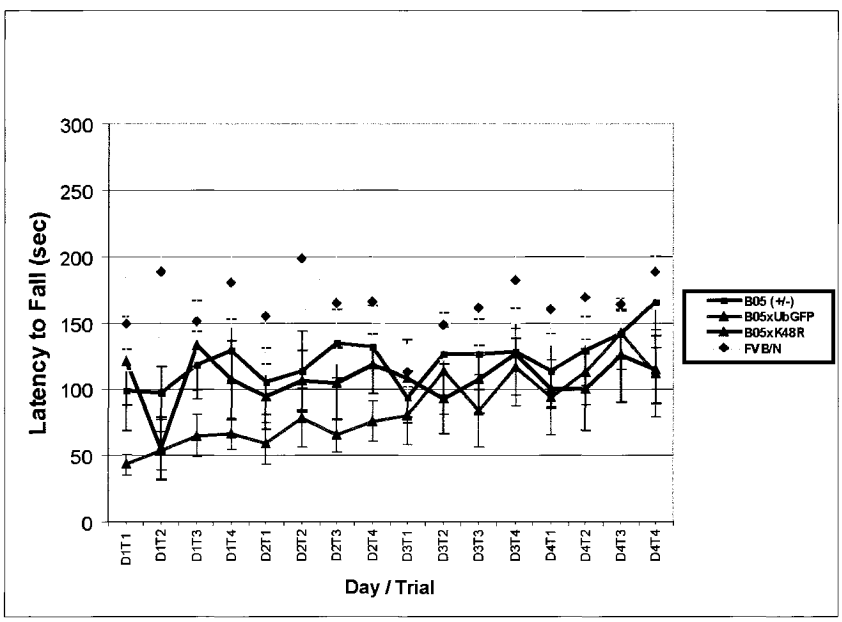

300
250
200
150
100
50
0
Latency to Fall (sec)
D1T1
D1T2
D1T3
D1T4
D2T1
D2T2
D2T3
D2T4
D3T1
D3T2
D3T3
D3T4
D4T1
D4T2
D4T3
D4T4
Day / Trial
B05 (+/-)
B05xUbGFP
B05xK48R
FVB/N

Figure 41. Analysis of the protein levels of Heat shock proteins in 3 month old mouse cerebella

Western analysis of mouse cerebella at 3 months of age with antibodies raised against A) HSP40, B) HSP70 and C) Bip. No difference in the protein levels were noted in the various strains. D) Tubulin served as a control for equal loading of protein

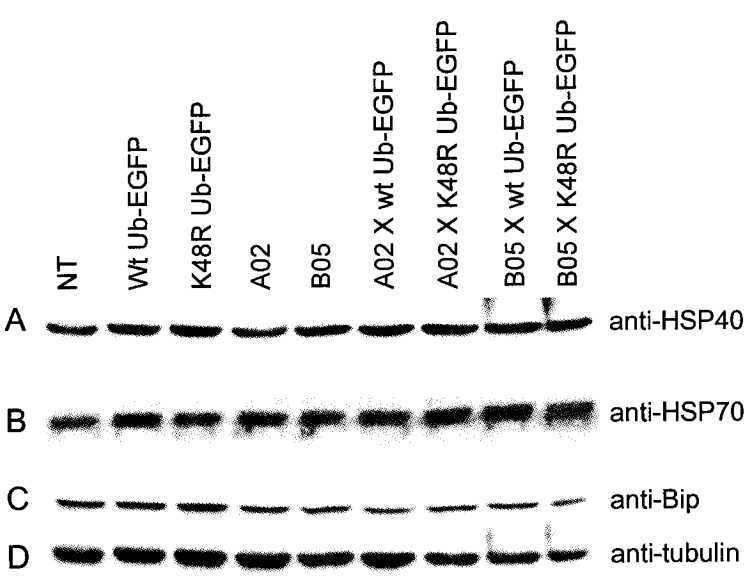
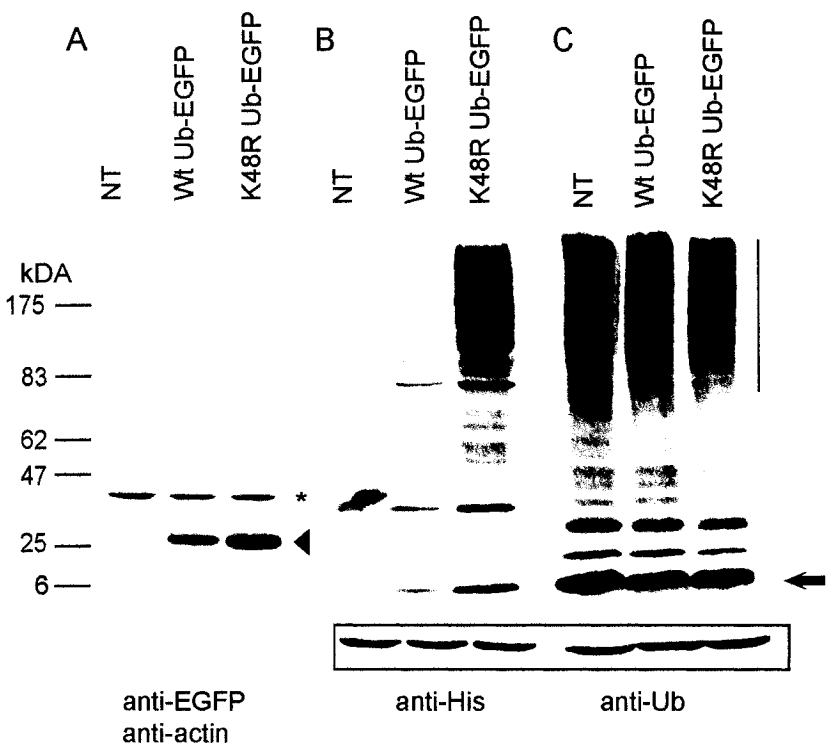

NT
Wt Ub-EGFP
K48R Ub-EGFP
A02
B05
A02 X wt Ub-EGFP
A02 X K48R Ub-EGFP
B05 X wt Ub-EGFP
B05 X K48R Ub-EGFP
A
anti-HSP40
B
anti-HSP70
C
anti-Bip
D
anti-tubulin

Figure 42. Detection of transgene-derived ubiquitin and respective conjugates in the cerebella of 3 month old mice

A) Western analysis with the EGFP specific antibody. 6xHis antibody. HMW conjugates (vertical bar) and transgene-derived monomeric ubiquitin were detected by the antibody. B) Same lysates as in A) analyzed with the ubiquitin specific antibody. No difference in the intensity of the HMW conjugates was observed in the various strains. C) Same lysates as in A) and B) analyzed with the EGFP-specific antibody. The processed EGFP marker is denoted by an arrowhead.


A
B
C
NT
Wt Ub-EGFP
K48R Ub-EGFP
kDA
175
83
62
47
25
6
*
anti-EGFP
anti-actin
anti-His
anti-Ub

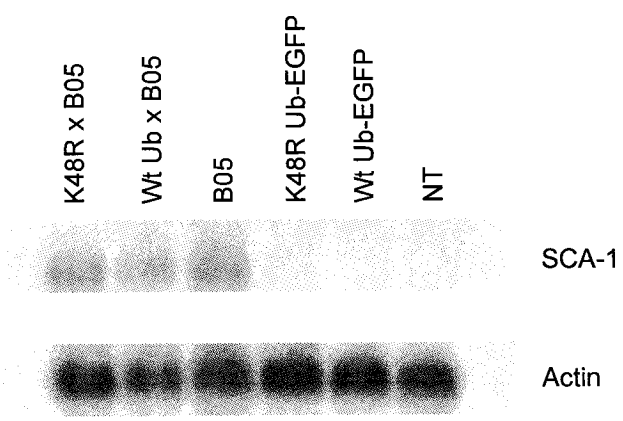
in expression of ataxin-1 in the genetic crosses, the mRNA levels of ataxin-1 were determined by Northern blot analysis; expanded polyQs cannot be quantitatively extracted for Western blot analysis and immunohistochemical analysis would only be semi-quantitative. No differences were noted in the genetic crosses when compared to BO5 (Fig 43).

3.5.4 K48R mutant ubiquitin stabilizes a subset of cellular proteins including p300/CBP

The expression of K48R mutant ubiquitin in stably transfected murine neuroblastoma cells increases the stability of natural and artificial UPP substrates. It was therefore conceivable that the neuroprotective effect of K48R ubiquitin could be mediated through stabilization of some key substrate(s) whose loss figured in the normal course of SCA-1 disease. The γ isotype of protein kinase C (PKC γ) is a neuron-specific, calcium activated enzyme involved in receptor-mediated phosphoinositide signaling cascades (Saito and Shirai, 2002). PKC γ is abundant in Purkinje cells and is clearly important in the normal functioning of the cerebellum; spinocerebellar ataxia type 14 is thought to arise from a missense mutation in the gene encoding PKC γ (Yabe et al., 2003). In BO5 mice, PKC γ expression in the cerebellum is profoundly perturbed through a posttranslational mechanism that is thought to involve enhanced proteolysis (Skinner et al., 2001). To determine if K48R mutant ubiquitin could partially or totally restore PKCγ protein levels in the diseased cerebellum lysates were prepared from mice at 3 months of age and analyzed by western blotting. Consistent with published results, PKCγ was found to be almost undetectable in B05 mice at this time point. In a genetic cross with mice expressing wild-type ubiquitin there was partial restoration of PKC protein levels, but a more substantial increase was noted for the B05xK48R mutant ubiquitin cross (Fig 44A). The stabilization of UPP substrates was not

Figure 43. Analysis of the mRNA levels of SCA-1 in 3 month old mouse cerebella

Northern blot analysis of total RNA from 3 month old straight genotypes and genetic crosses with a cDNA fragment encoding the non-expanded ataxin-1 protein (Q30). The mRNA levels of the transgene-derived pathological ataxin-1 (Q83) did not appear to differ significantly in the cerebella of genetic crosses when compared to B05 mice. Endogenous ataxin-1 mRNA was not detected by the probe. A human ß-actin cDNA fragment served as a control for loading.


K48R x B05
Wt Ub x B05
B05
K48R Ub-EGFP
Wt Ub-EGFP
NT
SCA-1
Actin

global, as evidenced by constant levels of p35 (a known UPP substrate, (Patrick et al., 1998) in all stains tested (Fig 44B). In considering proteins whose stabilization by K48R mutant ubiquitin would be expected to exert a profound effect on cellular homeostasis are those involved in the regulation of neuronal homeostasis. The sequestration of transcription factors by expanded polyglutamine proteins has been proposed as a mechanism of polyQ toxicity in the Huntington's model model of disease (Schaffar et al., 2004). Among those that are sequestered are p300/CBP acetyltransferases whose ubiquitination and degradation was previously shown to be enhanced in the presence of expanded polyglutamine proteins (Jiang et al., 2003). It was therefore conceivable that the expanded ataxin-1 may play an analogous role in enhancing the clearance of p300/CBP and that the incorporation of a K48R chain-terminating ubiquitin variant (in the poly-ubiquitin chain assembled on p300/CBP) would serve to maintain the protein levels of these acetyltransferases. To test this supposition, the protein levels of p300/CBP were analyzed by western blot analysis which revealed a dramatically reduced level in B05 cerebella at 3 months of age as compared to nontransgenic animals. p300/CBP protein levels were increased in transgenic mice expressing wild type ubiquitin relative to nontransgenic controls, with an even greater increase noted in the K48R strain. The influence of mutant ubiquitin was sufficiently profound to elevate the level of p300/CBP in the B05xK48R cross beyond that of nontransgenic controls (Fig 45A). The increase in p300/CBP protein levels was reflected by an increase in acetylated histone H4, as detected by an antibody specific for H4 acetylation on lysine 8 (Fig 45B). In the K48R strain a highly elevated level of H4 acetylation was detected.

Figure 44. Western analysis of PKCγ and p35 protein levels in the cerebella of 3 month old mice

A) PKCγ was almost undetectable in B05 heterozygous mice, was partially restored in the B05 x Wt cross, and reached levels comparable to or greater than A02 heterozygous in the B05 x K48R Ub cross. The membrane was simultaneously probed for tubulin to confirm equal loading. B) Levels of p35 did not vary among cerebella of the different genetic crosses.

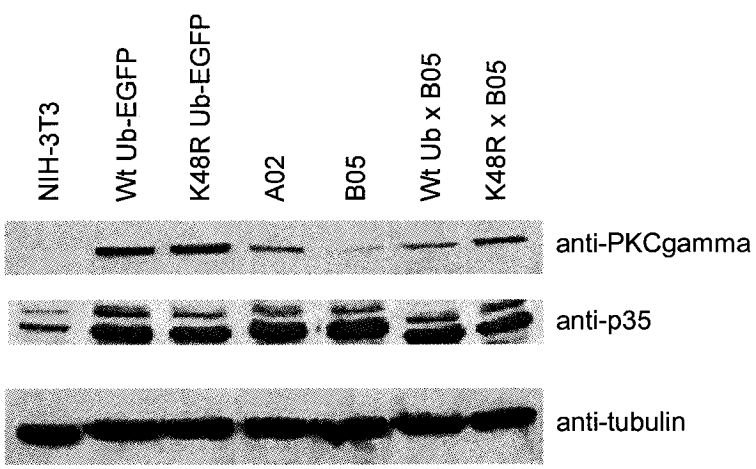
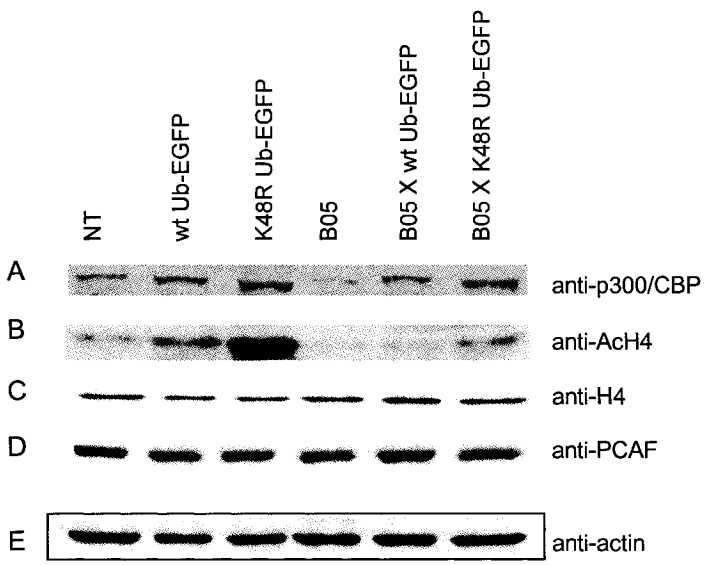

NIH-3T3
Wt Ub-EGFP
K48R Ub-EGFP
A02
B05
Wt Ub x B05
K48R x B05
anti-PKCgamma
anti-p35
anti-tubulin

Figure 45. Western analysis of acetyltransferases in 3 month old mouse cerebella

Western blot analysis with a p300/CBP antibody revealed that the levels were greatly diminished in the cerebella of B05 heterozygous mice but were restored to FVB/N levels or greater by K48R mutant ubiquitin. P300/CBP levels in K48R strain exceeded those of the FVB/N strain as did the HAT activity as evidenced by acetylated histone H4 (Ac-H4). Levels of the PCAF histone acetyltransferase and total histone 4 were unaffected by Ataxin-1 protein in the B05 strain or by expression of mutant ubiquitin.


NT
wt Ub-EGFP
K48R Ub-EGFP
B05
B05 X wt Ub-EGFP
B05 X K48R Ub-EGFP
A
anti-p300/CBP
B
anti-AcH4
C
anti-H4
D
anti-PCAF
E
anti-actin

3.5.5 K48R-mediated stabilization of CBP results in increased CREB-mediated transcription.

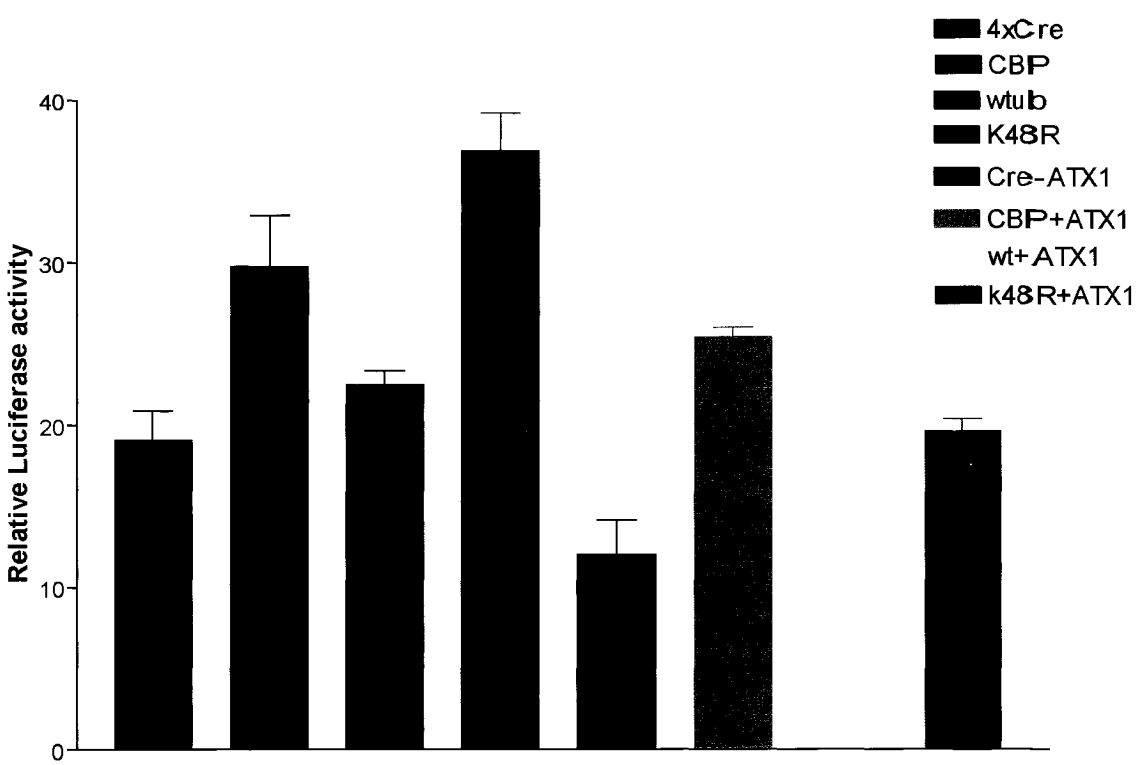
To determine if the postulated stabilization of p300/CBP by K48R mutant ubiquitin had functional consequences with respect to gene expression, a reporter assay was used wherein luciferase activity was dependent on a synthetic promoter incorporating multiple cyclic AMP response elements (CRE). These elements bind CREB (the cAMP element binding protein), thereby recruiting p300/CBP, whose HAT activity increases gene expression (as detected by luciferase activity in this assay system). It was found that transfection of K48R mutant ubiquitin construct elevated luciferase activity in forskolin-treated cells beyond what was observed for cells transfected with a CBP expression vector (Fig 46), perhaps through stabilization of both endogenous CBP and the closely related p300 HAT enzyme. Consistent with previous reports of polyQ-mediated downmodulation of p300/CBP (Jiang et al., 2003) reduced reporter activity was noted in cells transfected with ataxin-1 bearing an expanded polyglutamine tract (Fig 46). This effect could be overcome by cotransfection with CBP or K48R mutant ubiquitin (Fig 46).

3.6 *In vivo* analysis of EGFP-Ubb^{+1} transgenic mice

The data generated from the mutational analysis of the C-terminus of mammalian ubiquitin suggested that the effects of mild perturbations in ubiquitin pools (below the level of detection by western blotting) may not be obvious in cultured cells, but might only be manifest in cells such as the neurons of long-lived mammals expressing mutant ubiquitin. To test this hypothesis in an *in vivo* setting, a transgenic mouse model expressing an ubiquitin variant with an altered C-terminus was generated. The UbC promoter was used to drive ubiquitous expression of the transgene consisting of an N-terminal EGFP marker fused in frame with Ubb^{+1}. The EGFP marker was appended at the N-terminus of ubiquitin

Figure 46. CREB activation of a CRE-luciferase reporter

NIH 3T3 cells stimulated with forskolin were assayed for expression from a CRE-luciferase reporter using a dual luciferase assay system. As expected, transfection with CBP elevated luciferase expression, but transfection of K48R mutant ubiquitin elevated luciferase levels to an even greater extend. Transfection with ataxin-1 (Q82) resulted in a decrease in cre-mediated transcription that was negated by co-transfections of the expanded polyglutamine with either CBP or K48R mutant ubiquitin.


4xCre
CBP
wtub
K48R
Cre-ATX1
CBP+ATX1
wt+ATX1
k48R+ATX1
Relative Luciferase activity
40
30
20
10
0

(thus replacing the 6xHis epitope tag) to facilitate the *in vivo* detection of the transgene otherwise lacking an epitope tag (a schematic representation of the expression vector is shown in Fig 47A). Unlike the expression constructs (K63R and K48R) previously described the EGFP-Ubb^{+1} fusion protein is not expected to serve as a substrate for enzymes with ubiquitin cleaving activity; the EGFP marker serves as a proxy for detection of the ubiquitin moiety. The expression of the transgene was found to be uniformly expressed as assessed by fluorescence microscopy of dissected brains from progeny of a nontransgenic crossed with an EGFP-Ubb^{+1} founder mouse (Fig 47B). Neuronal expression of the transgene was also visualized by immunohistochemistry with an antibody specific for EGFP which revealed cytoplasmic and nuclear expression in neurons of the cerebral cortex (Fig 48A and 48B) of a 5 month old founder mouse. The presence of the neoepitope was confirmed by tissue staining with an antibody specific for the neoepitope; levels of cytoplasmic and nuclear expression were observed in the hippocampus (Fig 49) and the cerebral cortex (Fig 48C-D and Fig 50). The analysis of progeny from a non-transgenic crossed with an EGFP-Ubb^{+1} mouse by western blot with the EGFP antibody revealed the presence of the fusion protein while no higher order conjugates were observed in brain lysates from the transgenic animals (Fig 51A). Analysis of the same lysates with the ubiquitin-specific antibody revealed no difference in the levels of endogenous ubiquitin or respective conjugates suggesting that the expression of mutant ubiquitin does not result in a compensatory up-regulation of the stress-inducible endogenous ubiquitin genes (Fig 51C). In agreement with findings in cultured mammalian cells, *in vivo* expression of mutant ubiquitin in young animals (2 months of age) was not found to alter the chymotrypsin-like and peptidyl-like activities of the 20S core proteasome when compared

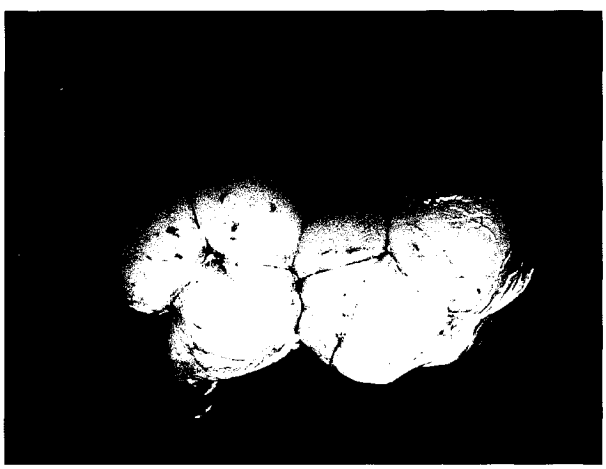
Figure 47. Detection of the EGFP-Ubb^{+1} fusion protein in dissected brain

A) Schematic representation of the EGFP-Ubb^{+1} transgene. B) dissected brains from a nontrangenic mouse and its transgenic littermate. When viewed under fluorescent light, the transgenic mouse brain was found to be uniformly fluorescent.

A

+/- -/-

B



C

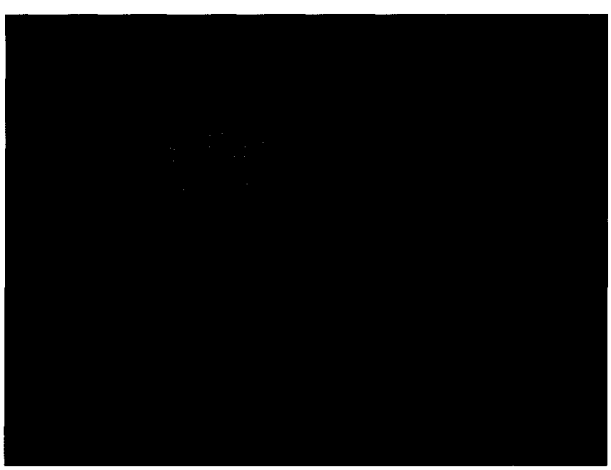


Figure 48. Immunohistochemical detection of the EGFP-Ubb+1 transgene in a 5 month old transgenic

Immunohistochemistry of coronal sections using A) the EGFP specific antibody and B) the antibody raised against the neoepitope. Both antibodies revealed cytoplasmic and nuclear expression of the transgene in cortical neurons in the cerebral cortex. Nuclei were visualized by counterstaining sections with hematoxylin.

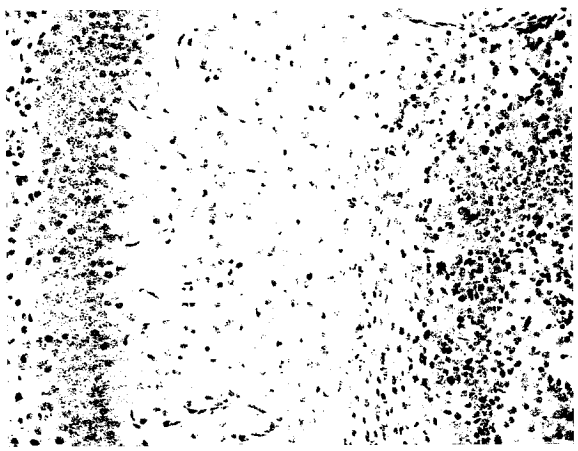
A

B

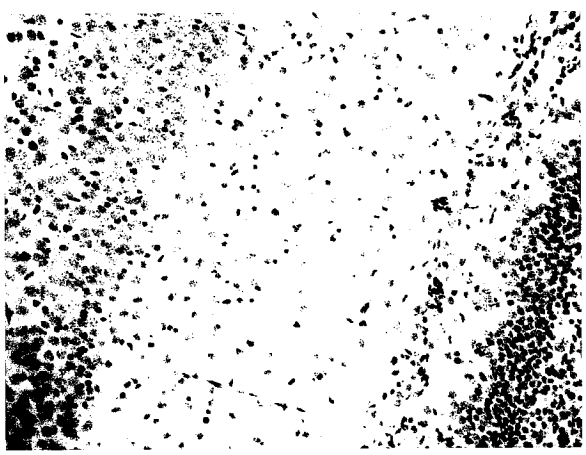
Figure 49. Analysis of the EGFP-Ubb^{+1} transgene in the hippocampus of a 3 day old mouse brain

A) Immunohistochemical analysis using an antibody specific for the neoepitope demonstrating uniform expression of the transgene in the CA1 neurons of the hippocampus. B) aged-matched nontransgenic littermate was found to be negative. C) secondary antibody control.

A



B



C

Figure 50. Detection of the neopitope in the cerebral cortex of a 3 day old EGFP-Ubb^{+1} transgenic mouse

Immunohistochemistry with the neoepitope specific antibody. Low magnification of the cerebral cortex of A) transgenic EGFP Ubb+1 mouse and B) Nontransgenic littermate. High magnification of the same regions of C) a transgenic mouse and D) a nontransgenic control. The transgene was found to be expressed in the cytoplasmic and nuclear compartments of neurons. E) Secondary antibody alone control.

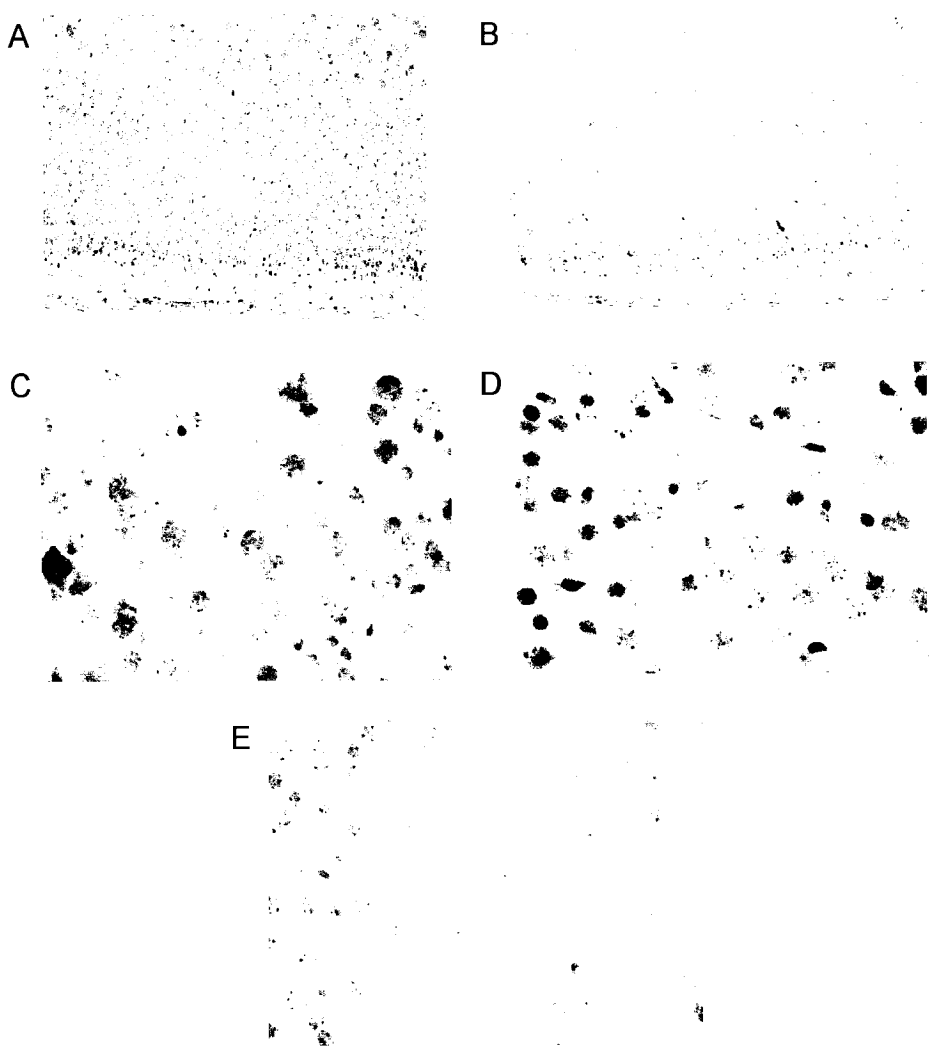
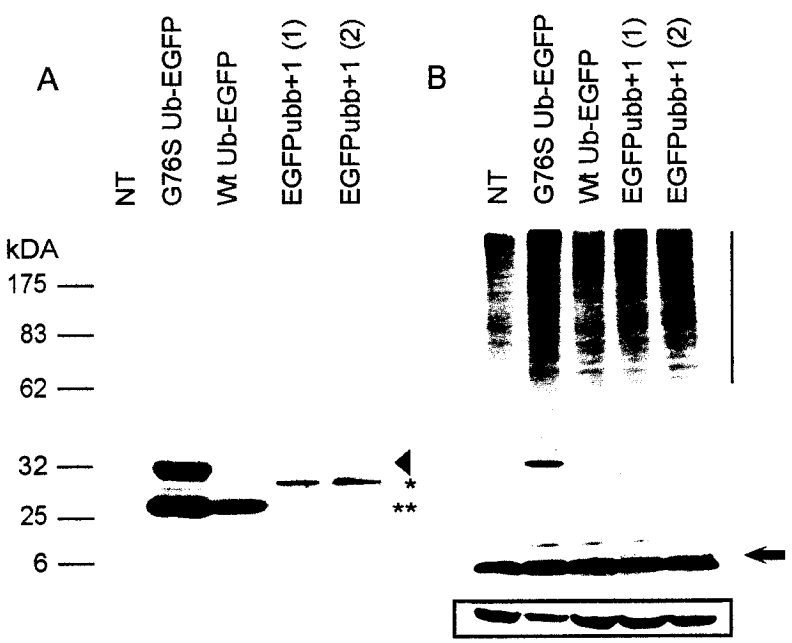

A
B
C
D
E

Figure 51. Detection of the EGFP-Ubb^{+1} transgene in the brains of transgenic mice by western analysis

A) Western blot analysis with the EGFP specific antibody of total brain lysates prepared from two heterozygous EGFP-Ubb+1, an aged matched wt Ub-EGFP transgenic and a non-transgenic littermate. An extract from cells transfected with the G76S Ub-EGFP expression vector served as a control to indicate the expected size of the Ub-EGFP fusion protein. The EGFP-Ubb+1 fusion protein and the processed EGFP marker derived from the processing of the wt Ub-EGFP fusion protein are indicated by an (*) and (**) respectively. The Ub-EGFP fusion protein in lysates from cells expressing G76S Ub-EGFP is indicated by an arrowhead. B) Same membrane as in A) analyzed with the ubiquitin-specific antibody. No difference in intensity of HMW conjugates (vertical bar) or in the levels of the ubiquitin monomer (arrow) were noted. Actin served as a loading control.


A
NT
G76S Ub-EGFP
Wt Ub-EGFP
EGFPubb+1 (1)
EGFPubb+1 (2)
kDA
175
83
62
32
25
6
*
**
B
NT
G76S Ub-EGFP
Wt Ub-EGFP
EGFPubb+1 (1)
EGFPubb+1 (2)

to an age-matched wt Ub-EGFP expressing mouse as assessed by processing of fluorogenic substrates (Fig 52). In accordance with the postulate of delayed effects due to expression of a C-terminal ubiquitin variant, was the preliminary data obtained by H&E staining of brain sections of a founder mouse at 5 months of age which revealed the presence of dark neurons in the dentate gyrus region of the hippocampus (Fig 53).

3.7 The case of a lysine-less ubiquitin variant

The unexpected observation that K48R mice were not only viable but conferred a protective phenotype suggested that the inhibitory effect on degradation by the introduction of a chain-terminating version of ubiquitin may provide beneficial effects. The increase in intensity of smears in K48R cell and brain lysates may represent the assembly of un-conventional linkages not assembled through K48 (other lysines that are present on the ubiquitin monomer can serve in conjugation reactions) which can be the basis for the protective effect observed in genetic crosses (the un-orthodox or mixed chains may preclude disassembly by de-ubiquitinating enzymes or may serve non-proteolytic functions). In order to eliminate this possibility a chain- terminating mutant isoform of ubiquitin in which the assembly of poly-Ub chain formation is completely abolished was generated, introduced into cultured mammalian cells and utilized in the generation of transgenic mice. This plasmid was designated noK Ub-EGFP; all lysine residues were mutated to arginines. Prior to the generation of transgenic mice, the expression construct was tested by transient transfection in 293T cells. Western blot analysis with the EGFP-specific antibody not surprisingly revealed that the Ub-EGFP fusion protein was efficiently recognized and processed by deubiquitinating enzymes (Fig 54C). The analysis of the same extracts with the 6xHis antibody revealed the presence of higher order ubiquitinated

Figure 52. Expression of mutant ubiquitin does not impair the activity of the 20S core proteasome *in vivo*

Total brain extracts were prepared from transgenic and nontransgenic littermates and assayed for their ability to process two fluorogenic substrates measuring the chymotrypsin like and peptidyl-glutamyl-like (PHG) activities of the proteasome. No significant difference in cleavage of the substrates were noted when compared to an aged matched wt Ub-EGFP brain extract. Lysates were assayed in triplicates. Proteasome inhibitor I was used as a control for the chymotrypsin-like activity whereas Epoxomycin (a more general inhibitor of the proteasome) served as a control for the PHG activity.

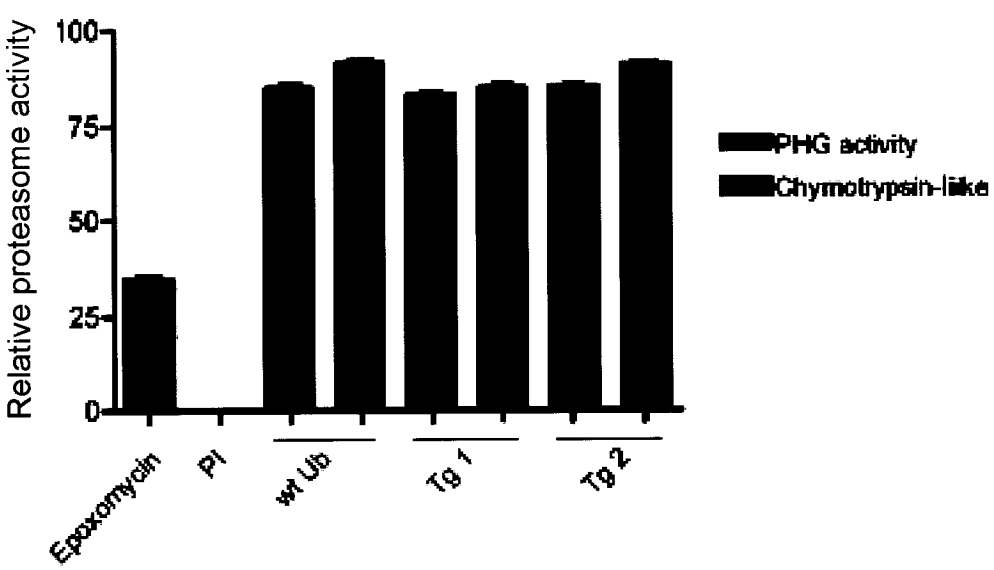
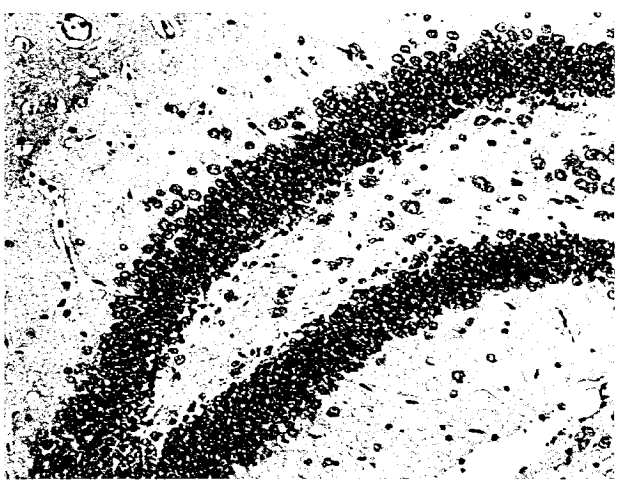

Relative proteasome activity
100
75
50
25
0
Epoxomycin
PI
wt Ub
Tg 1
Tg 2
PHG activity
Chymotrypsin-like

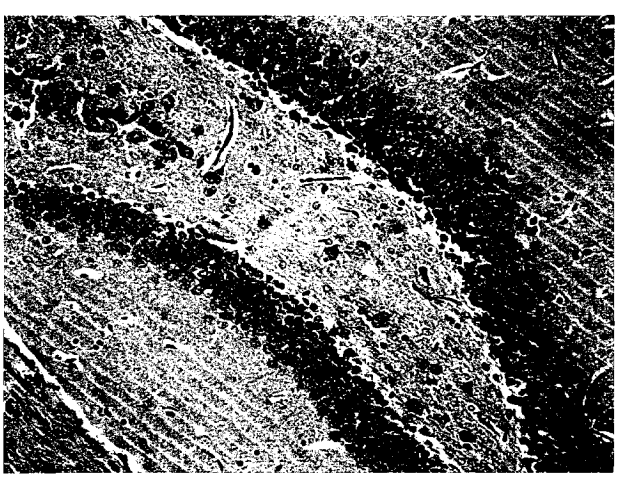
Figure 53. Neuropathology in the brains of a EGFP-Ubb^{+1} founder mouse

A) Immunohistochemical analysis using H&E of paraffin-embedded sections of brains derived from an EGFP-Ubb^{+1} founder at 5 months of age. Apoptotic or necrotic neurons were observed in the dentate gurus region of the hippocampus of the EGFPubb+1 transgenic mouse that were not observed in an aged-matched nontransgenic control (shown in panel B).

A



B



species whose detection was enhanced by pre-treatment with proteasome inhibitor for 3 hours prior to harvesting the cells (Fig 54A). The presence of ubiquitin in the HMW conjugates was confirmed by probing the membrane with an ubiquitin-specific antibody (Fig 54B). Transgene-derived and endogenous monomeric ubiquitin were not detected with the antibodies raised against the 6xHis epitope or ubiquitin (Fig 54A and B). The data, although unexpected, suggested that noK Ub may result in the depletion of ubiquitin pools, a feature proposed to contribute to neurodegenerative diseases. In transgenic mice, the fusion protein was found to be ubiquitiously expressed with high levels of expression detected in brains and testis as assessed by fluorescent microscopy (Fig 55) of dissected tissues and that the noK Ub-EGFP fusion protein was as processed as it was in cells (as assessed by western blot analysis with the EGFP-specific antibody, Fig 56C). The analysis of the same extracts with the antibody raised against the 6xHis epitope revealed the presence of the transgene-derived monomeric ubiquitin (Fig 56A). High molecular weight conjugates were not found to incorporate mutant ubiquitin (Fig 56A). The absence of these HMW conjugates in extracts from noK Ub transgenics suggested that the ubiquitin monomer may be found in the detergent-insoluble membrane fractions of the cell (mono-ubiquitination serves as a signal for endocytocis). To test this supposition, cell extracts from noK and non-transgenic controls were prepared in the presence of SDS. Western analysis of the extracts with the 6xHis epitope-tag antibody did not reveal a significant difference with respect to conjugate detection when compared to the extracts prepared in the absence of SDS (Fig 57). When the proteasomes of mice expressing noK Ub-EGFP were analyzed for their ability to process fluorogenic substrates, it was found to that their activity was dramatically increased when compared to mice expressing the wt Ub-EGFP

transgene (Fig 58). This suggested that the presence of noK Ub-EGFP may be affecting the expression of proteasome subunits.

Figure 54. Detection of noK Ub-EGFP in transiently transfected 293T cells

A) Western analysis of extracts with an antibody raised against the 6xHis epitope-tag. The antibody revealed the presence of high molecular weight conjugates (vertical line) in transfected cells. The transgene-derived ubiquitin monomer was not detected in noK Ub-EGFP expressing cells but was observed in lysates from cells expressing the wt Ub-EGFP isoform (arrowhead). B) Same lysates as in A) analyzed by western blot with the antibody raised against ubiquitin. The endogenous ubiquitin monomer was not detected in noK Ub-EGFP expressing cells but was observed in wt Ub-EGFP and untransfected cell lysates (arrow). C) Same lysates as in A) and B) analyzed with the EGFP specific antibody which confirmed the efficient processing of the Ub-EGFP fusion protein and was indicative of the levels of expression.

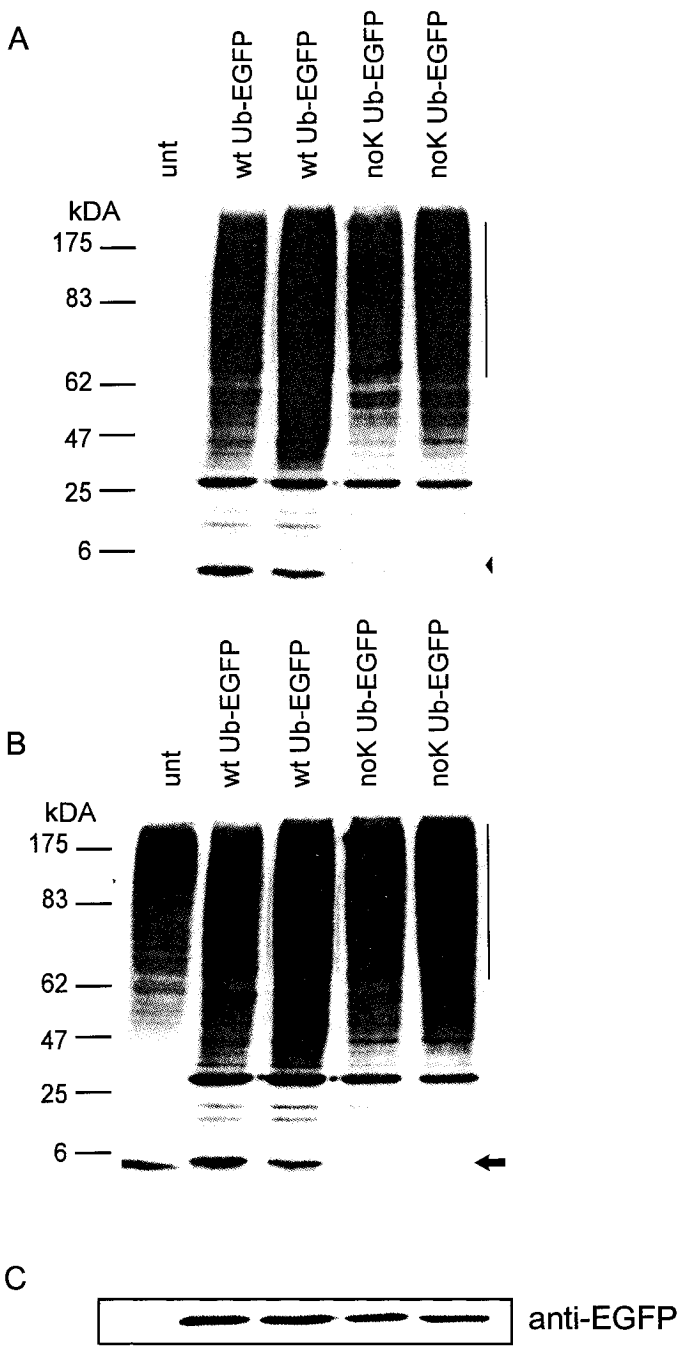

A
unt
wt Ub-EGFP
wt Ub-EGFP
noK Ub-EGFP
noK Ub-EGFP
kDA
175
83
62
47
25
6
B
unt
wt Ub-EGFP
wt Ub-EGFP
noK Ub-EGFP
noK Ub-EGFP
kDA
175
83
62
47
25
6
C
anti-EGFP

Figure 55. *In vivo* detection of the EGFP marker in noK Ub-EGFP transgenic mice

Dissected brains and testis from a heterozygous transgenic mouse. When viewed under fluorescent light (lower panel), the transgenic mouse brain and testis were found to be intensely fluorescent.

A

B

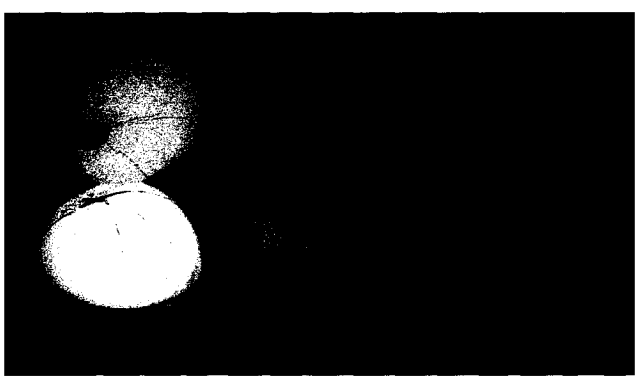


Figure 56. Detection of epitope tagged ubiquitin in noK Ub-EGFP transgenic mice

A) Western analysis with the 6xHis antibody revealing the presence of the transgene-derived ubiquitin monomer (arrow, anti-His). HMW conjugates were not detected. B) Same extracts as in A) analyzed with the ubiquitin-specific antibody. An arrow denotes the endogenous ubiquitin monomer (anti-Ub). HMW conjugates are indicated by a vertical line. C) Western analysis with the EGFP-specific antibody of the same lysates as in A) and B). The processed EGFP marker derived from the processing of the Ub-EGFP fusion proteins is denoted by an arrowhead. EGFP levels served as an indicator of expression levels; the noK Ub-EGFP transgene was found to be expressed at lower levels when compared to the wt Ub-EGFP fusion protein.

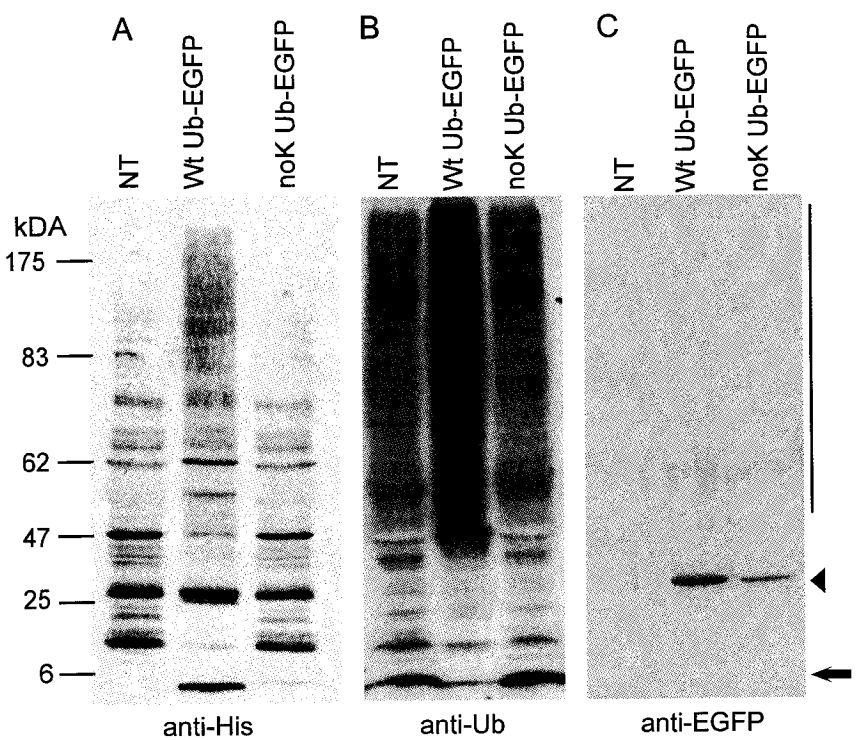

A
B
C
NT
Wt Ub-EGFP
noK Ub-EGFP
kDA
175
83
62
47
25
6
anti-His
anti-Ub
anti-EGFP

Figure 57. Analysis of the insoluble extracts from the brains of noK Ub-EGFP expressing mice

Western blot analysis with the 6xhis antibody of cell extracts from a noK Ub-EGFP and nontransgenic littermate in the presence of SDS. A lysates from mice expressing the wt Ub-EGFP transgene served as a control. Monomeric ubiquitin (arrowhead) was detected by the antibody whereas HMW conjugates (vertical line) were not observed in noK Ub-EGFP expressing mice.

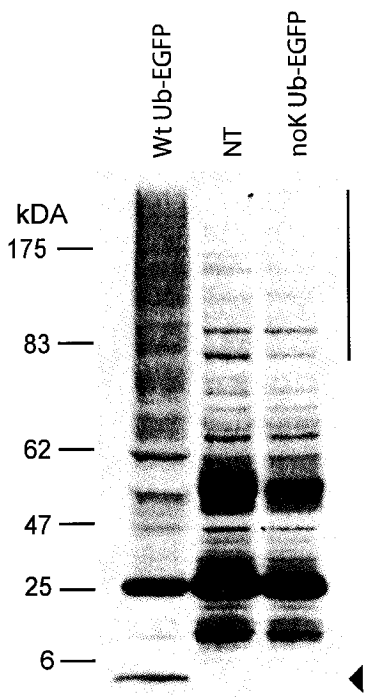
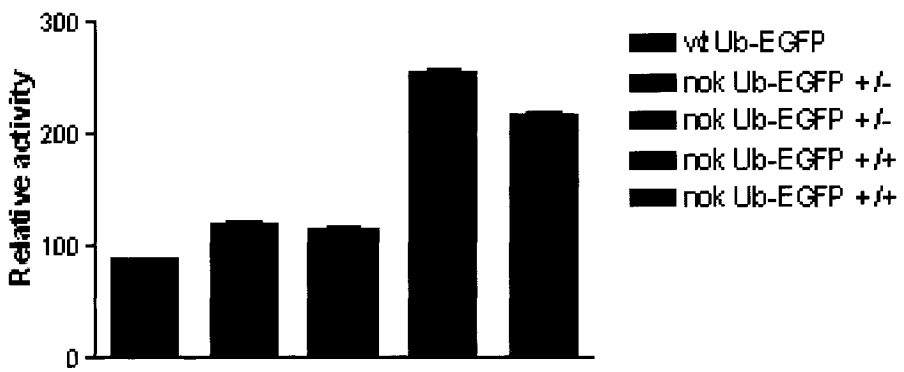

Wt Ub-EGFP
NT
noK Ub-EGFP
kDA
175
83
62
47
25
6

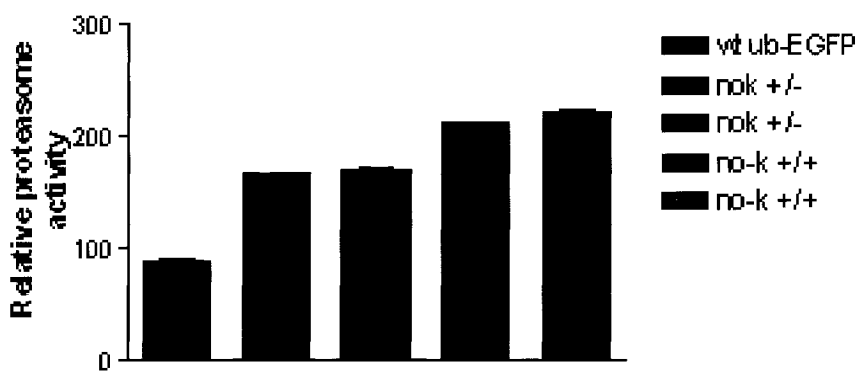
Figure 58. Expression of no-k Ub-EGFP enhances the activity of the 26S proteasome *in vivo*

Brain extracts from transgenic and nontransgenic littermates were assayed for their ability to process two fluorogenic substrates measuring in A) the chymotrypsin like and in B) the peptidyl-glutamyl-like (PGHP) activities of the proteasome. Both activities were found to be significantly increased in the brain lysates of two heterozygous and two homozygous no-K Ub-EGFP transgenics when compared to lysates from an aged-matched wt Ub-EGFP transgenic. Lysates were assayed in triplicates. Proteasome inhibitor I and Epoxomycin served as controls for the chymotrypsin-like and PHG activities respectively.

A


300
200
100
0
Relative activity
vt Ub-EGFP
nok Ub-EGFP +/-
nok Ub-EGFP +/-
nok Ub-EGFP +/+
nok Ub-EGFP +/+

B


300
200
100
0
Relative proteasome activity
vt ub-EGFP
nok +/-
nok +/-
no-k +/+
no-k +/+

Chapter 4
Discussion

4.1 Epitope-tagged ubiquitin in mammalian cells and in transgenic brains

The use of epitope-tagged ubiquitin to study the fate of wild-type or mutant ubiquitin in conjugation and proteasomal targeting is well established in the yeast model system (Ellison and Hochstrasser, 1991). More recently, epitope-tagged ubiquitin has proved of great utility in transfected cells of higher eukaryotes (Treier et al., 1994; Ward et al., 1995), and transgenic plants have been generated in which tagged ubiquitin can be used to retrieve ubiquitinated substrates (Ling et al., 2000). A similar strategy was employed herein to express 6xHis epitope-tagged wild type or variants of ubiquitin as linear fusions with EGFP, thereby replacing the ribosomal subunit with an easily detectable marker (Fig 6). The objective in creating these expression constructs was to study the effects of mutant isoforms of ubiquitin in higher eukaryotic cells and in transgenic mice. In agreement with the prediction, fusions of ubiquitin with EGFP were found to be efficiently recognized and processed in vitro (Fig 7 and 54) and *in vivo* (Fig 34 and 56) and that the ubiquitin moieties so generated were incorporated into HMW conjugates (Fig 7, 33 and 54) with the exception of noK Ub-EGFP which was not detected in high order conjugates in mice (Fig 56). The lack of detection of higher order ubiquitinated conjugates was not due to a localization of the transgene-derived ubiquitin to the membrane; no additional conjugates were observed in the insoluble membrane fraction (Fig 57) by western analysis with the epitope tag specific antibody. The basis for this is currently unclear and clouded by the lower levels of expression of the transgene as assessed by western blot with the EGFP specific antibody (Fig 56C). It is conceivable that the transgene-derived ubiquitin serves in the assembly of polyubiquitin chains that are below the level of detection by western

analysis. Alternatively, it may serve in mono-ubiquitination of substrates (rather than poly-ubiquitination) and such mono-ubiquitinated structures may be difficult to observe by western blot analysis without prior stimulation with an appropriate stimulus; it is well documented that the mono-ubiquitination of cellular substrates destined for lysosomal degradation require ligand-induced ubiquitination ((Burgdorf et al., 2004), (Haglund et al., 2002; Haglund et al., 2003)). The data suggest that high levels of expression of no K Ub may be too deleterious in vivo (a selection for low expressers may have occured) and the effects of its expression may only be manifest in animals subject to additional stress such as aging or in the presence of a burden of misfolded or toxic proteins (for example polyglutamine). In support of the adverse effect exerted by noK Ub is the increase in both the chymotrypsin and peptidyl-glutamyl activities of the 26S proteasome in 1 month old animals which was markedly pronounced in homozygous mice (Fig 58). Considering that the levels of endogenous ubiquitin were not found to differ significantly when compared to a nontransgenic control littermate (the wt Ub-EGFP lysate used in figure 56 was prepared from a non aged-matched control), it is likely that the expression of noK ubiquitin induces *de novo* synthesis of proteasome subunits which serve in the assembly of functional proteasomes to counteract the effects of the mutant. It is conceivable that the increase in proteasome activity may be masking the phenotype *in vivo* and it is possible that higher levels of expression would have recapitulated the findings in cultured mammalian cells (i.e. mainly a phenotype of ubiquitin deprivation, Fig 54). It is currently unclear how the expression of noK Ub depletes endogenous ubiquitin pools; it is possible that chains capped by noK Ub are not efficiently disassembled. Mutations in the globular domain of the ubiquitin monomer have been shown to affect its processing/recycling ((Johnsson and

Varshavsky, 1994a), (Johnsson and Varshavsky, 1994b)). In support of this supposition is the absence of both endogenous and transgene-derived ubiquitin from lysates of transfected cells (Fig 54). By utilizing in vitro assembled chains consisting of wild-type and noK ubiquitin moieties it should be possible to decipher whether the chains that have incorporated the latter isoform are refractory to disassembly by enzymes with ubiquitin recycling activity.

In brain lysates of other ubiquitin transgenics (K48R and K63R) it was found that the endogenous levels of ubiquitin in all animals (as assessed by western blot analysis with an ubiquitin-specific antibody) did not differ significantly between the different strains suggesting that the expression of mutant isoforms of ubiquitin does not result in a compensatory induction of endogenous ubiquitin genes (Fig 33). Using standard affinity methodologies, ubiquitin conjugates were recovered from cells (Fig 10) and from brains of transgenic mice (Fig 35). In the latter, the patterns of conjugates appeared to differ in pups versus adults in a manner reminiscent of endogenous ubiquitin conjugates, suggesting that the tagged ubiquitin was recognized and utilized in the same fashion as endogenous ubiquitin (Fig 34). With the probable exception of histone H2A, an abundantly ubiquitinated protein in brains, the identity of these ubiquitinated conjugates is unknown. It is presently unclear whether the observed conjugates represent a small number of different proteins with variable levels of ubiquitination or a more complex assortment of ubiquitinated proteins. SDS-PAGE analysis of the pattern, following the removal of ubiquitin chains by a deubiquitinating enzyme, would resolve this issue, but enzymes that can perform this function *in vitro* have yet to be identified. A candidate approach was adopted to detect a given ubiquitinated protein in total brain eluates; the lack of detection

of such abundantly expressed proteins (p35 and tau) suggests that the conjugates represented in the high molecular weight smears are probably composed of a large repertoire of ubiquitination proteins rather than a small number of different proteins with variable levels of ubiquitination. It is not unexpected that these conjugates would be represented by a complex assortment of substrates considering that the transgene-derived ubiquitin moieties can equally participate in poly or mono-ub chain assembly. This supposition is supported by the findings by Layfield *et al* demonstrating that a complex mixture of poly-ubiquitinated proteins can be successfully purified on an immobilized glutathione-S-transferase (GST)-S5a fusion protein from pig brains (Layfield et al., 2001). The purification strategy employed herein would serve admirably in elucidating ubiquinated protein structures whose ubiquitination levels and/or abundance may be up-regulated in response to an external stimulus (for example virus infection or cytokines).

The incorporation of the EGFP marker has allowed the determination of not only the distribution of transgene expression in pups and adult animals, but also the onset of transgene expression (of considerable importance for the analysis of ubiquitination in a developmental context). It was found that the UbC promoter became active in the early preimplantation embryo, with fluorescence first detectable at the morula stage (Figure 32). It is conceivable that the transgene is activated even earlier, because there is a known lag between translation of EGFP and its fluorescence (Heim et al., 1994). These data suggest that the UbC promoter is a suitable element not only to achieve widespread expression as has been reported previously (Schorpp et al., 1996), but also to achieve early embryonic expression. In this sense the UbC promoter is at least comparable to the cytomegalovirus (CMV) immediate early enhancer/chicken β-actin promoter that has also been used to drive

ubiquitous expression of an EGFP marker (Hadjantonakis et al., 1998). It must be noted that in the latter case the route of transgene incorporation was via electroporation of ES cells (clones were pre-selected for EGFP expression in aggregation chimeras (Hadjantonakis et al., 1998). The CMV enhancer element has been used in conjunction with various promoter elements to drive the ubiquitous expression of transgenes delivered by pronuclear injection, with results that are somewhat variable (Schmidt et al., 1990; Koedood et al., 1995; Baskar et al., 1996a; Baskar et al., 1996b; Sawicki et al., 1998; van den Pol and Ghosh, 1998). What is remarkable about the UbC promoter is the near perfect concordance between genotypic positivity and transgene expression *in vivo* as assayed by fluorescence. For the wt Ub-EGFP construct 9 out of 10 founders receiving the transgene expressed EGFP at detectable levels (we have not determined if the transgene of the remaining animal was silent, or was active at a level below the limits of detection). It would seem that at least in the context of this construct the UbC promoter is relatively insensitive to 'position effects' (i.e., the strong influence of insertion site on transcriptional activity (Robertson et al., 1995; Koetsier et al., 1996). We have not attempted to correlate fluorescence to copy number. The expression of 6xHis-Ub and of EGFP from the morula stage onward did not seem to affect preimplantation or postimplantation embryonic viability as assessed by the development of cultured embryos to the blastocyst stage and by litters sizes, respectively. These findings, and the apparently normal recognition of the tagged ubiquitin by the cellular enzymes responsible for conjugation suggest that the epitope tagged ubiquitin can efficiently be employed in higher eukaryotic cells and in the *in vivo* setting of transgenic mice.

4.2 Phenotypes of cells expressing ubiquitin variants bearing globular mutations

The objective in creating dominant-negative (K48R and K63R) mutant ubiquitin isoforms was to investigate the role of the ubiquitin/proteasome pathway in response to oxidative damage in mammalian cells. It has been previously reported that yeast cells deficient in the ubiquitin-conjugating enzyme Ubc7 are hypersensitive to cadmium-induced protein damage (Jungmann et al., 1993). Mammalian cells respond to cadmium-mediated oxidative damage by triggering a ubiquitin/proteasome response, and previous studies in the HT4 line have demonstrated the accumulation of ubiquitin-protein conjugates in a time- and dose-dependent manner (Figueiredo-Pereira et al., 1997). Cadmium was found to deplete cellular pools of glutathione (the cell's primary defense against oxidation), resulting in an accumulation of mixed protein thiols (Figueiredo-Pereira et al., 1998). The observation of accumulated high molecular weight ubiquitin conjugates in cadmium-treated cells, detectable with the epitope tag-specific antibody or with a ubiquitin-specific antibody (Fig 12) suggest that transgene-derived ubiquitin becomes incorporated into ubiquitin chains in response to oxidative stress. The cadmium-induced ubiquitination of proteins was found to occur in all of the transfected HT4 cell lines (Fig 12). The differences in intensity observed between HT4 cells expressing wild-type *versus* K63R mutant ubiquitin are attributable to the lower level of K63R mutant ubiquitin, and it is speculated that higher level expression of the K63R Ub-EGFP fusion would result in comparable levels of the high molecular weight smear of ubiquitinated proteins detected by Western blot analysis. Although the differences in expression levels among the lines were not significantly altered, there were profound differences in the sensitivity of the lines to cadmium as evidenced by the striking morphological changes observed under light microscopy. The cells expressing K48R mutant ubiquitin were more severely affected than the cells expressing exogenous

wild-type ubiquitin or untransfected control cells, but the most pronounced effects were observed in the K63R mutant ubiquitin-expressing cells (Fig 13). Cadmium is known to damage multiple targets in cells, including protein, lipids, and DNA (Stohs et al., 2001). Our data suggest that in neuroblastoma cells exposed to cadmium, DNA may be a more important target than protein, given the relative sensitivity of K63R *versus* K48R mutant ubiquitin-expressing cells. There is clear evidence from yeast (Spence et al., 1995) and more recently from mammalian cells (Hofmann and Pickart, 1999) demonstrating a role for Lys^{63}-linked ubiquitin chains in DNA repair, particularly in the pathway that mediates replication bypass through lesion-containing DNA. It is our supposition that HT4 cells expressing K63R mutant ubiquitin have a compromised ability to assemble the Lys^{63}-linked chains that are required for the repair of cadmium-induced DNA damage. Lys^{63}-linked chains have been reported to be involved in other pathways in the cell, including ribosome assembly (Spence et al., 2000) and IκB degradation (Deng et al., 2000a), and we cannot exclude contributions to the cadmium toxicity we have observed in K63R mutant ubiquitin-expressing cells from mechanisms unrelated to DNA repair. To reduce the complicating effects of DNA damage and to increase the component of toxicity due to protein damage, cells were treated with canavanine, an analog of arginine whose incorporation during protein translation leads to misfolding. Interference with the ubiquitin proteolytic pathway is known to sensitize yeast to canavanine (Heinemeyer et al., 1991), whereas overexpression of wild-type ubiquitin enhances the survival of yeast in the presence of this arginine analog (Chen and Piper, 1995). Yeast cells expressing K48R mutant ubiquitin have an impaired ability to degrade canavanyl proteins (Finley et al., 1994), which is

probably due to their diminished ability to assemble the Lys^{48}-linked chains that are the primary signal for proteasomal targeting (Pickart, 1997).

In accordance with the findings in yeast, HT4 cells expressing K48R mutant ubiquitin showed greater sensitivity to canavanine than cells expressing K63R mutant ubiquitin (Fig 14). As had been previously reported in yeast (Chen and Piper, 1995), overexpression of wild-type ubiquitin conferred some level of resistance, as evidenced by the viability of cells transfected with the wild-type expression vector compared with untransfected control cells. The relative sensitivity of K48R mutant ubiquitin-expressing cells cannot be explained by higher levels of transgene expression in K48R mutant ubiquitin expressors *versus* the other cell lines because expression of wild-type ubiquitin and K63R mutant ubiquitin conferred resistance, not sensitivity (Fig 14). The 20S core proteasome has been shown to degrade oxidized proteins in an ubiquitin-independent manner and it is possible that canavanyl proteins are eliminated in this fashion (Shringarpure et al., 2003). Given the differential sensitivity of cells to canavanine and the well documented requirement for ubiquitin in the degradation of canavanyl proteins in yeast, it is more likely that the basis for the sensitivity of cells expressing K48R mutant ubiquitin is the interference with the assembly of proteasomal targeting signals on canavanyl proteins. An accumulation of high molecular weight ubiquitin conjugates in cells expressing K48R mutant ubiquitin was observed even in the absence of additional stress (Fig 7). This phenomenon may also occur in the transgenic *Arabidopsis* model and may contribute to the enhanced recovery of ubiquitinated proteins by nickel chromatography that was reported for K48R mutant *versus* wild-type ubiquitin (Ling et al., 2000). Experiments performed on clonal populations (four different clones were tested, one of which is shown in Fig 16) revealed that lysates of K48R mutant

ubiquitin-expressing cells consistently contained elevated levels of high molecular weight conjugates relative to cells expressing the wild-type ubiquitin transgene; EGFP levels were equivalent as assessed by Western blotting with an antibody directed against EGFP or by flow cytometry. The data suggest that even in the absence of any stress, K48R mutant ubiquitin either caps the growing ubiquitin chains, resulting in stable subthreshold length chains, or directs the formation of unconventional linkages not competent to target proteins for proteolysis. The western blot data do not allow the discrimination between these possibilities and given the complexity of potential substrates, it is possible that both types of chains are represented in the high molecular weight smear. The assembly of nonfunctional chains would have the effect of depleting ubiquitin pools; and in some experiments, a decrease in the intensity of monomeric ubiquitin was noted in cells expressing K48R mutant ubiquitin. Depletion of ubiquitin pools would compromise the ability of cells to cope with the burden of aberrant protein presented by agents like canavanine and may be the basis for the increased detachment and cell death observed in K48R mutant ubiquitin-expressing cells treated with canavanine (Fig 14). All of these mechanisms (formation of sub-threshold chains, assembly of chains with unusual linkages, and depletion of monomeric ubiquitin pools) would have global effects on ubiquitin-mediated proteolysis, and such effects have been documented using two unrelated proteasome substrates (Fig 11). The present data do not specify which of the inhibitory mechanisms is operative in cells expressing K48R mutant ubiquitin. Further experiments will be required to determine this. The data demonstrate that ubiquitin is cytoprotective for mammalian cells of neural origin and mutations in ubiquitin predispose cells to the toxic effects of misfolded proteins.

4.3 In vivo modulation of SCA-1 by K48R mutant ubiquitin

From their colocalization with chaperones and UPP components it is likely that expanded polyglutamine proteins are recognized by the cell as improperly folded and subjected to a molecular triage that would include attempted refolding by chaperones, degradation by the proteasome, and if all else fails sequestration into inclusion bodies (discussed in (Gray, 2001). Evidence from transgenic fly (Fernandez-Funez et al., 2000) and mouse (Cummings et al., 2001) models of SCA1 strongly support such a triage, and one might predict that interference with the assembly of ubiquitin chains through expression of K48R mutant ubiquitin should exacerbate the situation by accelerating the accumulation of the abnormal ataxin-1 protein. From the observations we report here no such exacerbation occurred; indeed mutant ubiquitin exerted a protective effect that was not attributed to a potent stress protein response (Fig 41) or to a decrease in the SCA-1 transcript levels (Fig 43) both of which would clarify the alleviation of the SCA-1 phenotype in the genetic crosses. The most plausible explanation for the ability of mutant ubiquitin to delay the pathogenic progression of SCA1 would be that some key cellular protein or proteins normally subject to ubiquitin-mediated degradation in SCA1 is stabilized by mutant ubiquitin. Although the order of events in SCA1 is still not entirely clear, there is ample evidence that perturbation of transcription plays an early and important role. Through a subtractive cloning approach Lin *et al.* identified genes whose downregulation occurred before overt pathology in the B05 mouse model and in SCA1 patient tissues (Cummings and Zoghbi, 2000). These genes included PCCMT, IP3R1 (intracellular ER Calcium release channel), SERCA2 (ER calcium pump), type 1 inositol polyphosphate 5-phosphatase (inactivating enzyme of IP3), transient receptor potential type 3 (TRP3 calcium channel) and excitatory amino acid transporter type 4 (EAAT4 glutamate

transporter) all pointing to a dysregulation in calcium and glutamate homeostasis reported in numerous mouse models of disease. By the time pathology is apparent microarray analysis demonstrates a substantially altered pattern of gene expression in B05 cerebella as compared to age-matched controls (Tang and Gray, unpublished). If a key transcriptional regulator exists whose stabilization by K48R mutant ubiquitin contributes to the improved outcome of the B05xK48R mice it should meet the criteria of being a UPP substrate, being affected by polyglutamine proteins, and being required for neural homeostasis. p300/CBP clearly meets the first criterion (Li et al., 2002b; Jiang et al., 2003) and there is accumulating evidence that the expanded polyQ tracts found within the huntingtin and atrophin-1 proteins interact with and adversely affect the activity of CBP (Steffan et al., 2000; Nucifora et al., 2001) either by a mechanism of sequestration or through enhanced degradation, both of which would result in an alteration in transcription of important neuronal genes. A dissenting view is presented by recent results wherein mutant huntingtin increased expression of a CRE-lacZ reporter gene (Obrietan and Hoyt, 2004); the enhanced transactivation potential of the acetyltransferases by the Huntingtin's protein may reflect a compensatory mechanism to restore the loss of p300/CBP target genes during the early stages of pathology (the age of the mice in this study was not revealed). The requirement of p300 and/or CBP for neural homeostasis is clouded by the functional redundancy of these closely related proteins and the embryonic lethal phenotype of null mutants (Yao et al., 1998; Oike et al., 1999). Consistent with the postulated homeostatic function of CBP in neural tissues, elimination of CREB in the forebrain using a Cre/lox system has been found to induce neurodegeneration of the hippocampus and striatum, with features reminiscent of Huntington's disease (Mantamadiotis et al., 2002). The sequestration of CBP and therefore

the loss of CBP-mediated transcription by Purkinje-specific expression of CREB (CBP interacting protein) has recently been reported to be the basis for impaired performance of mice on the rotarod apparatus (Brodie et al., 2004).

p300 and CBP are histone acetyltransferases whose activities are in opposition to HDACs (histone deacetylases). Reasoning that the loss of HAT activity in polyQ disease could be offset by inhibition of HDACs, several groups have administered pharmacological agents targeting these enzymes in fly (Steffan et al., 2001; Ghosh and Feany, 2004) or mouse transgenic models of polyQ disease (Ferrante et al., 2003; Hockly et al., 2003) and have observed delayed disease progression. It seems that a beneficial effect can be achieved either through inhibition of HDACs or through stabilization of HATs.

The data reported herein clearly do not exclude possible effects on transcription through mediators other than p300/CBP. It is likely that the ubiquitin/proteasome pathway intersects with transcriptional regulation at many points (Muratani and Tansey, 2003). The expression of K48R mutant ubiquitin elevates cre-mediated transcription of a luciferase reporter beyond the level of exogenously expressed CBP (Fig 46) and the levels of acetylated histone H4 surpass the acetylation observed in the nontransgenic controls (Fig 45). It is conceivable that the stabilization of other transcriptional factors or co-factors by K48R mutant ubiquitin exerts a potent and beneficial effect on transcription of genes whose loss figures in the course of the disease; preliminary data has suggested that genes whose loss figures in the progression of SCA-1 are partially restored by K48R mutant ubiquitin (Tang and Gray, unpublished).

Atx-1 (82Q) was recently shown to interact with SMRT (silencing mediator for retinoid and thyroid hormone receptors) in mammalian cells (Tsai et al., 2004). Such an interaction

could promote the formation of transcriptional repressor complexes involving HDACs, perhaps directed to genes through chromatin binding activity of Atx-1 (Tsai et al., 2004). By simultaneously directing the formation of repressor complexes and enhancing the proteolysis of p300/CBP the abnormal Atx-1 protein could exert a powerful effect on Purkinje cell transcription. Left unresolved is why these neurons should be selectively vulnerable. Also unexplained is the partial restoration of p300/CBP and PKCγ protein levels by overexpression of wild type ubiquitin (Fig 44 and 45). One would expect that elevation of wild type ubiquitin pools could only enhance the efficiency of proteolysis, but this was clearly not the case. Whatever the molecular benefit in terms of p300/CBP protein levels in B05 x wild type ubiquitin crosses, there appears to be a threshold for this benefit to be realized at the cellular and organismal level that has not been reached. Wild type ubiquitin did not delay manifestations of SCA1 by histological (Fig 36) or behavioral measures (Fig 39); for this it appears that the inhibition of proteolysis by mutant ubiquitin is required. The chain-terminating ability of K48R mutant ubiquitin would serve in prolonging the signaling of these acetyltransferases and may be the basis of the neuroprotective effect apparent from the data. Polyubiquitin chains assembled through G76-K48 linkages represent the structural recognition unit for the proteasome. A recent report has suggested that the activity of the met4 transcription factor is inhibited by the presence of a K48 linked poly-ubiquitin chain, which acts through a non-proteolytic mechanism (Flick et al., 2004). Although this appears to be an exception rather than a rule, it highlights the complexity of targeting by ubiquitin and suggests that modification of proteins by K48 linked polyubiquitin chains may serve transient, non-proteolytic roles. It is presently unknown whether transcription factors in higher eukaryotes are subject to

similar regulation, but if this were the case it is conceivable to imagine how the expression of K48R mutant ubiquitin might serve a role in re-programming gene expression by modulating the activity of key transcriptional regulators. There is accumulating evidence suggesting transient ubiquitin-mediated activation of transcription factors may precede their proteolytic degradation (Muratani and Tansey, 2003). The identification of these potential regulators would prove very beneficial with respect to SCA-1 pathology and in elucidating the mechanism by which K48R mutant ubiquitin exerts its protective effect.

The polyglutamine disorders have tragically predictable outcomes currently beyond the reach of effective therapeutic interventions. The normalization of protein acetylation in transgenic model systems using HDAC inhibitors has provided some hope, with interventions affecting other polyQ-mediated perturbations currently under consideration (recently reviewed in (Beal and Ferrante, 2004)). Reduced levels of CBP and the subsequent reduction in expression of CREB/CBP target genes have been found to accompany the loss of presenilins in familial Alzheimer's disease (Saura et al., 2004), suggesting that the scope of therapies designed to recalibrate CBP-medited transcription may be very large. Small molecule inhibitors of the proteasome show promise in clinical trials for oncological disorders such as multiple myeloma (Adams, 2004), proteasomal inhibition has been shown to delay wallerian degeneration *in vivo* (Zhai et al., 2003) and more recently to suppress polyglutamine-induced nuclear inclusions in cultured post-mitotic neurons (Kim et al., 2004). The data presented herein suggest that the combinatorial effect of prolonging the life of key proteins or by modulating gene expression, K48R mutant ubiquitin may prove beneficial in the context of neurodegenerative diseases.

4.4 Functional constraints of the C-terminus of mammalian ubiquitin

The co-translational processing of endogenous ubiquitin fusions and the post-proteasomal recycling of the poly-Ub signal depend on the sequence identity of the C-terminus of ubiquitin (Ozkaynak et al., 1984; Wilkinson et al., 1995). It is therefore not unexpected that mutations affecting the G76 residue of ubiquitin have profound effects on ubiquitin metabolism in yeast (Ecker et al., 1987b; Hodgins et al., 1992). Forced expression of a G76A mutant isoform of ubiquitin has been shown to severely impair the growth rate of yeast cells and its expression has been associated with the accumulation of cellular substrates (Hodgins et al., 1992). Site directed point mutations and deletions affecting the C-terminus of ubiquitin resulted in vegetative growth defects with concomitant effects on global proteolysis (Hicke and Dunn, 2003) reminiscent to the ones observed in yeast cells expressing G76A mutant ubiquitin (Hodgins et al., 1992). A mechanistically related mechanism has been proposed to contribute to the pathogenesis of Alzheimer's disease wherein transcriptional frameshifting of the ubiquitin B mRNA generates an aberrant ubiquitin protein (termed Ubb+1) with an altered C-terminus (van Leeuwen et al., 1998). A comprehensive study of the C-terminus of ubiquitin was employed to investigate the constraints at the C-terminus of mammalian ubiquitin with regard to processing /conjugation and global proteolysis in higher eukaryotic cells. The data presented herein suggest that perturbations in the UPP pathway are not as easily achievable in higher eukaryotes as they are in the yeast model system and that such disruptions in ubiquitin homeostasis may be due to long term exposure to mutant ubiquitin.

C-terminal constraints on processing and conjugation of mammalian ubiquitin

Although there is limited crystallographic information available to predict which domains in ubiquitin are contacted by the enzymes charged with processing of fusions, it is evident that processing in higher eukaryotes is dependent on the globular domain of the protein as well as the protruding carboxyterminal region. Processing of yeast ubiquitin has previously been shown to be affected by substitution of isoleucine residues I3 or I13 within the globular domain in constructs where the C-terminus was wild type ((Johnsson and Varshavsky, 1994a). It is perhaps not surprising then that elimination of the globular domain resulted in total loss of processing in our system (Fig 23); it is likely that deubiquitinating enzymes rely on the globular domain to direct efficient processing at the C-terminus of ubiquitin fusion proteins. Recognition of the globular domain would presumably preclude illicit cleavage of proteins containing LRLRGG or similar sequences. In the current study it was determined that contrary to yeast, mutations in the stick region (R74A, R72A, R72S, L73D) or the region upstream of the stick (L69D) did not preclude processing or conjugation of mutant ubiquitin (Fig 24 and 25). Substitution of L67 to Leu partially abolished the processing of the Ub-EGFP fusion as did the insertion of amino acid residues into the LRLRGG 'stick' even in constructs that preserved the terminal glycine pair and the entire globular domain (Fig 23 and 24). These data suggest that, in addition to the globular domain and terminal glycines other constraints exist for the stick region of ubiquitin with regard to recognition and/or processing. Although steric hindrance of processing enzymes by the inserted isoleucines could not be formally excluded, a 'ruler' model in which the length of the extended C terminus is of primary importance is favored; it is likely that addition of amino acids to the C-terminus wrongly positions the glycine pair relative to the globular domain and precludes appropriate contacts with the catalytic

domains within the enzymes of the ubiquitin system. This model is supported by findings in a cell-free system (Ecker et al., 1987b) wherein deletion of an amino acid in the stick region of the ubiquitin monomer (delta73) resulted in loss of interaction of mutant ubiquitin with E1 ubiquitin-activating enzyme (interaction with E1 may also be dependent on the length of the C-terminus of ubiquitin).

The ruler model would place three constraints on ubiquitin: 1. contact of processing enzymes with key residues on the globular domain, 2. positioning of the glycine pair at a suitable distance from such globular domain contacts, and 3. accommodation of appropriately spaced residues into a catalytic site. The optimal configuration for proteolytic cleavage would consist in part of a glycine residue at either position 75 or 76. Substitution of both residues (even to highly conserved alanine residues) was shown to abrogate processing which was less affected by a single substitution (Fig 19). It is possible that the glycine pair at the site of cleavage confers considerable flexibility to fusion proteins, which may allow the proteolytic apparatus to be more insensitive to the subsequent residue(s) than would otherwise be the case.

It has not been formally excluded that the fusion proteins are subject to cleavage by enzymes that process ubiquitin-like molecules; given the requirement for the globular domain of ubiquitin (not a region of conservation in ubiquitin like molecules, (reviewed in (Schwartz and Hochstrasser, 2003)) in directing efficient processing it is more likely that Ub-EGFP fusion proteins are processed by enzymes with ubiquitin-like activity (ubiquitin-specific proteases and/or ubiquitin carboxyterminal hydrolases) resulting in at least partial processing of the fusion (Fig 20). In some cases, the transgene-derived monomeric ubiquitin was found to substitute for endogenous ubiquitin in the formation of HMW

conjugates and in conjugation to cellular protein substrates (Fig 21). The data do not allow discrimination between processing by cytosolic versus proteasome-bound deubiquitinating enzymes (DUBs) and it is speculate that the concerted action of both these types[2] of enzymes is responsible for the processing of Ub-EGFP fusion proteins. The ability of deubiquitinating enzymes to mediate the processing of fusions containing mutant isoforms of ubiquitin may reflect increased versatility and flexibility of these enzymes relative to their yeast counterparts. The processing of ubiquitin fusions by deubiquitinating enzymes in higher eukaryotes is remarkably insensitive to the identity of the first post-ubiquitin residue (Gilchrist et al., 1997) and will proceed even if that residue is a helix-distorting proline. Unlike yeast, mammalian cells have the ubiquitin-specific protease USP4, which efficiently cleaves the ubiquitin-proline bond (Gilchrist et al., 1997). USP4 clearly has the capacity to accommodate a variety of peptides into its catalytic site. This property of USP4 may be wholly or partially responsible for the observed difference in the ability of yeast and mammalian cells to process C-terminal mutants of ubiquitin. Likewise, it is conceivable that fusions of ubiquitin are processed at the level of the proteasome due to their ability to be recognized as substrates of the UFD pathway. In agreement with previous reports ((Stack et al., 2000) and (Yao and Cohen, 2002)) fusions of ubiquitin with EGFP were found to be targeted to the 26S proteasome presumably as a consequence of this pathway (Fig 27 and 28). In these studies, it was reported that the attached ubiquitin moieties were degraded by the 26S proteasome as part of the fusion protein. A minority of fusion proteins behaving in a similar manner (substitution mutants G75AG76I and G75IG76A, insertion mutants IGG and IIGG and G76V Ub, which has been previously reported to be degraded in this fashion) have been identified in the current

study. The half-lives of the substitution mutants G7AG76I and G75IG76A, the insertion mutants IGG and IIGG Ub-EGFP and some G76 mutants were relatively short in transfected cells treated with CHX, but could be restored by treatment with proteasome inhibitor (Fig 27). These data suggest that cells may utilize the proteasome to rapidly eliminate abnormal ubiquitin molecules, perhaps preventing perturbation of the primary proteolytic pathway through which damaged and/or misfolded proteins are eliminated. Some proportion of wild type ubiquitin may be degraded in the proteasome by failure of the deubiquitinating enzymes to cleave it from substrates entering the core particle (Hanna et al., 2003), but clearance of mutant ubiquitin may absolutely require the rapid proteolysis afforded by the proteasome. Also identified in the present screen were G76 fusion proteins in which the attached ubiquitin moiety was not efficiently destroyed by the 26S proteasome (Fig 28). It is speculated that the likelihood of an ubiquitin variant escaping proteolysis is dictated by the rate of deubiquitination versus degradation. The data are in accordance with a recent report in which ubiquitin-EGFP fusion proteins were found to be subject to hydrolysis by deubiquitinating enzymes intrinsically associated with the 26S proteasome (Guterman and Glickman, 2004).

Depletion of ubiquitin pools may occur in yeast expressing G76A mutant ubiquitin through formation of unanchored polyubiquitin chains (Hodgins et al., 1992) In a mammalian system it was found that G76A mutant ubiquitin was processed and was competent for conjugation to cellular protein substrates, further highlighting the differences between yeast and mammalian cells (Fig 17 and 18). The G76A Ub-EGFP plasmid has been stably-transfected in mouse neuroblastoma cells and a depletion of ubiquitin pools has not been observed by western blot analysis even in high passage cultures. Because no such

depletion of monomeric ubiquitin pools were noted, it is speculate that G76A is, like wild type ubiquitin, reversibly conjugated to protein substrates. It is possible that reduction in ubiquitin pools is counterbalanced by up-regulation of stress-inducible ubiquitin in the stable transfectants; lethality was only observed in yeast when mutant ubiquitin represented the sole source (Butt et al., 1988; Finley et al., 1994); for technical reasons an analogous experiment would be very difficult to perform in the mammalian system.

Finally, the ball and stick structure of ubiquitin appears to be a particularly useful device for covalent tagging of substrate proteins and as such the structure recurs in several ubiquitin-like proteins, including SUMO, ISG15, and NEdd8 (reviewed in (Schwartz and Hochstrasser, 2003)). Whereas the amino acid sequence of the globular domains of these proteins is not strongly conserved, two or more glycines occur at their C termini and can be considered 'signature' motifs of the ubiquitin-like proteins. The existence of at least one ubiquitin-like protein lacking terminal glycines (McNally et al., 2003) would indicate that possession of C-terminal glycines should not be considered a *sine qua non* for inclusion in the family. The looser constraints on the identity of the terminal residues in mammalian cells apparent from the data suggest that there may be additional ubiquitin-like proteins yet to be identified.

Consequences on proteolysis by expression of C-terminal ubiquitin variants

A decrease in the rate of proteasomal degradation (due to the degradation of mutant ubiquitin) has been proposed to account for the inhibitory effects on proteolysis in cells expressing G76 ubiquitin isoforms (Yao and Cohen, 2002). It is not unpredicted that proteasomes of cells presented with mutant ubiquitin would exhibit a diminished activity when assayed with fluorogenic or colorimetric based assays (ubiquitin is a stable protein and as such is difficult to dispose of). Wild-type ubiquitin in some cases has been shown to

be a substrate of the UPP (Hanna et al., 2003) and the data suggest that even the exogenous expression of the wt Ub isoform mildly affects the activity of the 20S core proteasome (Fig 28). The observed inhibition was more pronounced in cells expressing C-terminal ubiquitin mutants (Arg and Leu) when compared to cells expressing fusions of ubiquitin with EGFP that were efficiently destroyed by the proteasome as part as the fusion protein (Fig 28). The mechanism by which ubiquitin variants adversely affect proteasomal function is currently unclear. It has been reported that a functional 19S particle is required for the efficient degradation of fluorogenic substrates and mutations affecting the base component have been shown to impair the proteolytic activity of the 20S core particle (Glickman et al., 1998). It is then conceivable that the altered activity of the proteasome simply reflects an enhancement in the ability of mutant ubiquitin to bind to the 19S component of the proteasome. Also conceivable is an increase in the frequency at which ubiquitin is degraded (the mutation in the stick region of the C-terminus may preclude efficient disassembly by inherent deubiquitinating enzymes associated with the proteasome). A dissenting view would be represented by a mechanism of proteasomal antagonism through the accumulation of unanchored polyubiquitin chains (incorporating mutant ubiquitin) that are refractory to disassembly by recycling enzymes; changes in activity of the 20S core resulting from dominant-negative inhibition of the proteasome by these unanchored chains would be reflected by an accumulation of UPP substrates. The abundance of E2F-1 was not found to differ in cells expressing stick mutants (as assessed by western blot analysis) or in cells expressing G76Y Ub-EGFP which argues against the aforementioned and against irreversible conjugation of mutant ubiquitin (Fig 28 and 29). The data presented herein are not irreconcilable with published literature; they simply

suggest that the inhibitory effects on proteolysis imposed by mutant ubiquitin are not sufficient to result in a detectable alteration in the protein levels of UPP substrates. The effects of mild perturbations in ubiquitin pools (below the level of detection by western blotting) may not be obvious in cultured cells, but might only be manifest in cells such as the neurons of long-lived mammals expressing mutant ubiquitin. Consistent with this supposition is the observation that mice expressing EGFP-Ubb^{+1} do not exhibit pathology at an early age arguing against Ubb^{+1} behaving as a potent proteasome inhibitor. In agreement with this finding is the apparent normal processing of fluorogenic substrates in brain extracts prepared from 2 month old heterozygous EGFP-Ubb+1 mice (Fig 52). It is conceivable that the level of expression of mutant ubiquitin in mice is not elevated enough to result in early onset pathology (the expression levels seem significantly lower as assessed by western blot analysis with the EGFP antibody when compared to mice expressing the wt Ub-EGFP transgene, Fig 51) or that an upregulation of proteasomes is camouflaging the effect; whatever the mechanism, the expression of the EGFP-Ubb^{+1} transgene in animals (at least in young ones) does not result in observable effects on proteolysis suggesting that unlike yeast, higher eukaryotic cells can tolerate C-terminal ubiquitin mutants.

The data seem to suggest that the deficiencies in proteolysis do not arise from a decreased rate of degradation or an inability of cells to degrade mutant ubiquitin but rather a mechanism involving the accelerated targeting of C-terminal ubiquitin mutants to the 26S proteasome and the concomitant depletion of ubiquitin pools. The targeting of mutant ubiquitin to the 26S proteasome (by the addition of UFD sequence motifs) has been shown to result in a severe inhibition of proteasomes that surpasses that observed in the presence

of C-terminal variants (Lindsten et al., 2002). It was found that the expression of UFD modified ubiquitin moieties resulted in a robust accumulation of protein substrates even in cells in which the peptide was introduced as a fusion with wild-type ubiquitin (Fig 29). The accumulation of E2F-1 in these cells is ascribed to a peptide rather than an ubiquitin effect. It is speculated that the peptide (even in its cleaved form) has the ability to entice components of the UPP pathway and impose pressure on the available ubiquitin pools forcing the formation of poly-Ub chains on itself at the expense of other UPP substrates. The combination of polyubiquitin chain assembly on ubiquitin and the peptide in the case of G76Y CL1 would account for the more pronounced effect observed on the turnover of UPP substrates in these transfected cells. In the absence of such "enhancing signals" the expression of mutant ubiquitin is not deleterious to mammalian cells. This assertion is supported by the finding that the replacement of the EGFP marker with a $^{+1}$ peptide sequence (a non-enhancing motif when appended on the EGFP protein) altered the kinetics of degradation of the fusion protein (Fig 31). It was found that G76Y extension proteins were subject to processing and were insensitive to the presence of proteasome inhibitor which would argue against their being substrates of the UPP (Fig 30 and 31). The data are in agreement with a previous report wherein the stability of a G76V isoform of ubiquitin (in the absence of a C-terminal reporter protein) was found not to differ from the wild-type ubiquitin isoform (Qian et al., 2002) and the findings in transgenic mice expressing EGFP-Ubb+1; the presence of the transgene in dissected brains (Fig 47) and by immunohistochemistry (Fig 48, 49 and 50) argue against Ubb^{+1} being a substrate of the UPP (it would simply be degraded). This is the case in a transgenic mouse expressing G76V Ub-EGFP, an uncleavable and bonafide substrate of the UPP; EGFP fluorescence

has been shown to accumulate in primary cultures treated with proteasome inhibitors or presented with a burden of misfolded protein (Lindsten et al., 2003). This finding is not surprising when one considers that poly-Ub chain assembly relies on the globular domain of the ubiquitin monomer rather than the sequence identity of its carboxyl-terminus (Pickart, 1997; Sloper-Mould et al., 2001). The consensus in the literature is that the formation of unanchored poly-Ub chains is a rare event given the efficiency of polyubiquitin chain disassembly by the cell (Hadari et al., 1992; Wilkinson et al., 1995). In considering polyubiquitin chain assembly one can speculate that cells would be predisposed to the toxic effects of mutant ubiquitin if G76-substituted ubiquitin variants (such as the ones described herein) engaged in the formation of such chains (the mutation at their C-terminus would preclude their disassembly by isopeptidases). It is rather evident that aberrant chains would over the course of time exert extreme pressure on ubiquitin pools and antagonize the function of proteasomes which would be continuously occupied by unanchored polyubiquitin chains. The data seem to suggest that by simultaneously engaging in the formation of un-anchored polyubiquitin chains (depleting ubiquitin pools) that antagonize proteasomes, C-terminal ubiquitin variants (such as Ubb^{+1}) serve to enhance the phenotype of individuals affected by disease. This may be the basis for the detection of apoptopic and/or necrotic neurons observed by H and E in the hippocampus (an area previously reported to be affected in the pathogenesis of AD) of a five month old EGFP-Ubb+1 founder mouse. A correlation between transgene expression and onset of symptoms has not been attempted and it is conceivable that the basis for the aforementioned is a higher level of expression of the transgene in that founder mouse. Nevertheless, the in vitro and in vivo compiled data suggest that the combination of

ubiquitin deficiency and the occupancy of proteasomes in cells persistently presented with mutant ubiquitin may be the mechanism by which Ubb^{+1} contributes to the pathogenesis of Alzheimer's disease and suggest a consequential rather than a causative role for Ubb+1 in the pathogenesis of AD. Consistent with this supposition is the well documented accumulation of Ubb^{+1} protein levels in mice and humans with neurodegenerative diseases ((de Pril et al., 2004) and (Fischer et al., 2003)} and in brains of non-demented elderly (at a time in which proteasomal dysfunction is well documented) despite the presence of its mRNA in non-diseased areas and/or young controls (Fischer et al., 2003) and are consistent with a recent report suggesting that Ubb^{+1} is a sensor of proteasomal inhibition (Fischer et al., 2003). It is conceivable that the inhibition of proteasomes resulting from an unrelated phenomena featuring in disease (aging and neurodegeneration) or an increase in the frequency of translational errors would culminate in the accumulation of Ubb^{+1} at the protein level and may be the basis for its detection in NFTs in brains of patients suffering from disease and in non-demented elderly. By examining progeny of EGFP-Ubb^{+1} mice it should be possible to gain insight into the role of Ubb^{+1} in the physio-pathological process of aging and in the pathogenesis of Alzheimer's Disease.

4.5 Conclusion

It is believed that the conservation of ubiquitin in eukaryotic cells is highly suggestive of an essential role for each residue in the function of ubiquitin in mammalian systems. One might expect then that mutations that adversely affect ubiquitin metabolism in yeast may also have phenotypic and cellular consequences in higher eukaryotic cells. In this study it was determined that this was not the case; higher eukaryotes have evolved mechanisms (at least short term) to bypass mutations in ubiquitin (presumably to avoid perturbation of the primary proteolytic system charged with the responsibility of eliminating damaged and/or

misfolded protein) and in certain cases mutation in ubiquitin proved beneficial in delaying a pathological state. It was found that the *in vivo* expression of a K48R chain terminating version of ubiquitin mutant delayed (rather than exacerbated) the phenotype in a mouse model of SCA-1 and that the effects of expression of a C-terminal ubiquitin variant were only manifest after long term exposure. The former observation suggests that the inhibition of proteolysis may prove beneficial in the context of neurodegeneration and that the chain terminating ability of K48R mutant ubiquitin or possible effects on gene-reprogramming may be the basis for its neuroprotective effects; further experiments will be required to decipher the possible effects of K48R mutant ubiquitin on gene regulation and/or protein stability. By generating genetic crosses of K48R mice with other known neurodegenerative models (as an example AD or ALS in which the loss of p300/CBP has not been documented) it should be possible to dissect the molecular mechanisms by which K48R mutant ubiquitin exerts its protective effects and determine whether the exogenous expression of K48R can be considered as a therapeutic intervention in neurodegeneration.

The data generated with the mutational analysis of the C-terminus of ubiquitin suggested that the toxic effects of C-terminal ubiquitin variants may be manifest after long term exposure and are consistent with an exacerbating role for Ubb^{+1} in the late stages of disease. One possible mechanism may involve an increase in the repertoire of enzymes associated with the UPP which would serve in the rapid processing of engineered (as the ones described here) or naturally occurring ubiquitin fusions (such as the Ubb^{+1} mutant arising from molecular misreading) in which the G76 residue has been mutated. The second mechanism would involve the elimination of the fusion protein by directing it to the

26S proteasome. Both of these mechanisms would serve in maintaining cellular ubiquitin pools by precluding the formation of anchored poly-ubiquitin chains that antagonize proteasomes. The data also suggest that the expression of C-terminal ubiquitin variants would predispose cells (or mice) to the toxic effects of mutant ubiquitin by perturbing ubiquitin homeostasis. By treating EGFP-Ubb^{+1} mice with agents that affect protein turnover or ubiquitin pools it should be possible to test this hypothesis *in vivo*.

The observable differences apparent from the data between mammalian and yeast cells with regard to ubiquitin biology would caution against extrapolating results from unicellular organisms to highly complex tissue and organ systems. It is indisputable that yeast cells have served admirably in deciphering the enzymology of the UPP but it is clear that much information about ubiquitin biology remains to be elucidated in higher eukaryotes. The animal models described in this thesis should prove beneficial in deciphering the functions of ubiquitin in both normal and disease states.

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Statement of contribution of collaborators

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