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ALTERATIONS IN ACTIVITY AND SPECIFICITY OF INTRACELLULAR PROTEOLYSIS IN DISEASE PATHOGENESIS

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1. SUMMARY

The ubiquitin-proteasome pathway (UPP) plays a fundamental role in many basic cellular processes. Virtually all events in cellular physiology include control by the ubiquitin-proteasome pathway. Ubiquitinated substrates are degraded by the **26S proteasome**, a large multicatalytic protease complex. Through the degradation of ubiquitinated proteins all eukaryotic cells vary the protein concentration of regulatory proteins within minutes, which is the key to the very sophisticated cellular regulations of e.g. the cell cycle and division, differentiation and development, apoptosis involvement in the cellular response to stress and extracellular effectors, morphogenesis of neuronal networks, modulation of cell surface receptors, DNA repair, transcriptional regulation, regulation of the immune and inflammatory responses and so on. Low proteasomal activity can be compensated by increased activity of **TPPII** (Tri-peptidyl-peptidase II), another large cytosolic peptidase. Over-expression of TPPII can be sufficient to maintain cellular viability although the proteasome is inhibited. It may suggest a crucial role for the observed compensation, since it in part substitutes for one of the major regulatory systems of mammalian cells. The aim of the work described in the Licentiate study is to understand the regulation of cellular physiology performed by cytosolic proteolysis in Cell- and Immuno-biology, with respect to proteasome and TPPII. The results are summerized in the three papers:

Paper I: Tumors Acquire Inhibitor of Apoptosis Protein (IAP)-mediated Apoptosis Resistance through Altered Specificity of Cytosolic Proteolysis (J.Exp Med, Vol.197, 2003)

Paper II: Decreased cytosolic 26S proteasomal activity contributes to chromosomal aneuploidy. (Manuscript)

Paper III: Redistribution and Reduced Efficiency of the Ubiquitin-Proteasome Pathway in Response to Stable Poly-Glutamine Expression in Lymphoma Cells. (Manuscript)

In the first paper, we have put a question related to the increased activity of TPPII in EL-4 Lymphoma cells when the proteasome is inhibited and examined if this state has any consequences for pathways controlled by the ubiquitin-proteasome proteolysis, and asked whether this has any impact on tumor progression. We have studied apoptosis control in tumor cells with high TPPII activity, which can grow despite proteasomal inhibition. Several studies have implicated a high expression of 20S proteasomes in tumor cells, in line with a high demand for proteolysis in rapidly proliferating cells. However, death by apoptosis balances cellular proliferation, and resistance to apoptosis may be at least as important as the rate of proliferation for growth of the tumor tissue. In this study, we find that EL-4 lymphoma cells that can grow in the presence of low proteasomal activity acquire apoptosis resistance due to a failure in degradation of inhibitor of apoptosis proteins (IAPs). The rate of *in vivo* tumor growth of such cells was strongly increased. Rapid tumor growth, as well as a delayed degradation of IAPs, could be induced by transfection of TPPII. In addition, we observe a slower degradation of IAPs in cells derived

from large *in vivo* tumors, as well as a reduced activity of the proteasome in combination with up-regulated TPPII activity. Our data suggest a novel mechanism for apotosis resistance in tumors.

In paper II, we studied whether increased expression of TPPII was an essential response to stress, and redistribution of proteasomes. Here we found that suppression of the ubiquitin-proteasome pathway during stress, mediated at least in part by expression of TPPII, was crucial for cellular survival. Besides, we observed that TPPII expression was essential to adapt cellular proliferation to the condition of the microenvironment during stress. Our data give further support for nuclear translocation of proteasomes in tumor cell resistance to stress, and suggest that TPPII has an essential role in this process. TPPII-mediated control of proteasome distribution may be a crucial control of the rate of ubiquitin-dependent proteolysis, which is relevant to many pathogenic processes. We find cellular stress up-regulates TPPII, and that TPPII is required for nuclear translocation of proteasomes during stress. Furthermore, expression of TPPII was important for induction of growth arrest in response to cellular stress. Here, we established a link between redistribution of proteasome complexes and control of the rate of ubiquitin-dependent proteolysis.

Several aberrant gene products involved in pathogenesis of neurodegenerative diseases interfere with function of the ubiquitin-proteasome pathway. Proteins containing extended repeats of Glutamine (Q) cause inhibition of the ubiquitin-pathway and apoptotic cell death. To study the mechanism of these effects (Paper III), we have investigated how stably expressed poly-Q proteins affects the ubiquitin-proteasome pathway in lymphoma cells *in vitro* by using a poly-Glutamine-extended N-end rule GFP reporter. We find a significantly reduced ability to degrade ubiquitin-conjugates in poly-Q expressing cells and an alteration of the distribution of proteasome from the cytosol into perinuclear location in the presence of stable expression of poly-Q proteins. These cells failed to resist low concentrations of proteasomal inhibitor, whereas they had delayed apoptosis during exposure to several other apoptotic stimuli. This study shows that poly-Glutamine proteins may affect distribution of proteasomes to alter the efficiency of the ubiquitine-proteasome pathway and give further support for a link between cellular protection responses and pathological mechanisms during expression of poly-Q proteins.

All these data suggest a novel mechanism that controls the ubiquitin-proteasome pathway during stress. These are relevant in relation to new therapies for multi-therapy-resistant clinical tumors and other human diseases.

Key words: Proteasome, Ubiquitin, Neuro-degeneration, Poly-Glutamine, Aoptosis, Tripeptidyl-Peptidase II, Stress, Cancer, IAP

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2. LIST OF PAPERS

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II. Decreased cytosolic 26S proteasomal activity contributes to chromosomal aneuploidy. (Manuscript)

III. Redistribution and Reduced Efficiency of the Ubiquitin-Proteasome Pathway in Response to Stable Poly-Glutamine Expression in Lymphoma Cells. (Manuscript)

Abbreviations

ATP	adenosine triphosphate
APF-1	adenosine triphosphate -dependent proteolytic factor 1
BrAAP	branched-chain amino acid preferring
CCK	cholecystokinin
CP	core particle
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzymes
E3	ubiquitin ligases
ERAAP	ER aminopeptidase associated with antigen processing
GFP	green fluorescent protein
HD	Huntington´s disease
IAP	inhibitor of apoptosis protein
IFN	interferon
MHC	major histocompatibility complex
RP	regulatory particle
SCA	spinocerebellar ataxia
siRNA	small interfering RNA
SNAAP	small neutral amino acid preferring
TAP	transporters for antigen presentation
TPP II	tripeptidyl-peptidase II
UCH	ubiquitin C-terminal hydrolase
UBP	ubiquitin-specific processing protease
UPP	ubiquitin-proteasome pathway

4. INTRODUCTION

4.1 The Ubiquitin-proteasome Pathway

Protein degradation is essential for many cellular functions, including maintenance of homeostasis, ordered degradation of cell cycle regulating proteins, activation of transcription factors, removal of incorrectly folded of damaged proteins and generation of peptides presented by major histocompatibility complex class I molecules (MHC class I; reviewed in 1). The highly conserved **ubiquitin-proteasome pathway (UPP)** is the major intracellular system for extra-lysosomal protein degradation (2, 3). The UPP was discovered more than 20 years ago, the major task of the system is the regulated degradation of cytosolic and nuclear proteins that occurs within minutes or hours, a process that is vital for cell survival. In eukaryotes the ubiquitination system most often serves as a triggering signal for degradation. It accounts for targeting selected proteins for degradation by tagging them with chains of a small heat stable protein, the adenosine triphosphate (ATP)-dependent proteolytic factor 1 (APF-1), which was later identified to be ubiquitin, a ubiquitously expressed protein with previously unknown function (4). Together with the ubiquitin conjugation cascade, the proteasome controls the lifetime of most cellular proteins (5). This system is widely conserved through evolution and even though prokaryotes do not contain a ubiquitin homologue or orthologue some prokaryotes do contain proteasomes, however in a less complex form (6).

Proteolysis by the UPP involves two steps (**Figure** 1):
(**a**)covalent attachment of multiple ubiquitin molecules to the protein substrate (ubiquitination process), and (**b**) degradation of the targeted protein by the 26S proteasome complex (proteolysis process).

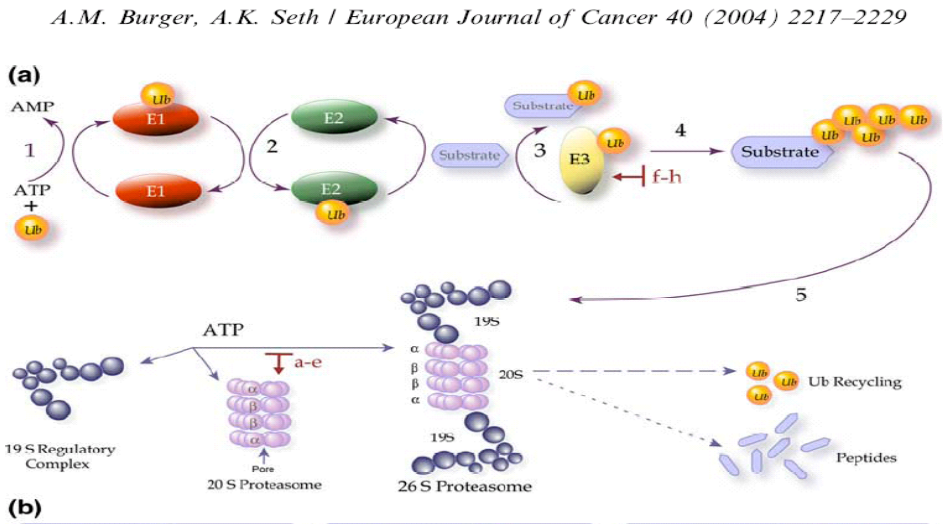


Figure1. Overview of the ubiquitin–proteasome pathway and possibilities for intervention. 1 = Ubiqutin activation; 2 = Ubiquitin (Ub) transfer from a ubiquitin activating enzyme (E1)via a thioester linkage to a ubiquitin conjugating enzyme (E2), 3 = E3 ubiquitin ligase which facilitates positioning and transfer of ubiquitin from the E2 directly onto the substrate and 4 = ubiquitin chain elongation by conjugation of Ub to lysine residues of ubiquitin at position 48 (or alternatively Lys29 and 63) by an E3 ligase. 5 = Transfer

of Ub-tagged proteins for adenosine triphosphate (ATP)-dependent substrate degradation in the 26S proteasome, which assembles from a 20S core and two 19S regulatory complexes.

Hershko and Ciechanover found that ubiquitin could be conjugated to Lys residues of proteins by an energy-dependent process requiring ATP in a sequential reaction that was named "**ubiquitination**". The primary function of ubiquitination is to serve as a reusable selective tag sorting out those proteins that are destined for proteasomal degradation. There are several key enzymes required for accomplishing the ubiquitin conjugation, including a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3).In a sequential manner, these enzymes collaborate to covalently conjugate ubiquitin first to proteins and next to one of the Lys within the ubiquitin until a chain of conjugated ubiquitin is formed, which is recognized by the proteasome resulting in degradation of the ubiquitinated protein (7).Conjugation of ubiquitin to the substrate proceeds via three-step mechanism. Firstly, ubiquitin is activated by the ubiquitin-activating enzyme (E1), then transferred by one of the ubiquitin-conjugating enzymes (E2s) to a member of the ubiquitin ligases (E3s), which catalyze the covalent attachment of ubiquitin to the substrate. Following the attachment of multiple molecules and the formation of a polyubiquitin chain, the ubiquitinated protein is deubiquitinated by the proteasome, and the ubiquitin is released and recycled by specific enzymes, the deubiquitinating enzymes (DUBs). These enzymes are divided into two major categories: the ubiquitin C-terminal hydrolases (UCHs), which are involved in the generation of ubiquitin monomers from polyubiquitin precursors and rescue activated ubiquitin from inappropriate conjugation to abundant intracellular nucleophiles, thereby maintaining the intracellular pool of free ubiquitin. The other group is the ubiquitin-specific processing proteases (UBPs), and they are involved in substrate deubiquitination and disassembly of ubiquitin chains (8).Once a substrate protein has been recongnized by the ubiquitin conjugation system and is modified by the covalent attachment of a multiubiquitin chain it becomes a target for proteasomal degradation. Ubiquitylation thus serves to translate the rather diverse degradation signals present on the individual substrates into a common `tag´ recognizable by the 26S proteasome.

The **26S proteasome,** often called "the proteasome", is a multicatalytic enzyme expressed in the nucleus and cytoplasm of all eukaryotic cells and specifically degrades ubiquitin-conjugated proteins in an ATP-dependent manner(Figure 2). It is comprised of two subcomplexes: a 20S core particle and two regulatory particles, the 19S caps (review in 9, 10). The **20S** core particle (CP) is located in between19S regulatory particles (RP) and consists of four heptameric rings: α_7 and β_7, forming the structure: $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ (containing 28 subunits, 14 of the α-type and 14 of the β-type). The two inner rings that consist of β subunits contain three proteolytic activities each. The β_1 subunit

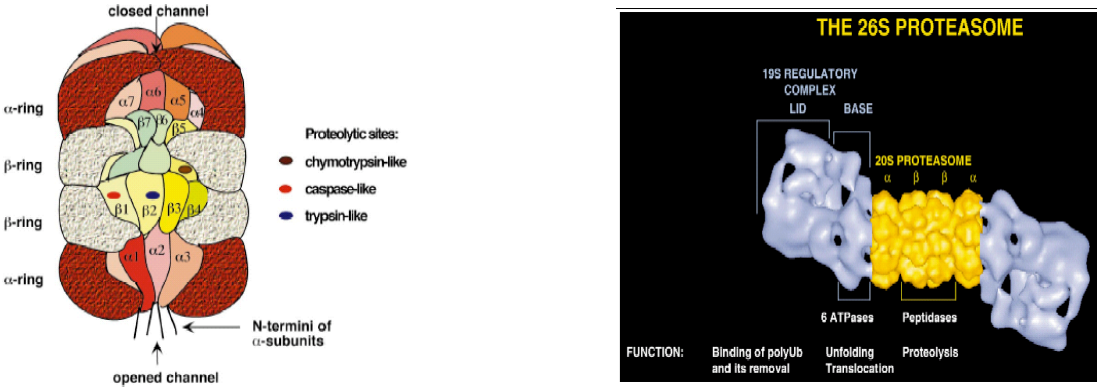


Figure 2. The 26S proteasome and its components (A.F. Kisselev, et. al. 2001).

display post-glutamyl peptide hydrolyzing (PGPH) activity, cleaving preferably after acidic amino acids. The β_2 subunit has a trypsin-like activity, cleaving preferably after tryptic amino acids. The last one, β_5 subunit is responsible for the chymotrypsin-like activity, which cleaves preferably after hydrophobic amino acids (11). Through cleavage of an N-terminal leader sequence that occurs during the assembly of the proteasome complex, the three catalytic sites are activated. The cleavage results in N-terminal *Thr* residues that exert the nucleophilic attacks on peptide bonds (12). In addition, there are two less characterised proteolytic activities: the "branched-chain amino acid preferring"(BrAAP) activity and the "small neutral amino acid preferring"(SNAAP) activity (13). The two **19S** regulatory particles (RPs) associate with the CP and form the 26S dumbbell-shaped complex and appear to be the regulatory subunits that control the recognition, deubiquitination, unfolding and translocation of substrates into the proteolytic chamber. One important function of the 19S complex is to recognize ubiquitinated proteins and other substrates of the proteasome. A second function is to open an orifice in the α ring that will allow entry of the substrate into the proteolytic chamber of the 20S CP. And also, since a folded protein would not be able to fit through the narrow proteasomal channel, it is assumed that the 19S particle unfolds substrates and inserts them into the 20S CP. Both the channel opening function and the unfolding of the substrate require metabolic energy and indeed, the 19S RP contains six different ATPase subunits (reviewed in 14).A unique feature of the mammalian proteasome has is that it has three exchangeable proteolytic β-subunits that are induced in the presence of cytokine IFN-γ. IFN-γ is produced by activated CD4 and CD8 positive T-cells and natural killer cells and plays a major role on mobilizing the host defence against infectious pathogens. The β_1, β_2, and β_5 subunits are exchanged to iβ_1/LMP2, iβ_2/MECL1 and iβ_5/LMP7, respectively, resulting in the formation of the **immunoproteasome complexes** (15). Another event that is observed upon IFN-γ stimulation is the exchange of the RP for another regulatory structure called 11S or PA28 (16).All these modification cause an altered preference of cleavage sites favoring the generation of peptides with hydrophobic C-termini and reducing the output of peptides with acidic C-termini. As for the 11S complex, this may allow release of 20S proteasomal degradation products of increased length. The peptides with a hydrophobic C-terminus are more likely to be suitable for antigen presentation by MHC class I molecules. This kind of alteration of cleavage specificity for the C-terminus is of most importance for the proteasome (17) because the N-terminus can later be further trimmed to fit the binding groove of

MHC class I by the ER-resident protease ERAAP (ER Aminopeptidase Associated with antigen Processing)(18).The peptides generated by proteasome vary in size between 3-22 amino acids with a median of 6(19). These are short lived in the cytosol and nucleus as they are subject to further degraded by other proteases and aminopeptidases (20,21). A minority of peptides will also be translocated by the transporters for antigen presentation (TAP) into the ER, where they can be loaded on MHC class I molecules and further transported to the cell surface and display to the immune system (22).

4.2 Tripeptidyl-peptidase II (TPPII)

Tripeptidyl-peptidase II is an aminopeptidase with endoproteolytic activity that removes tripeptides from a free N-terminus of oligopeptides (23). TPPII has a wide distribution and is present in the cytosol of a number of different cells of several different species, from fruit flies to humans. The 138kDa subunits of TPPII form large, oligomeric complexes (>1000 kDa) that are localized in the cytoplasm (23, 24) or the plasma membrane (25). The complex is larger than the 26S proteasome and has a rod-shaped supramolecular structure (Figure 3).

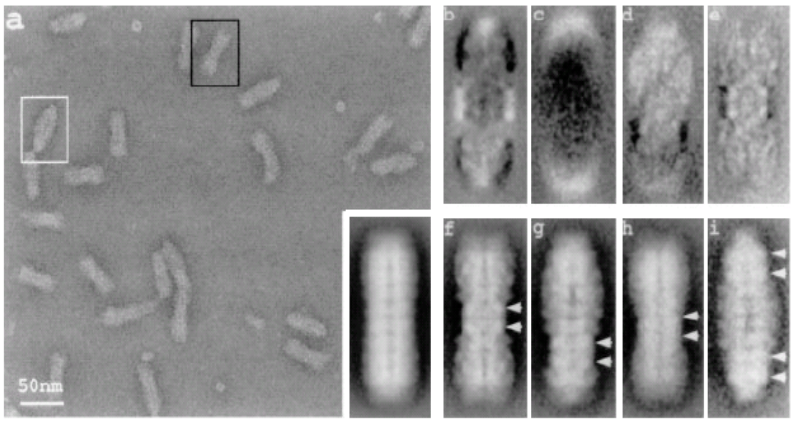


Figure3. Electron micrograph of a TPPII preparation negatively stained with uranyl acetate. White box, particle with an ovoid shape; Black box, dumbbell-shaped particle. (Geier et al., 1999)

The enzyme has been classified as a serine peptidase with an active site of the subtilisin type and the proposed physiological role for this enzyme is to participate in general protein turnover, probably in concert with the proteasome and other exopeptidases (26). The formation of tripeptides as intermediates during degradation of proteins to free amino acids may accelerate the process by increasing the concentration of protential substrates for other exopeptidases (27). A number of different tripeptides can be released sequentially from the N-terminus of longer peptides by the enzyme. TPPII has a slight preference for hydrophobic amino acid in the P_1-position and cannot cleave before or after proline residues.

It has been demonstrated that TPPII can compensate, at least in part, for the

loss of the proteasome in proteasome-inhibitor-adapted cells. Over-expression of TPPII also protected EL-4 cells from the effect of proteasome inhibitors (24, 28). TPPII is also upregulated in BL and BL-like cells that have impaired proteasome activity (29). In addition to this general function, more specific functions have also been suggested, e.g. an involvement of a membrane-bound form of TPPII in the inactivation of the neuropeptide cholecystokinin (CCK) (25), and a role upstream of caspase-1 in Shigella-induced apoptosis (30). It is therefore not surprising that when an efficient proteolytic system has evolved, it will be used for specific degradation of certain targets as well as functioning in less specific processes. This appears to be the case not only for the proteasome but also for TPPII, which shows that also exo-peptidases are important in protein degradation (27). In addition, several studies have shown a major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation (31). It appears that TPPII has a substantial effect on epitope generation. With its endoproteolytic and exoproteolytic activities, TPPII may act "downstream" of the proteasome and relies on products released by the proteasome (reviewed in 32). Furthermore, it proposes that TPPII can act in combination with or independent of the proteasome system and can generate epitopes that evade generation by the proteasome-system (33).

4.3 The ubiquitin-proteasome pathway (UPP) in human disease

The ubiquitin-proteasome pathway (UPP) plays a pivotal role in protein homeostasis and most cellular processes. Ubiquitin-mediated proteolysis of a variety of cellular proteins are involved in many basic cellular processes. Among these processes are lots of important regulators, such as cyclins, tumor suppressors, transcriptional activators and their inhibitors, cell surface receptors, etc. Besides, mutated and denatured/ misfolded proteins are recognized specifically and are removed efficiently by UPP. Considering the broad range of substrates and processes in which the ubiquitin-proteasome pathway is involved, it is not surprising that aberrations or impairment in the system have been implicated in the pathogenesis of several diseases such as cancer, malignancies, genetic and neurodegenerative diseases, as well as immune surveillance.

Most of them exhibit abnormal accumulation and altered composition of components of the pathway. And also, cellular responses to stress are crucial to allow cell survival in face of a changing microenvironment. In most of responses to stress, a proper response through the ubiquitin-proteasome pathway is necessary to ensure removal of damaged proteins.

In this study, we focused on the involvement of UPP and TPPII in the mechanism of neurodegenerative diseases and cancer.

A number of neurodegenerative disorders, including Huntington's disease (HD) and the spinocerebellar ataxias, are caused by the genetic insertion of expanded and unstable glutamine repeats (34, 35). A number of studies have focused on the potential role of protein aggregation and disruption of the proteasome proteolytic pathway in polyglutamine-mediated neurodegeneration. However, at present it is not clear whether polyglutamine-mediated protein aggregation is sufficient to induce cell death, nor has it

been clearly determined whether proteasome inhibition precedes, coincides, or occurs as the result of the formation of polyglutamine-associated protein aggregation.

Accumulating evidence indicates that the UPP plays an essential role in malignant transformation. The UPP regulates with exquisite specificity such diverse cellular processes as cell cycle progression, inhibition or execution of apoptosis, and activation or expression of transcription factors. Additionally, ubiquitin–proteasome-mediated proteolysis plays a major role in responses of cancer cells to stimulatory signals. All of these factors are critical for the development of cancer. The UPP also regulates the cellular sensitivity to apoptosis by adjusting the intracellular levels of several proteins involved in the control of apoptosis, including Bcl-2 family members, inhibitor of apoptosis proteins (IAPs) and some caspases (36-39).

5. Aim of the study

The general aim of this work is: To understand the regulation of cellular physiology performed by the ubiquitin-proteasome pathway (UPP) in Cell- and Immuno-biology, with respect to Proteasome and Tri-peptidyi-peptidase II (TPPII) in disease pathogenesis.

More specifically my we set out to investigate:

1. The state of the ubiquitin-proteasome pathway and the alteration in activity and specificity of intracellular proteolysis during cellular stress.

2. To study substrate selection of TPPII – possibility to mediate limited assistance of proteasomal activity.

6. Results and Discussion:

6.1 Apoptosis control in tumor cells with altered specificity of UPP (Paper I)

Apoptosis resistance mediated by IAP molecules is frequently observed in tumor cells, and is regarded as a major problem during tumor therapy. Here, we studied if tumor cells can alter the specificity of cytosolic proteolysis in order to acquire apoptosis resistance that promotes growth of tumor.

Cells with Low Proteasomal Activity show Resistance to Apoptosis and Failure in Degradation of IAP Molecules.

We tested whether cells with low proteasomal activity were able to properly control apoptosis induction, as this normally follows when cells are grown in the presence of proteasomal inhibitors. We used EL-4ad cells, a variant cell line of EL-4 that adapted to grow in the presence of a covalent proteasome inhibitor. EL-4ad cells were obtained by seeding control EL-4 cells at 10^5 /ml in 12-well plates and continuously incubated in 10 μM NLVS which is a treatment that blocks all catalytically active β-subunits except for Z/MECL-1, and kills most cells within 24–48 h. However, a subpopulation adapts to grow progressively within 2–3 wk (28). The concentration of NLVS is then gradually increased to 50 μM NLVS (6, 24). We observed that EL-4ad cells failed to undergo apoptosis when exposed to several apoptotic stimuli, such as serum starvation, TNF-_ and etoposide in comparison to EL-4 control cells, as shown by inefficient activation of caspases 3, 8, and 9 as well as absence of DNA fragmentation. Many reports describe that inhibitor of apotosis proteins (IAPs) must be degraded by the proteasome subsequently to mitochondrial cytochrome c release to allow activation of caspase 9, and further progression into apoptosis. We also found degradation of XIAP, an endogenous antagonist of caspase 9, in response to etoposide treatment in EL-4 cells, whereas this was very slow in EL-4ad cells. Meanwhile we saw the similarly reduced degradation of c-IAP-1 in EL-4ad cells after etoposide treatment. These data suggest that EL-4 cells may acquire apoptosis resistance through an altered specificity of cytosolic proteolysis. The apoptosis resistant phenotype was reversed by increased expression of Smac/DIABLO an IAP inactivator(40-42). These observations suggest that the inadequate degradation of IAP molecules inhibit the transduction of apoptotic signals in EL-4ad cells.

EL-4 Cells Adapted to Low Proteasomal Activity Form Rapidly Growing In Vivo Tumors by an Upregulation of TPPII Activity.

Subcutaneous tumor growth in vivo may expose growing cells to more stress (e.g., competition for nutrients and oxygen), and we therefore tested in vivo growth of EL-4 control and EL-4ad cells by inoculation into irradiated syngeneic C57Bl/6 mice. We found that tumors of EL-4ad cells were rapidly growing. In contrast, growth of EL-4 tumors was significantly slower. Using a lower cell dose than initially used, the difference in growth rate between EL-4 and EL-4ad tumors was even more evident. We also killed animals and excised cells from large in vivo tumors for later analysis (denoted EL-4/tumor cells). Importantly, EL-4 lymphoma cells with an altered specificity of cytosolic proteolysis showed a growth advantage in vivo over control EL-4 cells. We next studied whether proteolytic pathways accessory to the proteasome, that are up-regulated in EL-4ad cells, could alter the degradation of IAP molecules as well as whether increased TPP II activity regulated the growth of EL-4 tumors in vivo by using EL-4 wild type cells transfected with either control vector, pcDNA3, or with pcDNA3-TPP II, described previously (26). TPP II transfection conferred a reduced reliance of proteasomal proteolysis, since EL-4.TPP II proliferated in the presence of NLVS as well as AdaAhx$_3$L$_3$VS (a novel amino-terminally extended vinyl sulphone that inhibits all-subunits (β1, β2, and β5) with comparable efficiency, whereas as EL-4pcDNA3 cells did not. In western blot analysis, EL-4.TPP II cells displayed a failure in degradation of both XIAP and c-IAP-1 compared with EL-4pcDNA3 cells during etoposide treatment. And also, TPP II transfected cells showed a strongly increased rate of *in vivo* tumor growth compared with tumors transfected with pcDNA3 control vector. This supports the notion that apoptosis resistance linked to TPP II was responsible for the observed effects on growth of EL-4 tumors *in vivo*. In peptidase assays, we tested cleavage of peptide substrates preferred by either TPP II (AAF-AMC) or the proteasome (succ-LLVY-AMC). High molecular weight peptidases in EL-4/tumor cells had increased cleavage of AAF-AMC and reduced cleavage of succ-LLVY-AMC, compared with EL-4 control cells. This activity profile corresponded to increased TPP II activity and reduced proteasomal activity, as determined by specific inhibitors of either the proteasome or TPP II (24, 40). At the same time, we observed an inefficient degradation of both XIAP and c-IAP-1 in EL-4/tumor cells whereas these IAPs were effectively degraded in EL-4 control cells as treated these cells with etoposide, as well as a weak induction of DNA fragmentation in EL-4/tumor cells in comparison to EL-4 control cells.

Cellular Growth with Insufficient Nutrition Increases TPP II Activity and Resistance to Apoptosis.

It has been proposed that poor nutritional conditions in the tumor microenvironment contributes to selection of apoptosis resistant cells (43). As increased expression of TPP II conferred a partial protection from apoptosis of EL-4 cells, and induced rapid tumor growth in vivo, we adapted EL-4 cells for long-term survival in starvation medium with reduced content of amino acids and growth factors (25) in order to see if poor nutritional conditions may affect the activity of TPP II in tumor cells. By western blot analysis with anti-TPP II serum and enzymatic activity assays, we found high levels of

TPP II protein in EL-4 cells growing in starvation medium, which correlated with a strong increase in enzymatic activity cleaving AAF-AMC (Fig.4).

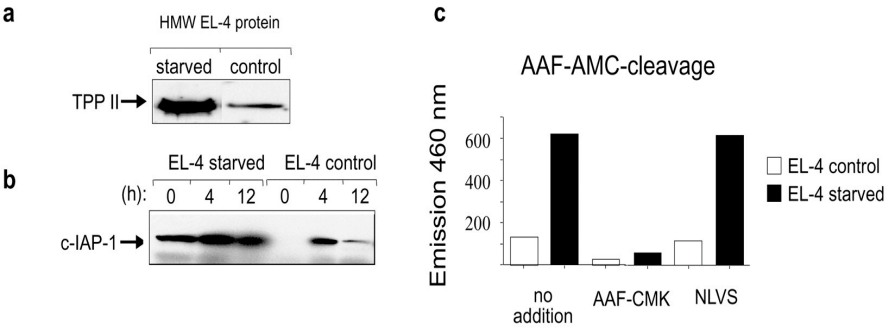


Figure4. Induction of TPP II activity and rapid tumor growth by adaptation to cellular starvation (a) EL-4 cells incubated in cell culture medium diluted with PBS were lysed, and high molecular weight cytosolic proteins were analysed by Western blot for TPP II expression. (b) EL-4 cells and EL-4 cells growing in starvation medium were exposed to etoposide and degradation of c-IAP-1 was followed by Western blotting analysis. (c) The high molecular weight cytosolic fractions in (a) were analyzed for cleavage of AAF-AMC. The inhibitors NLVS and AAF-CMK were included as controls.

Further, EL-4 cells adapted to proliferation in starvation medium were resistant to apoptosis and expressed high levels of c-IAP-1. These data fit well with the notion that apoptosis resistance mediated by IAP molecules allows a limited proliferation during serum starvation. Besides, EL-4 cells adapted to starvation medium displayed an increased rate of tumor growth, especially in inoculates with lower cell numbers which implicated a substantial influence of TPPII on EL-4 tumor growth in vivo, and that TPPII is also regulated in response to the nutritional state of the microenvironment.

All data suggest that an alteration in the activity of proteolytic pathways that are responsible for cellular protein turn-over contributes to apoptosis resistance in tumor cells. EL-4ad cells were resistant to apoptosis, at least in part due to a failure in efficient degradation of IAP molecules, and were able to grow rapidly as tumors in vivo. Besides, we find that adaptation to growth in starving culture conditions in vitro causes a phenotype in EL-4 cells including TPP II up-regulation, poor degradation of c-IAP-1, and rapid tumor growth in vivo. This may suggest a link between the microenvironment and signals that alter the specificity of intracellular proteolysis.

6.2 The link between UPP and chromosomal aneuploidy (Paper II)

In tumor cells, there are frequent mutations in genes that encode regulatory proteins controlling the cell cycle and apoptosis.The level of such proteins are also regulated by UPP (46). Virtually all tumors exhibit genetic instability, and the most common form is the instability of chromosome numbers (47). Chronic cellular stress, observed during chronic inflammation and tissue damage, or exposure to chemicals that cause oxidative stress (48-50), may

result in chromosomal aberrations. However, it is unclear how cellular stress translates into this genetic phenotype.

In this paper, we explored how cellular stress leads to aneuploidy, which may be caused by mutations that impair the mitotic machinery, and additionally by exposure to agent that causes cellular stress (48-50).

Partial Inhibition of UPP by Cellular Stress Contributes to a Failure in the Control of Chromosome Numbers.

By using EL-4 lymphoma and Hela cervical carcinoma cells expressing the fluorescent reporter proteins, UbGV76-Green Fluorescent Protein(GFP) and Ub-R-GFP (which are rapidly ubiquitinated and degraded by the proteasome, ref.51) we monitored the activity of the UPP in live cells by flow cytometry on normal conditions versve several forms of cellular stress. We observed a gradual accumulation of fluorescence over several days after exposure to both γ-irradiation and starvation, which was comparable to that induced by sub-lethal concentration of NLVS suggesting reduced substrate degradation by proteasome. Then, we tested if cell growth with this phenotype affected chromosomal activity. Counting Giemsa-stained metaphase chromosomes in culture of EL-4 and EL-4ad cells showed that most metaphases of EL-4 cells contained approximately 40 chromosomes, while in EL-4ad cells there was a marked increase in variability of chromosome numbers. Also, we found the similar variation in chromosome numbers of several independent clones prepared from the EL-4ad bulk culture by limiting dilution. These data implicated that a partially inhibited UPP reduced chromosomal stability in EL-4 lymphoma cells under cellular stress.

Up-regulated TPPII by Cellular Stress Reduces Substrate Degradation by the Proteasome.

We next tested how cellular stress reduced degradation of our reporter substrates by UPP. Previous studies show that TPPII is up-regulated upon stress in EL-4 cells, either by starvation or inhibition of the proteasome and has a crucial role in the compensation for the low proteasomal activity (24, 28). To explore the role of TPPII during cellular stress, we generated EL-4 cells with inhibited TPPII expression through stable expression of siRNA complementary to TPP II (termed EL-4.TPPII ^{low}) (52) and co-transfected these cells with UbGV76-GFP, as described above (51). Upon _-irradiation, we observed an accumulation of fluorescence in EL-4-UbGV76-GFP cells, but not in cells co-expressing the siRNA encoding TPPII ^{low} plasmid. Direct inhibition of the proteasome with 25μM NLVS induced strong GFP-fluorescence in both groups of cells. Besides, we also found that the degradation of substrates regulated by UPP was altered by TPPII during stress through the measurement of degradation of XIAP, which is translated during stress inhibits apoptosis until its degradation by UPP (53-55). Besides, suppression of TPPII expression allowed complete removal of Ub-R-GFP-Q112, a reporter molecule with a poly-Glutamine repeat that accumulates during several neurodegenerative diseases. We performed a further study on the relationship between UPP and this kind of diseases in Paper III.

These observations supported that TPPII may regulate proteasomal substrate degradation in response to stress.

Increased Expression of TPPII is Essential for sub-cellular redistribution of proteasomes in Cellular Stress and Contributed to Failure to Control Chromosome Numbers.

Regulation of 26S proteasome activity is performed at the translational (56) as well as post-translational level (46, 57) through control of the catalytic cores (20S proteasome) assembly with regulatory complexes (19S proteasome). The subcellular location of proteasomes is not stable (58), and previous reports have shown that proteasomes leave the cytosol for translocation into the nuclei of tumor cells undergoing stress (59). So we tested the subcellular localization of 19S complexes that control ubiquitin-dependent degradation performed by the 20S catalytic core (46, 57). In immunochemistry assays, we observed a clear homogenous 19S proteasome-staining in the cytosol and nuclus in. However, in starved EL-4.pSUPER cells, that staining was detectable mainly in the nuclei. These findings were in line with the reduced cytosolic activity of UPP during stress in EL-4 cells that correlates with egress of 19S complexes from cytosol. In contrast, EL-4.TPPII ^{low} cells failed to translocate 19S complexes into nuclei. Therefore, we concluded that TPPII is required in the re-distribution of proteasome in response to cellular stress.

Finally, we determined if TPP II is an important component of the cellular stress response by using EL-4.TPP II cells (stably transfected cells with over-expressed TPPII) and EL-4.pcDNA3 cells (expressing only control vector). After exposure to heat shock, EL-4.pcDNA3 cells showed a proteasome-dependent removal of ubiquitin-conjugates, and subsequently failed to grow in vitro. But, EL-4.TPPII rapidly cleared ubiquitin-conjugates, even in the presence of NLVS, and resumed growth sooner after heat shock ended (Fig 5).

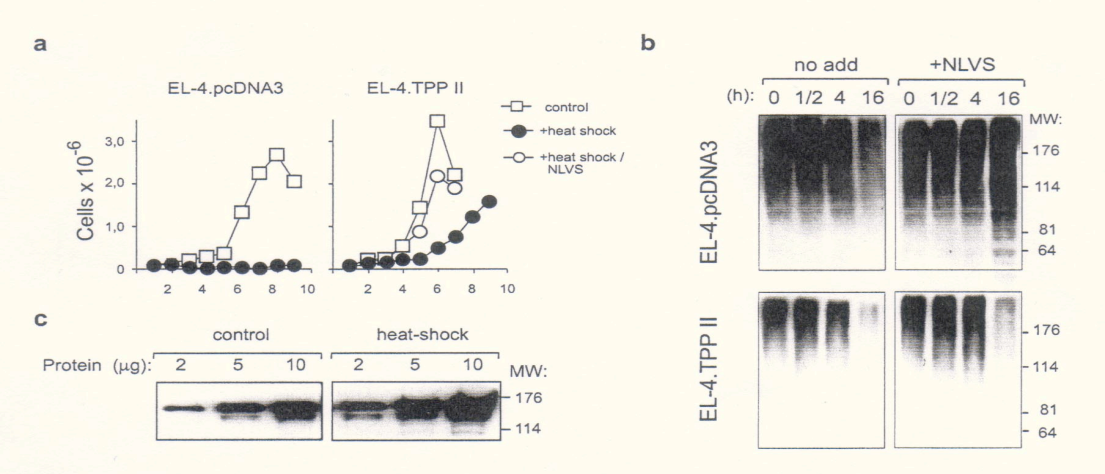
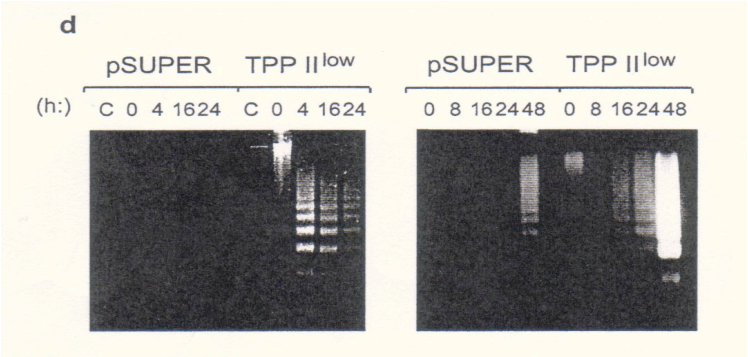


Figure5. Resistance to cellular stress requires TPP II expression (a-c, above) EL-4.pcDNA3 and EL-4. TPP II cells exposed to heat shock and analyzed for proliferation *in vitro* (a) and content of ubiquitinated proteins (b) in the presence or absence of proteasomal inhibitor (25µM NLVS), (c) EL-4 control cells left untreated or exposed to heat shock, blotted with anti-TPP II serum, (d, below), (d) EL-4. pSUPER and EL-4. TPP II ^{low} cells exposed to head shock or starvation and analyzed for DNA fragmentation.



Furthermore, TPPII was up-regulated 2- to 3-foldat the higher temperature confirming prior observations during conditions of cellular stress that TPPII is up-regulated by starvation and proteasome inhibitor treatment. We also exposed EL-4.pSUPER cells and EL-4.TPPII ^{low} cells to heat shock and starvation. EL-4.TPPII ^{low} cells failed to resist either heat shock or starvation showing by DNA fragmentation test, and did not survive without an exchange of medium. While EL-4.pSUPER cells were susceptible to starvation, their DNA fragmentation was significantly delayed, and persisted for days in the same overgrown conditions. Meanwhile, growth arrest and survival of EL-4.pSUPER cells correlated with an up-regulated TPPII, accumulation of UbGV76-GFP and reduction of DNA synthesis. But we did not see such responses in EL-4.TPPII ^{low} cells. And also, there was no significant effect on chromosome numbers in EL-4.pcDNA3 cells, while EL-4.TPP II cells showed increased numbers of chromosome and heightened variability of chromosome number. Thus, up-regulation of TPPII seemed to contribute to protection from cellular stress and but also promote aneuploidy.

Here, we established a link between control over chromosome number and activity of UPP. Cellular stress up-regulates TPPII to promote cellular survival, probably by reducing the rate at which cytosolic regulatory factory are degraded because of TPPII-mediated control of proteasome distribution due to the egress of 19S complexes from cytosol. We have explored a further study on the redistribution of proteasome within cells present in stress in Paper III.

6.3 Redistribution and Reduced Efficiency of the UPP in Protection against Stable Poly-Glutamine Expression (Paper III)

Proteins containing extended repeats of Glutamines are involved in the pathogenesis of Huntington´s diseas, SCA1 and eight other know neurodegenative diseases. Here, we investigated aggregate formation and the status of the UPP in proliferating cells with stable expression of a fluorescent poly-Glutamine (poly-Q)-extended GFP reporter targeted for proteasomal degradation. In order to study how the UPP was affected by a stable expressed poly-Glutamine protein, we made stable EL-4 cell transfectants of GFP-reporter constructs encoding Ub-R-GFP-Q112 and Ub-R-GFP (two GFP-reporter molecules designed for proteasomal degradation via N-erminal degradation signal) (51, 60).

Aberrant Protein Folding and the Inhibition of UPP induced by a stable expressed Poly-Q Repeat

Measured by flow cytometry, EL-4.Ub-R-GFP cells (as a control) expressed very slow steady level of fluorescence, but in the presence of NLVS over night, strong fluorescence was detected in these cells. These phenotype were confirmed by

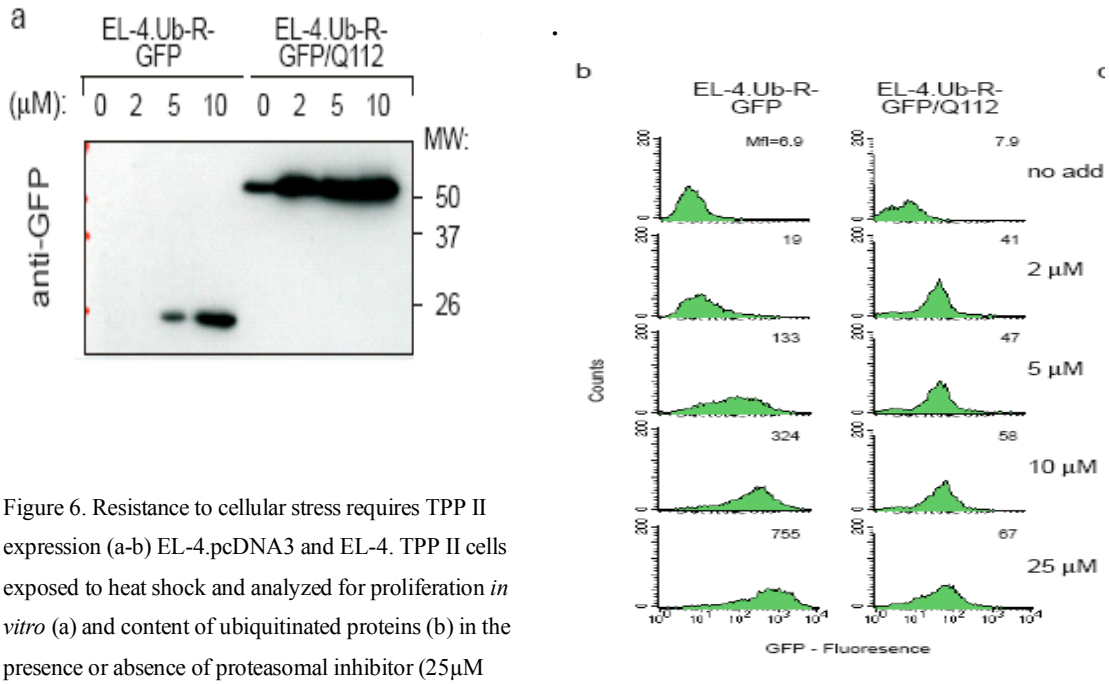


Figure 6. Resistance to cellular stress requires TPP II expression (a-b) EL-4.pcDNA3 and EL-4. TPP II cells exposed to heat shock and analyzed for proliferation *in vitro* (a) and content of ubiquitinated proteins (b) in the presence or absence of proteasomal inhibitor (25μM NLVS).

Western Blotting with anti-GFP serum (this method detects the presence of GFP independently of its state of folding. In contrast, EL-4.Ub-R-GFP-Q112 cells accumulated fluorescence but failed to reach more than 10-15% of the fluorescence detected in EL-4.Ub-R-GFP (Fig6.b). However, by western blotting assay (Fig6.a), we saw a substantial content of GFP substrates in untreated EL-4.R-GFP-Q112 cells as well as substrates a much stronger increase of R-GFP-Q112 during NLVS treatment .

Normally, GFP requires correct folding for emission of fluorescence, so we concluded that these R-GFP-Q112 substrates were misfolded (3). Having known that the R-GFP-Q112 substrate was not adequately removed in stably transfected EL-4 cells, we tested whether this had any effect on the UPP. We found that EL-4. R-GFP-Q112 cells proliferated normally *in vitro* in comparison to EL-4. R-GFP control cells. But treated with low concentration of NLVS, the growth of R-GFP-Q112 cells was prevented, whereas no effects on R-GFP control cells. Besides, the presence of DNA fragmentation in EL-4.R-GFP-Q112 cells treated with NLVS showed that these cells died by apoptosis. Besides, after we exposed both two cells to heat shock (42° 45min) in the presence or absence of 5 μM NLVS, EL-4.Ub-R-GFP control cells

displayed a strong accumulation of ubiquitinated proteins after 30 minutes, which was removed after 4 hours. However, R-GFP-Q112 cells failed to clear ubiquitin conjugates even after 24 hours, and the removal was completely inhibited in the cells treated with NLVS. These data implied poly-Q repeats mediate a partial inhibition of the UPP. We studied how stable expression of the poly-Q proteins affected transduction of apoptotic signals. Several previous reported have suggested that transient expression of toxic levels of poly-Q proteins may trigger apoptosis (62-64). However, these investigations may have used systems with transient poly-Q protein expression, whereby the pathogenic effects may be stronger than in our system. We exposed both EL-4.Ub-R-GFP and EL-4.Ub-R-GFP-Q112 cells to heat shock, etoposide and serum starvation, and detected the apoptotic response by measuring DNA fragmentation. EL-4.Ub-R-GFP-Q112 cells showed a significantly delayed apoptosis to all tested agents (except NLVS), compared with control cells, which suggested these cells mediated an effect similar to that of a low dose of proteasomal inhibitor, and confers reduced susceptibility to apoptosis (65).

And also, in the tumor growth experiment, we found that tumor growth of EL-4.Ub-R-GFP-Q112 cells *in vivo* was enhanced and tumors rapidly shrank after the mice were injected with MG132 (another inhibitor of proteasome). From these data, we concluded that a reduction of proteasomal activity may suppress apoptosis *in vivo*, but this results in increased susceptibility to proteasomal inhibitors.

Redistribution of Proteasomes in Cells with Stably Expression of Poly-Q Protein

To explore why the R-GFP-Q112 substrates were not degraded, we detected the subcellular localisation of both R-GFP-Q112 substrates and proteasomal complexes by immunohistochemical analysis as well as western blotting assay. We found that virtually all R-GFP-Q112 proteins resided in the fraction of lowest molecular weight (100.000_g/5h supernatant), i.e soluble. The data were similar in both untreated cells and cells treated with NLVS, although a minor poly-Q band appeared in high molecular weight fraction (100.000_g/5h pellet) in NLVS-treated cells (Fig.7).

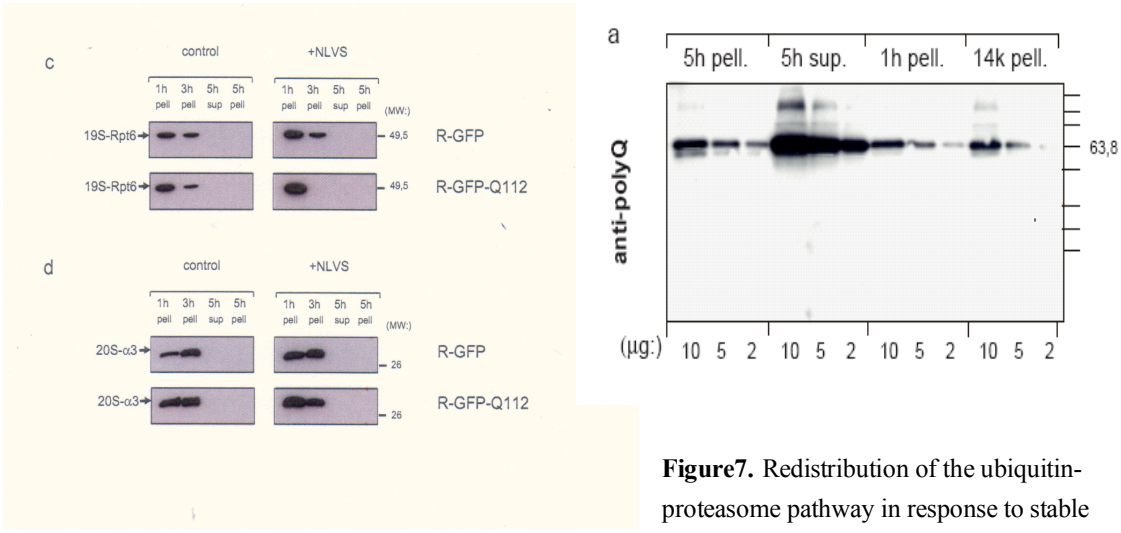


Figure7. Redistribution of the ubiquitin-proteasome pathway in response to stable poly-Q expression. (a) EL-4.Ub-R-GFP and (b) EL-4.Ub-R-GFP-Q112 cells were immunohisto-chemically analysed with by staining of fixed cells with anti-Rpt6 (a ATPase 19S proteasome sub-unit), with unclear stain included for comparison. (c,

d) Proteasomal sub-units of EL-4.Ub-R-GFP and EL-4.Ub-R-GFP-Q112 cells analysed by Western blotting for expression of 19S (c, Rpt6) or 20S (d, α3) sub-units in sub-cellular fractions.

Besides, we detected 19S (rpt6) complexes evenly over cytosol and nucleus in EL-4.Ub-R-GFP cells, but very few of 19S complexes in EL-4.Ub-R-GFP-Q112 cells, whereas19S complexes in R-GFP-Q112 cells confined to a perinuclear location. And meanwhile, we failed to observe significant differences in the expression of level of proteasomal 20S (α3) or 19S sub-units. These data suggest that virtually all poly-Q substrate in EL-4.Ub-R-GFP-Q112 cells may be present in soluble form (no inclusions in EL-4.Ub-R-GFP-Q112 cells were detected), and this implies that the inhibition of UPP here occurred largely independently of protein inclusion. So we concluded that an extended poly-Q repeat causes sub-cellular redistribution of proteasomes in EL-4 cells that does not overlap with most poly-Q substrate.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

- An altered specificity of cytosolic proteolysis, with reduced proteasomal activity and up-regulated activity of TPPII, disturbs the transduction of signals by ubiquitin-proteasome pathway.

- The suppression of ubiquitin-proteasome pathway during stress, mediated at least in part by the expression of TPP II, causes de-regulation of chromosome numbers.

- TPPII is effectively controlling the rate of cytosolic proteasomal substrate degradation.

- A stably expressed poly-Glutamine protein may partially impair the activity of the ubiquitin-proteasome pathway through redistribution of proteasoma complexes.

Our data give further support for a link among the ubiquitin-proteasome pathway, cellular protection responses and pathological mechanisms in human diseases.

The link between insufficient ubiquitin-proteasome pathway and genetic instability controlled by an altered rate of proteasomal proteolysis warrants further study in cancer cells and other models of pathogenesis. More specifically, we may address whether an altered specificity of cytosolic proteolysis contributes to the acquisition of malignant characteristics and explore the status of the ubiquitin-proteasome in human diseases.

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