Role of the ubiquitin-proteasomal system and HDAC6 in the maintenance of protein homeostasis

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Abbreviations

AMD	Age dependent Macula Degeneration
AP-1	activator protein 1
ATP	adenosine triphosphat
CHIP	carboxyl-terminus of Hsp70 interacting protein
CO	carbon monoxide
FDA	US Food and Drug Administration
GR	growth hormone receptor
HIF-1	hypoxia inducing factor-1
HIP	Hsp70 interacting protein
HNE	4-Hydroxy-2-nonenal
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
Keap-1	Kelch-like ECH-associated protein 1
MAPK	mitogen activated protein kinase
MDA	malondialdehyde
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLS	nuclear localization sequence
Nrf-2	nuclear factor erythroid 2-related factor 2
PI3K	phosphoinositol-3- kinase
PKC	protein kinase C
RISC	RNA-induced silencing complex
ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
USP	ubiquitin specific protease

1. Introduction

1.1 Protein homeostasis – proteostasis

Mammalian cells express about 10000 different proteins which are synthesized as linear, unfolded amino acid sequences on ribosomes. To gain the typical protein functionality it is necessary that these linear sequences are folded into their native state, comprising their three-dimensional structure. The individual folding pattern is partly predefined by the amino acid sequence. 20-30% of total proteins in mammalian cells have inherently lost their three dimensional order and rely on additional binding partners to adopt a conformational state [1]. Due to the possibility that one protein can take on more than one conformation, the protein folding process possesses high complexity. In search of the right protein conformation (the native state), exhibiting a thermodynamically stable order with low free energy-surfaces, it comes to folding intermediates (occurs for about 90% of all cellular proteins) [2]. Due to free hydrophobic amino acids, folding intermediates tend to build rapidly compact globular protein structures, which are not leading to the native protein structure. Most of these unwanted globular protein structures can be defined as misfolded proteins or partially folded proteins. To bring them into the right conformation, there is the need of molecular chaperones, which help to overcome some thermodynamic barriers [3]. Since partially folded or misfolded proteins exhibit access to hydrophobic residues, they tend to accumulate and form aggregates [4].

The prevention of protein misfolding and protein aggregation and maintaining the native protein structure, respectively protein functionality, is the definition of protein homeostasis (proteostasis). To cope with this challenge the proteostasis network comprises about 800 proteins, segmented into 200 chaperones and co-chaperones, that maintain folding, refolding and stabilization of proteins and 600 ubiquitin-proteasomal and autophagy components, which are responsible for the degradation of unfoldeable proteins and protein aggregates [5].

1.2 The proteasomal system

Maintenance of protein homeostasis is an essential part of keeping up cell functionality and cell survival [6]. As it was mentioned, unfolded and unfunctional proteins become refolded by molecular chaperones to regain their functionality. However, due to several

possible modifications, proteins become resistant to a refolding process. To cope with that challenge, the cell has installed two systems to degrade irreversibly misfolded proteins: autophagy and the ubiquitin-proteasomal system (UPS) [7]. Whereas autophagy is mostly responsible to store and sequester potentially toxic protein aggregates [8], the UPS is responsible for the highly regulated degradation of most cytosolic proteins and proteins that are located in the nucleus and the endoplasmatic reticulum (ER) [9-11].

1.2.1 The 20S core proteasome

The proteasome is a cylindric shaped, multi-catalytic protease with a molecular mass of about 700kDa. The core structure of this complex is called the 20S proteasome. Due to the fact that the 20S proteasome builds the core structure for all existing proteasomal forms, it is involved in a multitude of cellular processes, such as life span regulation of proteins [12,13], protein quality control [14,15], cell cycle regulation [16], carcinogenesis [17,18], DNA-repair [19,20], gene-expression [21,22], stress response [23,24], the immune response [25,26] and degradation of oxidized proteins [27,28].

The 20S proteasome consists of two α-rings and two β-rings, forming a barrel, whereas the two β-rings are arranged in the middle and the two α-rings form the closure on each side of the barrel **(Fig.1)** [29]. These αββα ring-construction forms three chambers inside the 20S proteasome: two ante- or forechambers, build of one α-ring and one β-ring and a core chamber, which is build by the two inner β-rings [30]. Every α/β-ring consists of seven α- or β- subunits ($\alpha_{1\text{-}7}/\beta_{1\text{-}7}$). While the α-subunits are responsible for substrate access and binding of the several proteasomal regulators, the β-subunits form the proteolytic center of the 20S core structure. The main task of β-subunits is to degrade the large variety of polypeptides and proteins which pass the α-ring. Therefore, three of the seven subunits have a threonine protease activity. The $\boldsymbol{\beta_1}$ subunits preferentially cleave after acidic residues (peptidyl-glutamyl-peptide-hydrolase activity or caspase like activity), the $\boldsymbol{\beta_2}$ subunit displays a trypsin-like activity (cleavage after basic amino acids) and $\boldsymbol{\beta_5}$ acts as a chymotrypsin-like protease (cleavage after neutral amino acids) [31,32].

Access into the proteolytic center of the proteasome is only possible if the substrate interacts with the outer α-subunits, followed by a conformational change, unlocking a gate that is formed by N-terminal ends of the α_2, α_3 and α_4 subunits. It was observed that detergents like SDS or hydrophobic molecules have the ability to open the closed conformation of the 20S proteasome, enhancing its proteolytic activity. Thus, substrates

of the 20S proteasome have to be unfolded, (modified) proteins, exhibiting their hydrophobic structure [31,33,34].

1.2.2 The 26S proteasome

Since proteolysis catalyzed by the proteasome comprises not alone unfolded and damaged proteins, but rather a large spectrum of short lived, regulatory, onco- and structural proteins [35], the 20S proteasome builds a complex with two 19S proteasome regulators (which is also called "PA700"). These two regulators bind to each side (to each α-ring) of the "20S cylinder", forming the 26S proteasome **(Fig. 1)**. In total the 20S proteasome and the two 19S regulators reaching a protein mass of about 2MDa [31,36]. The 19S regulator forms a ring shaped base, which builds the connection to the α-rings of the 20S proteasome and a lid structure, regulating the entrance of polyubiquitinated substrates [37]. The ring-shaped footing of the 19S regulator is made out of 6 different Regulatory particle of triple ATPase (Rpt) - and 4 different Regulatory particle of non-ATPase (Rpn) subunits (Rpt_{1-6}, Rpn_{1-2}, Rpn_{10} and Rpn_{13}). The base of the 19S proteasome has three important functions: (i) recognition of ubiquitin-tagged substrates, (ii) protein unfolding and (iii) opening of the translocation-channel in the α-ring [37]. All 6 Rpt subunits and Rpn1/2 are involved in protein unfolding and the gate opening process. Recognition and binding of polyubiquitinated proteins is conducted by Rpn10 and Rpn 13, which are closely located to the lid structure. The lid consist of nine Rpn subunits ($Rpn_{3,5-9,11,12,15}$), whereas Rpn 11 functions as a deubiquitinating enzyme, cleaving polyubiquitin chains at the proximal site of proteasomal substrates [38]. It is possible that the 19S regulator is also associated to external deubiquitinating enzymes, like Usp14 or Uch37. Some of them, for example Usp14 (in yeast: Ubp6), become activated by a lack of free available monoubiquitin, accelerating deubiquitination of proteasomal substrates and altering proteasomal function [39].

For the sake of completeness, it should be mentioned shortly that there are also two other common forms of the proteasome. Besides the 19S regulator the proteasome is also able to build a complex with the 11S proteasome regulator (also called PA 28). If the 11S regulator binds to both sides of the 20S cylinder and the normal β-subunits (β_1, β_2, β_5) are substituted by inducible β-subunits (β_{1i}, β_{2i}, β_{5i}), there is talk of the immunoproteasome [40]. Thus, cytokines like IFN-γ or TNF-α induces synthesis and activity of these inducible β-subunits, which leads together with the 11S regulator to a spectrum of produced peptides that are associated with MHC class 1 antigen

presentation [41]. Is the 20S proteasome bound to one 11S regulator on the one side and to a 19S regulator on the other side, arises the hybrid proteasome. Although, the function of the hybrid proteasome is not fully understood, it is suggested that it also participates in antigen processing, since it was demonstrated that the activity of the hybrid proteasome is also stimulated by IFN- γ. Due to the incapacity of the 11S regulator to degrade folded proteins, it is possible that the 19S regulator of the hybrid proteasome is responsible for the ATP-dependent unfolding process and the 11S regulator take care for the production of MHC-1 related peptides [42].

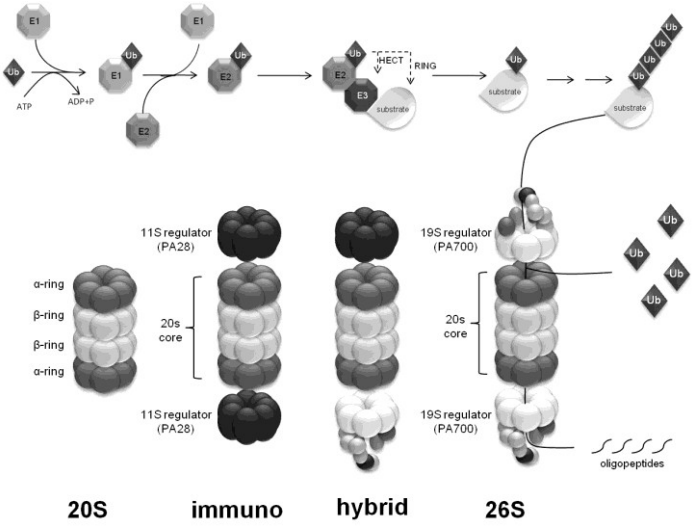

Figure 1: The ubiquitin proteasomal system
The figure depicts the several kinds of cellular proteasomes and the schematic mechanism of protein-ubiquitination ([43] modified).

1.3 Ubiquitin and the ubiquitination process

As it was mentioned, most proteins have to be polyubiquitinated for degradation by the 26S proteasome. A chain of at least four ubiquitin molecules is required to mark a protein for proteolysis by the 26S proteasome.

1.3.1 Ubiquitin

Ubiquitin is a highly conserved, small protein consisting of 76 amino acids [44]. Several consisting amino acids have important functions for the ubiquitination process. The C-terminal glycine (G76) of ubiquitin is essential for the most common way to attach the first ubiquitin moiety to an ε-amino group of a lysine residue of its substrate by forming an isopeptide bond [45]. It is also possible, that ubiquitin binds to the N-terminal

residue of a peptide, preparing a linear polyubiquitin chain or there is a generation of ester/thioester bonds by conjugation with a cystein, serine or threonine residue [46]. Furthermore, ubiquitin contains several lysine residues which are necessary for the formation of polyubiquitin chains. In total the protein sequence of ubiquitin possesses five lysine (K) residues – K6, K27, K29, K48 and K63. In dependency of the involved lysine residue, a polyubiquitin-chain is able to fulfill different functions. Polyubiquitin chains that are based on K63-linkages act as targeting sequences for autophagy and endocytosis, activates kinases or play a role in activation of DNA-repairing enzymes [47-49]. On the other hand, K48-polyubiquitin chains function as targets for proteasomal degradation [50].

1.3.2. The ubiquitination process

The mechanism of protein ubiquitination comprises 3 different groups of enzymes, which are the ubiquitin-activating enzymes (E1), the ubiquitin conjugating enzymes (E2) and the ubiquitin ligases (E3). The ubiquitination mechanism starts with the ATP-dependent activation of one ubiquitin molecule. This reaction is catalyzed by one of eight E1 enzymes, maintaining the bond between the C-terminal glycine of ubiquitin with a cysteine residue of E1. In a second step ubiquitin is transferred to cysteine residue of an E2 enzyme. In a last step, one of the several E3-ubiquitin ligases, the substrate and the E2 enzyme with the bound ubiquitin molecule build a complex, where ubiquitin is transferred to the substrate. Ubiquitin tagging of the substrate can be conducted by two possible reaction pathways. Either ubiquitin is transferred directly to the substrate or in a two step mechanism. It depends on the participating E3 ubiquitin ligase that transfers ubiquitin to the proteins. E3 enzymes with an RING-motif transfer ubiquitin directly to the substrate. whereas E3 enzymes with a HECT-motif bind the ubiquitin molecule first on themselves and afterwards to the substrate [51,52] (Fig.1).

1.4. The Heat Shock Protein family

Any protein that has the ability to stabilize and/or support another protein to obtain its functional conformation can be defined as a chaperone molecule [53]. Most of the known chaperones belong to the family of heat shock proteins (Hsps). Although Hsps are also constitutively expressed in mammalian cells, they are highly inducible by a diversity of proteotoxic stress stimuli, such as heat stress or oxidative stress [54,55]. The Hsp family can be subdivided into two groups of chaperones: first, the molecular

chaperones, including Hsp100, Hsp90, Hsp70, Hsp60/chaperonins and Hsp40 and second, the small Hsps (sHsps), comprising for example heme oxygenase-1 (Hsp 32) and Hsp27 [56]. Even though all of these Hsps have different protein sizes and functions, they are united by the similarity to have one or more heat shock elements (HSEs) in their promotor region, functioning as binding sides for the activating heat shock factors (HSFs) [57].

Molecular chaperones are responsible for protein (re)folding, stabilization of proteins within the cytosolic compartment and support of the ubiquitination process. In contrast to sHsps, enzymatic activity of molecular chaperones is highly ATP-dependent and co-regulated by several co-chaperones [58]. On the contrary, sHsps are ATP independent and do not interact with co-chaperones, but rather form large homo-oligomers to mediate their chaperone-function. Binding of this large sHsp-oligomers to proteins in a non-native state prevents substrate aggregation and creates a reservoir of unfolded proteins for subsequent refolding by interacting molecular chaperones [59].

1.4.1 Hsp90

Under normal physiological and constitutive conditions Hsp90 is already present at very high concentrations in the cytosol and can be further up-regulated in stress situations. High basal levels of Hsp90 allow augmented binding occurrence and short-lived interactions with client proteins. The Hsp90 chaperone is a flexible dimer, whereas every monomer consists of a N-terminal domain which is connected via a long linker sequence with a M-domain and ends up with a C-terminal domain [60]. The C-terminal domain is necessary for Hsp90 dimerization. The M-domain is responsible for substrate binding and interaction with the co-chaperone AHA-1 during one folding cycle. The ATP-bind site is located at the N-terminus of Hsp90 [61,62]. Binding of ATP results in a dimerization of the N-terminal domains and forms a “molecular clamp”, which is needed for substrate processing. Hydrolysis of ATP, which is the rate limiting step of the cycle, resolves the molecular clamp and leaves the Hsp90 dimer in an open conformation. Hydrolysis of ATP and therefore the efficiency of the Hsp90 processing cycle is influenced by some co-chaperones. AHA-1 stimulates ATP hydrolysis, whereas CDC37 and HOP inhibit the N-terminal ATP hydrolysis activity and block the dimerization of the N-terminal domains. In addition, p23 stabilizes the dimerized form of Hsp90 before ATP-hydrolysis [63,64]. Beyond protein (re)folding, Hsp90 is responsible for binding and stabilization of a special, defined group of proteins, called

clients. The wide field of client proteins comprises protein kinases, nuclear hormone receptors, cell surface receptors or transcription factors in the cytosol [65]. Therefore, Hsp90 plays also a role in RNA transcription, RNA processing, DNA replication, DNA recombination, DNA repair and DNA metabolism [66].

1.4.2 Hsp70

Due to its multiple functions, Hsp70 is the best examined heat shock protein. Function of Hsp70 comprises folding and stabilization of newly synthesized proteins, membrane translocations of secretory proteins, proofreading (in collaboration with Hsp40) and repair/refolding of damaged, unfolded or partly aggregated proteins [67-70]. To cope with all of these different obligations, the 70 kDa chaperone is expressed as a constitutive form (Hsc70; gene: HSPA8), maintaining the normal cell function, and as a stress inducible form (Hsp70/72; gene: HSPA1A, HSPA1B and HSPA1L), which is mainly responsible for the prevention of protein damage or protein aggregation as well as the re-establishment of functional proteins.

Although, there are different forms of Hsp70, the general structure of the chaperone is equal. The N-terminal domain is responsible for ATP binding and hydrolysis, whereas the C-terminal domain, consisting of a β-sandwich domain and an α- helical domain, is responsible for substrate binding [71]. The β-sandwich detects protein segments, which are enriched in hydrophobic amino acids, especially when they are close to positive charged residues, indicating an unfolded protein [72]. Substrate affinity of the β-sandwich and the α-helices depends on the ATP-status of Hsp70, which is coordinated by some co-chaperones. The ATP bound form of Hsp70 shows a low affinity to the bound substrate and is therefore responsible for a rapid binding, processing and release of the substrate, while the ADP-bound form possesses a much higher affinity to the bound substrate and thus binding and release of proteins expires in a very slow way [73]. In the ADP-bound state, one of the α-helices of the substrate binding domain (SBD) forms salt bridges and hydrogen bonds to the outer loops and thereby closing the substrate binding pocket like a lid [74]. Closure of the lid can strongly be accelerated by interaction of Hsp70 with the co-chaperone Hsp40, functioning as an ATP-hydrolysis activator. Additionally, Hsp40 is also involved in the detection of unfolded proteins and the recruitment of Hsp70 to such substrates [75,76]. There are also two other co-factors, influencing ATP-hydrolysis of the N-terminal Hsp70 domain. HIP binds to the N-terminal ATPase domain and stabilizes the ADP-bound state of Hsp70, promoting

therefore the folding activity the chaperone [77]. The opponent of HIP is Bag-1, which is a member of the Bag protein family. Bag-1 possesses like all members of the Bag protein family a Bag domain which is necessary for the interaction with the N-terminal ATPase domain of Hsp70. Bag-1 functions as an acceleration factor of ADP release at the N-terminal ATPase domain and facilitates the start of a new ATP cycle of the chaperone [78].

1.4.3 Interaction of Hsp90, Hsp70 and the co- chaperone CHIP

As it was mentioned Hsp90 is responsible for the binding and stabilization of client substrates. Among these clients are kinases like Raf, enzymes like NOS, signaling proteins like VEGF, and growth factor receptors like GR or the polyglutamine androgen receptor [79,80]. Before these client proteins become substrates of Hsp90 stabilization, they have been partly folded by Hsp70. In a complex made of Hsp70 and Hsp90, which is linked by another co-chaperone called HOP, Hsp70 delivers the folded client to Hsp90. This process is connected to several conformational movements of the chaperones and is therefore ATP-dependent.

Substrate proteins, which are despite of several folding cycles not foldable, become ubiquitinated by the co-chaperone CHIP. CHIP is a RING domain - E3 ubiquitin ligase with a TPR domain at its N-terminus, enabling the interaction with EEVD-domains of Hsp70 and Hsp90. On the C-terminus of CHIP there is a “U-box” domain that interacts with the UBCH5 family of E2 ubiquitin-conjugating enzymes [81]. Binding of CHIP to the TPR acceptor site of Hsp70 results on the one side in a retardation of the protein refolding process and on the other side in a transformation of the Hsp70 chaperone to a “chaperone- dependent ubiquitin ligase”[81]. To decelerate the ATP-cycle of Hsp70 and Hsp90, CHIP inhibits ATP hydrolysis, keeping the chaperones in a low substrate-affinity state. It is supposed that this “slowing down”-mechanism assists the loading of misfolded proteins from chaperones into the ubiquitin-proteasome machinery [81,82].

Due to its TPR domain, Hsp90 is also up to associate with CHIP [83]. Interaction of CHIP with Hsp90, results in a remodeling of the chaperone, slowing down of the ATPase cycle and thus to an inhibition of Hsp90 client proteins [81]. It seems that there are two possible scenarios for CHIP mediated ubiquitination and degradation of Hsp90 clients. Some client proteins, such as GR, are ubiquitinated by CHIP during they are bound to Hsp90 [81]. Others, like ErbB2, are first transferred back to Hsp70 before they become ubiquitinated by CHIP[84].

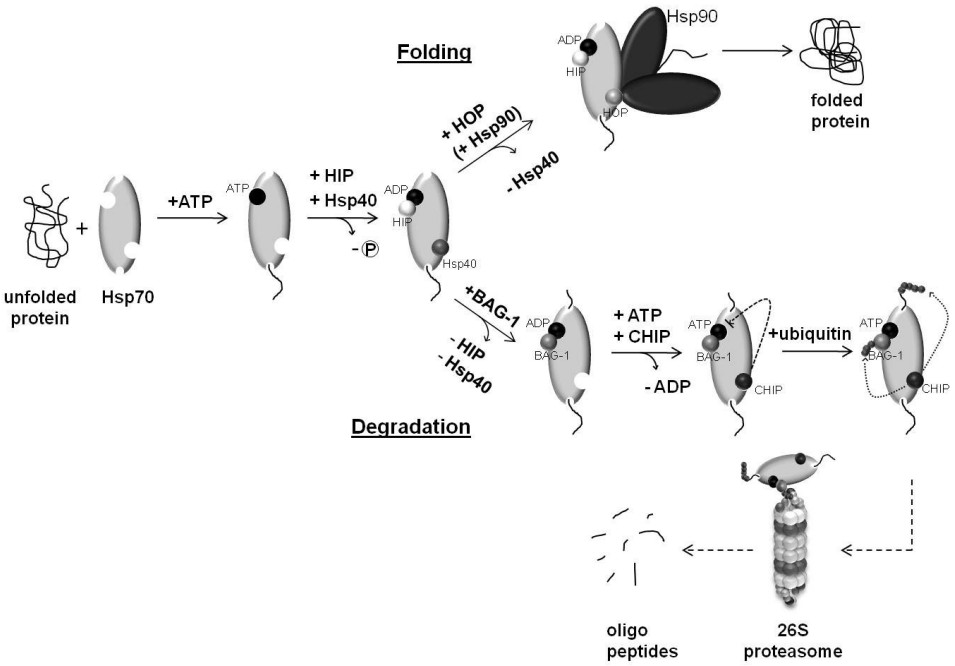

Figure 2: Folding or degradation of chaperone substrates.
Folding: The folding process of Hsp70 depends on ATP and several co-chaperones. After ATP and the substrate has bound to Hsp70, HIP binds to the N-terminal ATPase- domain and Hsp40 binds to the C-terminus, resulting in a fast hydrolysis of ATP to ADP and a stronger affinity to ADP by the chaperone. At this constitution Hsp70 has a strong binding affinity to the substrate, allowing proper folding of the protein. If interaction with Hsp90 is necessary HOP replace Hsp40 and functions as a linker molecule between Hsp70 and Hsp90. ***Degradation:*** In the state, when Hsp40 and HIP has bound to Hsp70 BAG-1 replace HIP at the ATPase-binding domain. Following the dissociation of Hsp40 and HIP, ADP is exchanged by new ATP and the TPR containing-protein CHIP binds to the C-terminal EEVD domain. CHIP inhibits the hydrolysis of ATP and ubiquitinates the bound substrate, as well as Hsp70 and BAG-1. K-11 linked ubiquitination of Hsp70 and BAG-1 functions as a binding signal to the 26S proteasome, where the ubiquitinated substrate becomes degraded ([81,85,86] modified).

1.4.4 Hsp32/heme oxygenase 1 and the Nrf-2 activation mechanism

As it is indicated in the protein`s name, the substrate of heme oxygenase-1 (HO-1) is heme. HO-1 catalyzes the degradation of free heme into three components: Fe^{2+}, biliverdin/bilirubin and CO [87]. HO-1 alias Hsp32 is as such not a typical heat shock protein. Although, HO-1 lacking conventional heat shock protein functions, such as protein stabilization or refolding of denatured proteins, it belongs to the heat shock protein family. This is due to the high stress activation of HO-1 and the occurrence of some HSE sequences in its promotor region [88-90]. Interestingly, HO-1 is inducible by several stress stimuli, comprising UV-irradiation, heavy metals or oxidative stress, but not by heat stress [91-93]. Besides HSF-1, the *hmox1* gene activation is also regulated by AP-1, NF-κB and in particular by Nrf-2.

Nrf-2 is a universal transcription factor (TF), which is activated during a multitude of cellular stress stimuli, like xenobiotics and oxidative stress. Therefore Nrf-2 regulates the gene expression of genes, involved in oxidative stress resistance and xenobiotics detoxification [94]. All of these target genes possess one or more stress response elements (StREs) (also called antioxidant or electrophile response element (ARE/EpRE)), which are the binding receptor of Nrf-2 in the up-stream-located enhancer regions of target genes. Usually Nrf-2 heterodimerizes with small Maf proteins in the nucleus to function as ligands for StREs [95]. The regulatory domains of hmox1 can be subdivided into three parts, whereas two of them are highly assembled with StREs [96].

In unstressed cells Nrf-2 is captured in the cytosol by its inhibitor Keap-1 (Fig. 3). Keap-1 binding site of Nrf-2 is the highly conserved region Neh2. Nrf-2 and Keap-1 function as a sensitive sensor of oxidizing electrophiles and other stress stimuli. In the cellular resting state, reactive cysteins (C273 and C288) of the Neh2 binding site of Keap-1 are in a reduced state, keeping the bound to Nrf-2 stable and sequester the TF in the cytosol. Exposure to oxidative stress leads to an oxidation of the C273 and C288 and the liberation of Nrf-2 [97]. Due to the Keap-1 supported ubiquitination of Nrf-2 and its degradation by the proteasome, the half-life of Keap-1 bound Nrf-2 is relatively short. During the ubiquitination process of Nrf-2, Keap-1 recruits the E3 ubiquitin ligase Cul-3/Rbx1 and functions as a substrate adaptor for Cul-3/Rbx1, resulting in the ubiquitination and degradation of Nrf-2 (Fig.3) [98].This is the reason for low basal levels of Nrf-2 in unstressed cells [99]. Treatment of cells with electrophiles significantly prolongs the half life of Nrf-2, increase the total amount of it and leads to a translocation into the nucleus of Nrf-2 [100]. Moreover, Nrf-2 activation is dependent on different signaling pathways, which are also responsible for Nrf-2 liberation and phosphorylation. Following signaling pathways/kinases are involved in Nrf-2 activation: PI3K-, PKC-, ERK and the MAPK pathway (Fig. 3) [101,102]. Depending on the respective stimulus one of these three pathways or kinases phosphorylates a serine residue in the Neh2 region of Nrf-2, supporting the disruption of Keap-1 and Nrf-2 [103].

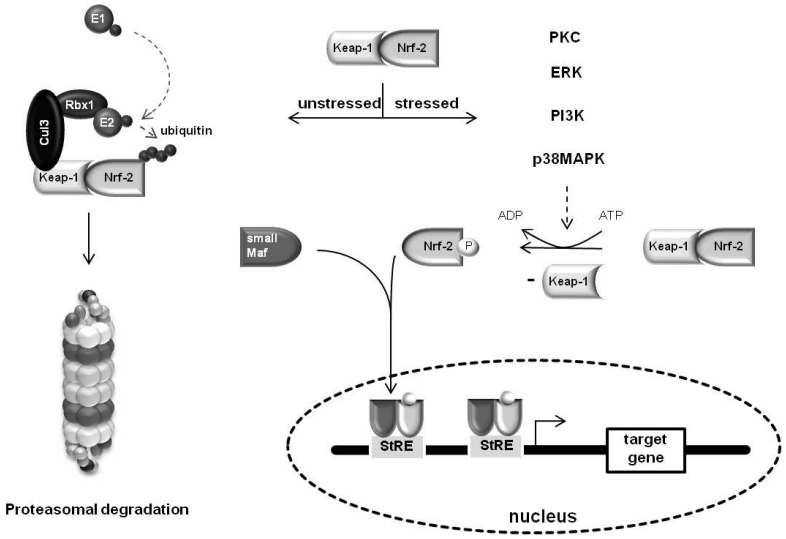

Figure 3: Nrf-2 Signaling Pathway.
In unstressed cells Nrf-2 is sequestered in the cytosol by Keap-1, leading to a Cul-3/Rbx1 dependent degradation of Nrf-2 by the proteasome. Under stress conditions Nrf-2 dissociates from Keap-1 and becomes phosphorylated by kinase activity. Phosphorylated Nrf-2 translocates into the nucleus and binds together with small Maf to several StREs, inducing gene transcription of several stress response proteins ([104] modified).

1.5 Histone deacetylase 6 (HDAC6)

Classical HDACs can be subdivided into two major classes. Class I HDACs include HDAC 1, 2, 3, 8 and class II HDACs comprise HDAC 4, 5, 6, 7, 9, 10. HDAC11, a recently found HDAC, is closely related to HDAC1, however, due to a too low overall sequence similarity, HDAC11 could not be allocated to class I or II HDACs [105]. Most HDACs contain a NLS, but can also be located in the cytosol as well. The four members of class I HDACs are primary located in the nucleus, whereas class II HDACs are able to switch between the nucleus and the cytosol. Especially HDAC6 is predominantly located in the cytoplasm [106]. The general function of HDACs is the removal of acetyl groups from histones comprising the nucleosome. Hypoacetylation results in a tighter wrapping of the DNA around nucleosomes and decrease the accessibility for transcription factors (TFs), which is tantamount with a transcriptional repression. Thus, HDACs play a crucial role in signal transduction, regulation in gene expression, cell growth and cell death [107].

Within the family of HDACs, HDAC6 is a rather unique enzyme. The identity of HDAC6 with other human HDACs is very low. With regard to the structure of HDAC6 it is remarkable, that the protein contains two catalytical deacetylation domains instead of one. Moreover, HDAC6 possesses in contrast to the other HDACs a C-terminal ubiquitin binding domain, enabling the deacetylase to recognize and bind ubiquitinated

proteins [108]. Due to cytosolic location of HDAC6, HDAC6 target proteins are mainly cytosolic proteins. Prominent substrates are tubulin [109], Hsp90 [110], Ku70 [111] and cortactin [112]. Thus, HDAC6 plays a critical role in the regulation of cell motility, apoptosis, autophagy and maintenance of proteostasis [113-117].

1.6 Oxidative stress and protein modifications

There are several reasons why proteins become modified and therefore unfunctional. Inherited mutations, UV-irradiation, heat stress, heavy metal intoxications and oxidative stress are all possible stress stimuli, leading to protein modifications [118]. Especially oxidants have the ability to react with nearly all amino acids, forming protein modifications like protein carbonyls or oxidation of sulfur containing groups.

1.6.1 Oxidative stress

Per definition, oxidative stress is the imbalance between reactive oxygen species (ROS) formationand the antioxidative system, whereas there is an overbalance on the side of ROS [119]. ROS includes a large group of different oxygen-containing molecules, which are separated into free radicals, such as superoxide anion ($^{\bullet}O_2^-$), hydroxyl – and lipid radicals ($^{\bullet}OH$) as well as oxidizing non-radical species like hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-) and singlet oxygen($^1O_2$) [120]. The main source of ROS is the respiratory chain of the mitochondria, where ROS are continuously produced by one - electron reduction of molecular oxygen. Enzymes like NAD(P)H oxidases, xanthine oxidase, cyclooxygenase and lipoxygenase are also key sources for cellular generated ROS [121]. But ROS are not only a product of cellular enzymes, environmental factors like UV-irradiation or metal-ions such as Fe^{2+} (Fenton-reaction) are also possible reasons for the generation of ROS [118].

Due to the continuous generation of ROS, cellular systems have a large arsenal of several antioxidants available to prevent oxidative damages. The antioxidative protection system can be divided into internal and external antioxidants. Cellular expressed enzymes like catalase, superoxide dismutase or the glutathione system count among the internal antioxidants. External antioxidants are antioxidative vitamins like ascorbic acid and tocopherols or secondary plant products like polyphenols [118]. Due to inherited alterations of antioxidants expression, adverse lifestyle conditions, malnutrition or the ageing process, the antioxidative protection declines, leading to an overbalance of ROS and oxidative stress [118]. The chronic exposure to oxidative stress

over years, is suggested to be a major reason for the ageing process [122] and many age-dependent diseases like Alzheimer's Diseases [123], Parkinson's Disease [124], Huntington's Disease [125], heart failure [126], cataract and AMD formation [127,128], skin ageing [129], atherosclerosis [130], type 2 diabetes [130] and cancer [131].

1.6.2 Oxidative protein modifications

Besides lipids and the DNA, proteins are the most vulnerable targets of oxidation reactions. Oxidative modifications of proteins affect single amino acids, peptides or whole proteins with the ability to accumulate and forming aggregates. The consequence of protein oxidation is an impairment of receptor functionality, antibodies, signal transduction pathways, transport proteins and enzymes. Therefore, oxidative protein damage is a far reaching problem, because it affects also other biomolecules, for example by raising Ca^{2+} levels [132] or increasing DNA mutations by faulty DNA repair or synthesis due to oxidative modified DNA polymerases.

Oxidative modifications of proteins can either occur by direct attack of ROS or by secondary reactions with end products of lipid peroxidation, such as MDA [133] or HNE, [134] or glycation products [135]. Very prone amino acid residues for oxidative modifications are the sulfur group-containing amino acids cysteine and methionine, amino acids containing an additional nitrogen group like arginine, lysine and proline, the amino acid glutamyl and the aromatic amino acids like phenylalanine or tyrosine, histidin, threonin [136]. Besides a multitude of special and unique single-amino acid modifications, as the generation of 2-Oxohistidine, 3-Nitrotyrosine or the production of methionine sulfoxide (MetO), the occurrence of protein carbonyls affects a larger group of amino acid residues. In particular, the oxidative reaction with arginine-, lysine, proline-, threonine- and glutamic acid results in the formation of protein carbonyls [137,138]. Due to the widespread distribution of protein carbonyls, they are common used marker for oxidative protein modifications. By derivatization of protein carbonyls with 2,4-dinitrophenylhydrazine, protein carbonyls can be detected using ELISA techniques, immunoblotting or immunochemistry [139]. Studies, which screened the subcellular distribution of protein carbonyls, found that protein carbonyls are co-localized with ROS generating organelles, like the mitochondria [140,141]. In contrast to methionine sulfoxide or sulfur-nitrosylation, protein carbonyls are non- reversible protein modifications. Thus, suchlike proteins have to be degraded by the proteasome.

1.7 RNA interference (RNAi) techniques for proteins suppression

Gene silencing by RNAi techniques is a modern and potent method to examining the function of distinct genes/proteins, the involvement of one protein in a complex pathway, drug target discovery/validation and therapeutics [142]. RNAi is a conserved and natural biological response, which can be found in nearly all eukaryotic organisms. In plants and some prokaryotes RNAi is an essential part in the defense mechanism against virus RNA [143]. In most multi-cellular organisms RNAi plays an important role in the regulation of gene-expression, especially for the coordination of the immune system [144]. Thus, any eukaryotic cell possesses a complete set of different enzymes, which are necessary for the processing of interfering RNAs. For gene silencing via RNAi there are two major possibilities, either by the application of short hairpin RNA (shRNA) or small interfering RNA (siRNA). siRNAs are short, double stranded RNA (dsRNA) sequences with the ability to interfere with coding mRNAs after a cellular processing procedure. After transfection the long dsRNA is processed in a first step by a dicer endonuclease into small, double stranded siRNA sequence with a length of about 21 nucleotides. 19 nucleotides of this processed RNA form a helix, whereas each 3'end remains unpaired. In a second step the double stranded siRNA interacts with the RNA-induced silencing complex (RISC).Within the RISC the double stranded siRNA becomes unwind by helicases, offering the sense strand of the siRNA for degradation. The loaded antisense strand of the siRNA and the RISC bound the target mRNA, resulting in a cleavage of the coding mRNA and a prevention of protein biosynthesis [144,145]. Commercially available siRNAs are already processed with bacterial recombinant Dicer and interacts after transfection directly with the RISC.

The mechanism of shRNA processing is very similar to the process of conventional siRNA. shRNA is a sequence of RNA nucleotides, forming tight hairpin turns inside the sequence. After processing by dicer enzymes and interaction with the RISC, shRNA functions like normal siRNAs, leading to the degradation or blockade of the complementary target mRNA strand [144]. But, in contrast to "ordinary" siRNA, shRNA is part of a vector, which is then incorporated in the target genome. Transfection of shRNA vectors is commonly conducted via electroporation [146] or lentiviral transfer [147]. In general the used vectors are equipped with a U6 or H1 promotor for shRNA transcription. These promoters regulate the synthesis of small, highly abundant

non-coding RNA and have not any intragenetic control regions [148]. During transcription via polymerase III, a self complementary RNA is produced, forming the shRNA.

2. Aim of the work

Maintenance of protein homeostasis is central for the functionality and survival of the cell. Impairment of protein homeostasis is closely connected to various diseases. Especially during the ageing process, when some of the protein homeostasis control mechanisms decline, disturbances in proteostasis lead to age dependent diseases as neurodegeneration, atherosclerosis, cataracts, skin ageing or diabetes. To preserve proteostasis there exist either the possibility to repair/refold damaged proteins or degrade misfolded proteins. The two major systems fulfilling this assignment are the ubiquitin-proteasomal system and the family of chaperones.

The role of the ubiquitin-proteasomal system in proteostasis during oxidative stress and the mechanisms after proteasome malfunction in regard to maintain proteostasis were the major goals of this work. The data can give a better understanding about age-dependent diseases or cancer therapies, using proteasome inhibitors.

The following aspects of proteasomal function were investigated:

- First a comprehensive overview on the current knowledge about the ubiquitin-proteasomal system, during oxidative stress, disturbances of the protein homeostasis by oxidative stress and the connection to the ageing process and age-related diseases has to be given **(Publication I)**.
- In a next step it should be determined how the proteasome and ubiquitin are involved in the degradation of oxidized proteins after the exposure to different oxidizing stress stimuli **(Publication II)**.
- Further on it was required to establish a system to isolate ubiquitinated proteins and test for their oxidation status **(Publication II and III)**
- In further experiments the identity of the polyubiquitinated proteins after oxidative stress of Publication II were determined and categorized **(Publication III)**.
- Additionally it should be elucidated, how these determined proteins are associated and what is the kinetic of their proteasome dependent degradation **(Publication III)**
- Due to the central part of the proteasome in the maintenance of proteostasis, it is a major point of interest what happen with proteostasis and the proteostasis control system during proteasomal failure. Therefore, the response of

chaperones to proteasome inhibition was studied by determination of gene expression- and protein expression levels **(Publication IV)**.

- Since it was demonstrated for the first time that the non classical heat shock protein HO-1 (Hsp32) is also up regulated after proteasome inhibition, the molecular pathways involved in HO-1 induction upon proteasome inhibition has to be elucidated **(Publication IV)**.

3. Publications

3.1 Publication I: Protein Oxidative Modification in the Aging Organism and the Role of the Ubiquitin Proteasomal System

Marc Kästle, Tilman Grune

Current Pharmaceutical Design, 2011, 17(36):4007-22

Oxidative stress plays a crucial role in the development of the ageing process and age-dependent diseases. The majority of these diseases are closely connected to disturbances of proteostasis by protein oxidation and an impairment of the ubiquitin-proteasomal system (UPS). This review summarizes the function of the UPS, protein oxidation and the ageing process. It clearly shows how protein oxidation leads to an impairment of the UPS and to several connected age-dependent diseases.

Own work:

- Literature research
- Writing of the manuscript
- Preparation of figures

Protein Oxidative Modification in the Aging Organism and the Role of the Ubiquitin Proteasomal System

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Abstract: Living in an oxygen containing environment is automatically connected to oxidative stress. Beside lipids and nucleic acids, especially proteins are very susceptible for oxidative modifications. These oxidative modifications comprise alterations of single amino acids, like the formation of protein carbonyls and methionine sulfoxide, or the aggregation of whole proteins. Due to the ongoing accumulation of protein aggregates during the aging process, the cellular protein quality control system becomes more and more overwhelmed.

One essential element of the protein quality control machinery is the ubiquitin proteasomal system which plays therefore a crucial part in the aging process, too. Ubiquitination of proteins is a three step mechanism to tag proteins with a polyubiquitin chain for the proteasome. The proteasome is a regulated, barrel-shaped multi-enzyme complex which is responsible for the degradation of proteins. Although there is no drastic loss of all proteasomal subunits during the aging process, there is a functional decline of the proteasome activity in aging organisms. Impairment of the ubiquitin proteasome system leads to increasing protein aggregation and cellular death.

A lot of age related diseases are closely connected to an inhibition of the proteasome and the formation of large protein aggregates. Especially skin aging, atherosclerosis, age-dependent macula degeneration, cataract formation and several neurodegenerative diseases are directly connected to the decline of proteasome function.

This review outlines the connections between aging, oxidative stress and protein oxidation, as well as the influence on the ubiquitin proteasomal system and several associated diseases.

Keywords: Proteasome, ubiquitin, aging, protein oxidation, protein aggregation, age-dependent diseases.

INTRODUCTION

In the last decades many theories about aging and the aging process were postulated. Until today no theory is fully accepted, but in contrast to many other theories, the free radical theory of aging, first established by D. Harman in 1956 [1] and often revised afterwards, is still one of the conclusive theories describing the aging process and its associated physiological changes and diseases [2]. The presence of free radicals and a resulting oxidative stress have a multitude of modifying and destroying effects to lipids, to DNA and most importantly also to proteins, leading sooner or later to cellular senescence, cancer and other age-dependent diseases. Therefore, cells possess a large arsenal of defending and repairing mechanisms, trying to keep cells undamaged and functional. To achieve this, two ways are possible: on the one hand cells could rely on anti-oxidative redox-systems like the glutathione system or the superoxide dismutase-catalase-system to detoxify oxidants or on the other hand there are systems which are responsible to repair or degrade damaged structures after an oxidative modification had occurred. With regard to modified proteins, the ubiquitin proteasomal system is the most important cellular pathway to degrade damaged, non-functional, misfolded and mutant proteins [3].

Therefore, we give here in this review an overview, about oxidative modifications of proteins, the role of oxidative stress during aging and the ubiquitin proteasomal system as one of the major player in protein maintenance in oxidative stress and aging. The review concentrates also on the impact of oxidative modified proteins on the proteasomal system in aging and age associated diseases.

THE UBIQUITIN PROTEASOMAL SYSTEM (UPS)

Despite autophagy, UPS is the major proteolytic machinery in the cell [3]. It is a system with a number of components. The central part of UPS is the proteasome itself. The proteasome consists of a proteolytic core structure, which can be regulated by different regulatory subunits. The second part of the system is the ubiquitin machinery. As it will be explained below, the ubiquitination system contains three different groups of enzymes which are necessary for ubiquitin binding and elongation to tag proteins for degradation (Fig. **1**).

THE 20S AND 26S PROTEASOMES

The proteasome is a multicatalytical protease with a molecular mass of about 700 kDa [4], which consists of the 20S core structure (20S proteasome). Therefore, the 20S proteasome is(at least as part of the other proteasomal forms) involved in a multitude of cellular processes, as life span regulation of proteins [5; 6], protein quality control [7; 8], cell cycle regulation [9], carcinogenesis [10; 11], DNA-repair [12; 13], gene expression [14; 15], stress [16; 17] and immune response [18; 19] and degradation of oxidized proteins [20; 21].

The 20S proteasome has a barrel shaped structure constructed by four protein rings. Subunits of two of the rings are homologous, the two α -rings and the two β –rings. The two β-rings form the middle of the barrel and the two α-rings are arranged on each end of the barrel, resulting in a αββα-ring-sequence (Fig. **1**) [22; 23]. The rings form, therefore, three chambers, two antechambers, build by one α- ring and one β- ring and one main-/or core-chamber, build by the two β- rings in the middle. Every ring consists of 7 subunits: $\alpha_{1\text{-}7}$ or $\beta_{1\text{-}7}$, respectively. The outer α-rings regulate the substrate access to the inner proteolytic chamber [24]. An additional function of the α-rings is the binding of the different regulators, which modify the purpose of the proteasome. Three regulator units are known and described. The 11S regulator, which is also called PA28 [25], the ATP-dependent 19S regulator (PA700) [26] and the third activator PA200 ($PA200_i$, $PA200_{ii}$, $PA200_{iii}$), which is only located in the nucleus, participating in spermatogenesis and DNA-repair (Fig. **1**) [4; 27; 28].

The proteolytic capability of the proteasome is located in the inner core-chamber. Three of the seven β- subunits have a

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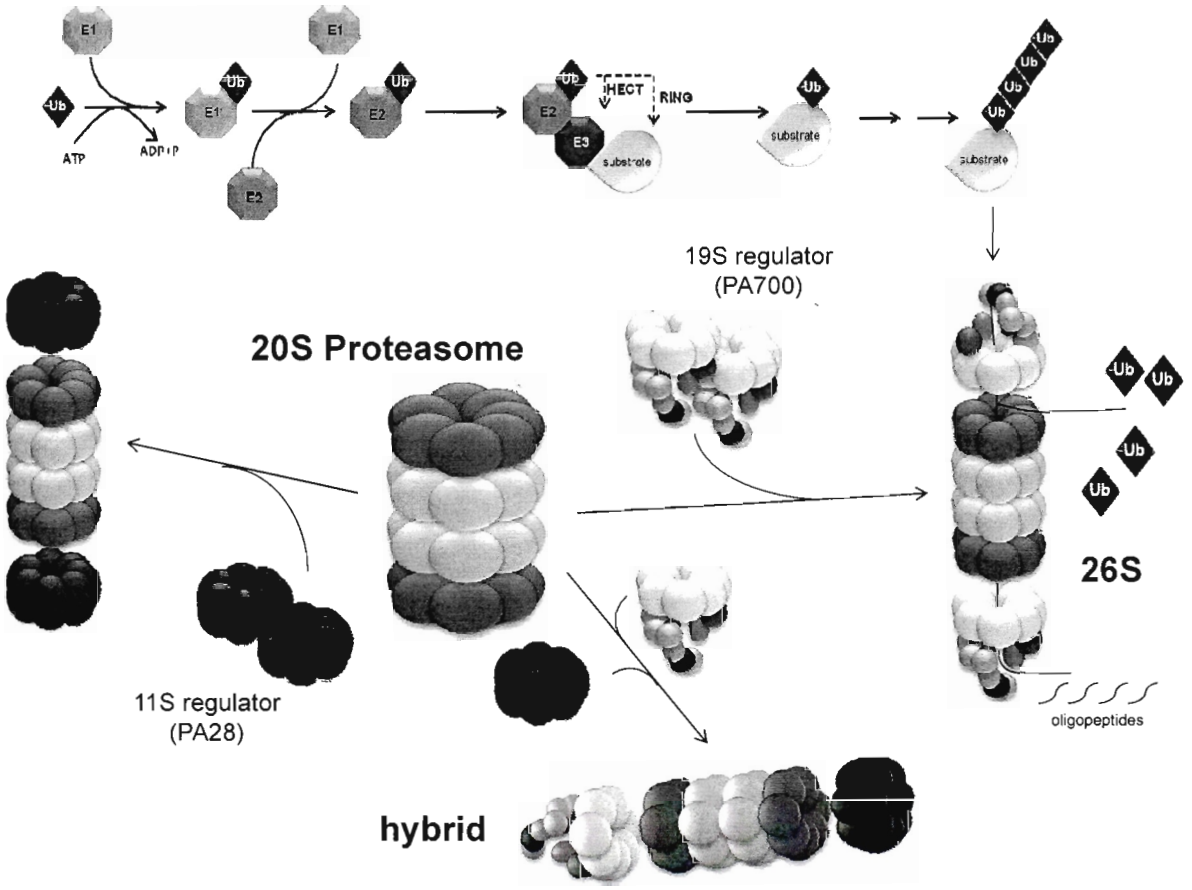

Fig. (1). The Ubiquitin-Proteasome-System

A scheme of the ubiquitination reactions and the 4 major forms of the proteasome is shown here. Ubiquitin is activated by an E1 enzyme under the consumption of ATP. In a second step Ubiquitin is transferred to an E2 enzyme which builds a complex with an E3 enzyme and the substrate. Depending on the motifs of the E3 enzyme ubiquitin is transferred directly to the substrate (*RING motif*) or first to the E3 enzyme and then subsequently to the substrate (*HECT motif*). Polyubiquitinated proteins/substrates are degraded by the 26S proteasome. Detailed description will be found in the text. The 20S proteasome is composed of two 19S regulators and the 20S proteasome. The 20S proteasome- the core of the proteasomal system- exits in a free form or might associate with 19S or 11S regulators, forming different variants of the proteasome. Note: other proteasomal regulators are described and more modifications of the 20S proteasome (subunits exchange, phosphorylation etc.) might occur. Ub -Ubiquitin; E1- Ubiquitin activating enzyme, E2-Ubiquitin conjugating enzyme; E3-Ubiquitin ligase.

threonine-like protease activity. The β_1 subunits preferentially cleave after acidic residues (peptidyl-glutamyl-peptide-hydrolase activity or caspase like activity), the β_2 subunit displays a trypsin-like activity (cleavage after basic amino acids) and β_5 acts chymotrypsin-like (cleavage after neutral amino acids) [4; 26]. According to the "bite and chew model" from Kisselev *et al.* the active β-subunits are in an allosteric relation [29]. Specifically the activation of the β_5 subunits by an artificial polypeptide (for example: SUC-LLVY-MNA) also leading to the activation of the caspase-like activity of β_1. Kisselev proposes a cyclic mechanism for protein and peptide degradation in the inner chamber: initially the peptide is cleaved by the chymotrypsin-like activity of subunit β_5, afterwards β_5 triggers further degradation by β_1, and β_5 becomes temporarily inactive. Are there no more cleavage sites for β_1, β_5 becomes reactivated and the cycle starts again [29]. Furthermore, it has been revealed that β_5 is the most important proteolytic activity of the proteasome, followed by β_2 and β_1. Mutant-cells lacking β_5 and β_2, are not able to survive [30].

Without bound regulator particles, the 20S proteasome is able to degrade proteins only in an already unfolded state, exposing hydrophobic surface structures [31]. Since proteolysis catalyzed by the proteasome comprises not alone unfolded and damaged proteins, but a rather large spectrum of short lived regulatory, onco- and structural proteins [32], the 20S proteasome is regulated by the 19S regulator/activator. Therefore, the 20S core particle is completed by one 19S regulator on each side (on each α-ring) of the 20S proteasome. In total the 20S proteasome and the two 19S regulators reaching a protein mass of about 2MDa. This mega - complex is called the 26S proteasome [4; 26]. Proteolysis by the 26S proteasome is a highly regulated and energy dependent mechanism. Proteins have to be marked by a polyubiquitin-chain to pass the 19S regulator and enter the 20S core particle. Energy dependent mechanisms are the ubiquitination reaction, which will be discussed later and the recognition of ubiquitin, cleavage of the ubiquitin chain and passing the protein to the 20S core particle by the 19S regulators (Fig. 1). Detection of the polyubiquitin chains by the 19S subunits, involving Rpn10, Rpn13, Rpn1, and Rpt5 subunits (for humans), results in a conformational reaction between the 19S regulator and the α-ring of the 20S proteasome, enhancing substrate access to the inner proteolytic chamber [33; 34]. After recognition of the ubiquitinated protein, the polyubiquitin chain is cleaved by the lid structure of the 19S regulator, involving the Rpn-11 subunit, which possesses a Zn^{2+} dependent proteolytic center that catalyzes the degradation of the polyubiquitin chain. The released monoubiquitin molecules can be reused for further protein ubiquitination [35].

In addition, ATP plays also an important role for the assembly of the 26S proteasome. Therefore it has been shown in earlier studies by Eytan *et al* [36] and Orino *et al.* [37] that the formation of the 26S proteasome is dependent on the ATP and Mg^{2+} levels in the cell. Low levels of these two metabolites result in a dissociation of the 19S regulator subunit from the 20S core unit [36; 37].

In total, the 19S regulator of the 26S proteasome is, therefore, responsible for recognition and capturing of polyubiquitinated proteins (subunits: Rpn10, Rpn 13), deubiquitination (subunit: Rpn11), unfolding of proteins and opening of the α-ring gate (subunits: Rpt1-Rpt6) [35].

Interestingly, the 26S proteasome is compared to the other subtypes of the proteasome not highly concentrated in cells [38]. In this line, Tanahashi *et al.* have shown in HeLa cells by Western blotting and immunoprecipitation, that there is a relative amount of 31% free 20S proteasome, 15% immunoproteasome (PA28-20S-PA28), 18% hybrid proteasome (PA700-20S-PA28), only 11% 26S proteasome (PA700-20S-PA700), 15% free PA28 regulator and 10% free PA700 regulator [38]. Furthermore, the concentration of the proteasome subtypes is not stable. After exposure to INF-γ a drastic increase of the immunoproteasome and the hybrid proteasome can be observed [38]. Why the 26S proteasome is the lowest fraction is not clear. It can be assumed that the relative amount of the 26S proteasome is lower than that of the other proteasome subtypes, due to its very high proteolytic activity and capability, which might compensate its low content. Localization of the 20S and the 26S proteasome seems to be almost equal. The 20S and the 26S proteasome has been found in the cytoplasm, the nucleus and associated to the ER membrane, whereas the content of 26S proteasome in the nucleus is higher than in the cytoplasm [31; 39].

UBIQUITIN AND THE ASSEMBLY OF POLYUBIQUITIN CHAINS

As already mentioned above, most proteins have to be polyubiquitinated for degradation by the proteasomal system. A chain of at least four ubiquitin molecules is required to mark a protein for proteolysis by the 26S proteasome.

The ubiquitin molecule is a highly conserved, small protein consisting of 76 amino acids arranged in a mixture of α and β structures [40]. Only 3 of the 76 amino acids differ between yeast and human, a fact, that demonstrates the strong selective, evolutionary pressure on the molecule [41]. There are several important, functional amino acids: the C-terminal glycin (G76), which is crucial for the attachment of ubiquitin to proteins and, furthermore, the different lysine residues for ubiquitin chain building. In total the protein sequence of ubiquitin possesses five lysine residues – K6, K27, K29, K48 and K63 [4]. Depending on the position of the bound lysine residue, the polyubiquitin-chain can fulfill different functions like targeting for degradation by the proteasome (K48) [4], the lysosome (K63) [42], endocytosis (K63) [43], kinase activation or DNA repair (K63) [44].

The ubiquitination reaction, that means the targeting of proteins for degradation by the 26S proteasome, is a three step mechanism that involves three different groups of enzymes (E1-E3). The first step, which is catalyzed by one of eight E1-enzymes, is the ATP-dependent activation of a ubiquitin molecule [45]. For that reason the C-terminal glycine of ubiquitin is linked to a cysteine residue of E1. The bound ubiquitin is transferred to an ubiquitin-conjugating enzyme (E2). As well as at the activation reaction the C-terminal G76 is transferred to a cysteine residue of E2 *via* a thioester bond. In a third step, E2 with the activated ubiquitin, one E3 ubiquitin ligase and the substrate protein build a complex, where ubiquitin is tagged to the substrate. During this reaction ubiquitin can be transferred *via* two principle reaction pathways. Either ubiquitin becomes directly bound with a lysine residue of the substrate or there is an intermediate step, where ubiquitin is transferred to E3 and from that E3 to the substrate. The two different reactions are catalyzed by two different types of ubiquitin ligases. The first reaction is mediated by RING-motif ubiquitin ligases and the second one by HECT-motif ubiquitin ligases [46]. However, both kinds of E3's function as allosteric activators of the E2 enzyme, resulting in the release of ubiquitin from the E2 and the final transfer of ubiquitin to the substrate (Fig. **1**) [46].

The elongation mechanism of the ubiquitin chain assembly is still under discussion. Today four different models are considered as possible mechanisms. The sequential addition model (the standard model) describes a stepwise elongation of the ubiquitin chain by one ubiquitin molecule per step. According to this model, the ubiquitin chain extension follows the same way like the initiation reaction, with the exception that the ubiquitin molecule is not bound to the substrate, but rather to an already bound ubiquitin molecule [46; 47]. However, this model becomes less reliable, if the substrate posses a long ubiquitin chain, because the distance between the end of the polyubiquitin chain and the reaction side of the E2/E3 complex seems too long. Additionally, there are a variety of different modifications or additions that should help to explain the sequential addition model. One of these additional hypotheses describes the involvement of chain elongation factors, which were described at the first time by Koegl *et al.* and were called E4s [48]. The mechanism how these E4s act is vague. In yeast the E4 enzyme Ufd2 binds to already existing ubiquitin chains and co-operate at the same time with an orchestra of ubiquitin binding factors. Furthermore, E4 enzymes have been shown to participate in the delivery of polyubiquitinated proteins to the 26S proteasome [49].

Another hypothesis, describing the long ubiquitin chain assembly is the indexation model from Noel and colleagues [50]. They demonstrated that the HECT domain of ubiquitin ligases includes three components. The N-lobe, which functions as a bottom-structure, is connected to a C-lobe. Interestingly this connection is mediated by a Hinge loop, allowing the C-lobe to rotate approximately 100° between two different conformations. Based on this observation the group of Noel has proposed that there is an ubiquitin chain assembly directly at the C-lobe of HECT-E3s. Due to the rotation ability of the C-lobe it is possible to build a tetra-ubiquitin chain without any conformational problem with the complexed E2 enzymes. The tetra-ubiquitin chain is then transferred to a bound substrate protein [50].

A third model is called the seesaw model. Here it is assumed that ubiquitin chain building is conducted by a dimer of E2 enzymes that are connected to the complex of a HECT E3 and the substrate. In contrast to the other models the seesaw mechanism places the recently added ubiquitin at the start of the chain and not at the end [46].

The last model is the hybrid model, which also includes the pre-assembly of the ubiquitin chain before it is transferred to the substrate. A non-covalent interaction between (poly) ubiquitin and a site in the E2 or E3 placed it for nucleophilic attack on the E2~ubiquitin thioester. In addition the free end of the chain must be activated by E1 and transferred to the E2 cysteine before the ubiquitin chain can be transferred to the substrate [46]. For detailed information about the different elongation hypotheses please see the review of Hochstrasser M. [46].

UBIQUITIN AND ITS FUNCTION BEYOND THE UPS

Tagging proteins for the degradation by the 26S proteasome is mediated by polyubiquitin chains that are linked *via* the lysine on position 48 of ubiquitin (K48 linkage). As mentioned before, linking the ubiquitin molecules on other lysine residues, mono- or polyubiquitination have other functions.

Therefore, the role of ubiquitination in protein degradation is not only required for the 26S proteasome, but also for the proteolysis *via* the lysosomes. Earlier it was assumed that protein degradation by autophagy is a random mechanism. Today there are evi-

dences, suggesting that autophagy is also partially regulated by ubiquitin signaling [51]. Support for this theory comes from proteins, being a part of the autophagic machinery, as, for example, the phagophore. p62 is one of the best investigated proteins of the phagophore, which possesses an ubiquitin associated (UBA) domain. p62 interacts directly with the microtubule-associated protein light chain 3 (LC3), a targeting signal for autophagic membranes and a well established marker for autophagy [52]. Ubiquitin-dependent signaling for autophagic degradation is not conducted by K48-linkage, but rather by K63- and K27-linked ubiquitin chains [51; 53].

Besides the important involvement in proteolysis, ubiquitin is also a crucial part of the DNA repair system. One example for ubiquitin and DNA maintenance includes the proliferating cell nuclear antigen (PCNA) and its role in error prone and error free DNA repair. Upon DNA damages by chemicals, oxidative stress or UV-A irradiation, PCNA becomes monoubiquitinated by Rad6 and Rad18. Monoubiquitinated PCNA activates the error prone DNA repair. In case of insufficient DNA repair, monoubiquitination of PCNA is extended to a K63-linked polyubiquitin chain, which is essential for PCNA mediated error free DNA repair [54; 55].

Like PCNA, the Faconi Anemia protein FANCD2 is a protein which is involved in DNA repair. Additionally the activity of FANCD2 is regulated by ubiquitin. Monoubiquitination on a conserved lysine residue enables FANCD2 to target chromatin associated nuclear foci, and recruiting BRCA1/2 and FANCN, which are essential for homology-directed repair (HDR) of double strand breaks [56].

For a long time it was thought that phosphorylation and poly(ADP)ribosylation were the only modifications involved in the nucleotide excision repair (NER) or double strand break (DSB) - DNA repair. But, it is becoming widely accepted that ubiquitination is crucial for all important steps of these DNA repair pathways. Due to polyubiquitination (whether K48 or K63-linked is not known) of DNA repair enzymes like DDB1/2, XPC, Polδ, Polε or several histones (H2A/B, H3 H4), ubiquitination is involved in the initiation, activation and elongation steps of NER and DSB repair [57-61].

PROTEIN MODIFICATIONS

As a consequence of oxidative stress or during the aging process, which is also accompanied by oxidative stress [1; 2; 62], proteins are, besides fatty acids [63] and the nucleic acids [64], abundant targets of oxidative modifications. These modifications affect single amino acids, peptides or whole proteins which tend to accumulate and build huge, insoluble protein aggregates.

MODIFICATION OF AMINO ACID SIDE CHAINS – SULFUR OXIDATION AND PROTEIN CARBONYLS

Basically all amino acids are vulnerable for oxidation. But due to their chemical structure and functional groups some amino acids are more susceptible for oxidative or nitrosative modifications, including the sulfur-containing amino acids methionine and cysteine, the nitrogen-containing amino acids arginine, lysine and proline, the aromatic amino acids tryptophan, phenylalanine, tyrosine, as well as histidine, threonine and glutamyl [65].

Virtually all kinds of redox-related stress are responsible for the oxidative modification of sulfhydryl groups of methionine- and cysteine [66]. Hydrogen peroxide [67], peroxynitrite [68], hypochlorus acid [69], metal catalyzed reactions [70], ozone [71] and UV irradiation [72] have been shown to convert methionine to methionine sulfoxide (MetO). In contrast to many other damaged or modified amino acids, MetO can be repaired by the thioredoxin TR(SH)2- dependent methionine sulfoxide reductases (Msr) [73]. There exist two Msr genes (MsrA and MsrB) and several splicing isoforms of MsrB. The oxidation to MetO and the following reduction of MetO to methionine by Msr is a potential antioxidant system [66]. Moskovitz *et al.* demonstrated this fact by comparing wild type mice with mutant mice lacking the MsrA gene. Mice with no MsrA expression, had reduced life span, were more vulnerable to oxidative stress and obtained higher tissue concentrations of protein carbonyls [74]. Certainly is has been observed by the groups of Friguet and Stadtman that there is a decline of MsrA activity during the aging process [74; 75], high oxidative stress [76] and UV irradiation [72].

The most familiar protein- or amino acid modification, which is connected to oxidative stress, is the formation of protein carbonyls. Therefore, protein carbonyls are a well-established biomarker for oxidative protein damage and the degree of oxidative stress [77-81]. Especially the oxidative reaction with arginine-, lysine-, proline-, threonine- and glutamic acid side chains leads to protein carbonyl formation [66; 82; 83]. But not only direct interaction of proteins with reactive oxygen species (ROS) is a reason for carbonylation, also the interaction with lipid peroxidation products, such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA) and the glycation reaction of proteins (Maillard reaction, AGE development) are responsible for protein carbonyl genesis [82; 84].

Because protein carbonyls are good markers for oxidized proteins and oxidative stress, they are also widely used as markers for age-dependent oxidative stress and protein damage. In fact, it has been shown in various publications and model organisms that there is an accumulation of protein carbonyls during the aging process or age-related diseases. Enhanced levels of protein carbonyl adducts were measured in physically exercised rats [85], aging yeast [86] and aging nematodes [87]. Furthermore, same findings could be observed for humans. Moskovitz *et al.* summarized protein carbonyl contents of human brain, human lens and human dermal fibroblasts, collected over a life span of about 80 years [82]. Beginning at the age of ten years, the content of protein carbonyls is very low. Further on, there is only a slow increase of protein carbonyls until the age of about 45. After this "mid-point", protein carbonyl content accumulates exponentially with increasing life span [82; 88-90].

MODIFICATION OF PROTEINS – ACCUMULATION AND AGGREGATION

Due to the single amino acid modification, proteins are at risk for unfolding and degeneration of their three dimensional structure, revealing the inner hydrophobic core structure of the protein. Such hydrophobic patches are very susceptible for interaction with other hydrophobic groups, triggering the aggregation of proteins (Fig. **2**) [91; 92].

There are different reasons that lead to the aggregation of proteins. One is mutations of proteins which render proteins prone to misfolding and aggregation. These inherited genomic mutations are among the other causes responsible for the familiar forms of several neurodegenerative diseases, including forms of Alzheimer Disease, Huntington Disease and Parkinson Disease [93-95]. Also mutations of several rescue and quality control proteins like the heat shock protein family (Hsp) can negatively affect protein maintenance and aggregation. A recent review from Laskowska *et al.* summarizes the influence of mutations of Hsps and small Hsps (sHsps) on protein aggregation and associated diseases [95]. sHsps are heat-inducible, molecular chaperones with a molecular mass between 12 and 40kDa, which contain a conserved α-crystallin domain on the C-terminal side. Like the ordinary Hsps, sHsps interact with unfolded and destabilized proteins, but it is suggested that sHsps do not require ATP for substrate binding [96]. In the review of Laskowska *et al.* it is described that an elevated formation of protein aggregates in case of Hsp/sHsp mutation, which includes deposited and incorporated Hsp proteins in such protein aggregates leads to cataracts, desmin related myopathy and the mentioned neurodegenerations [95]. Another reason for protein aggregation is the defect in protein biogenesis, which includes mainly translational errors. Misincorpo-

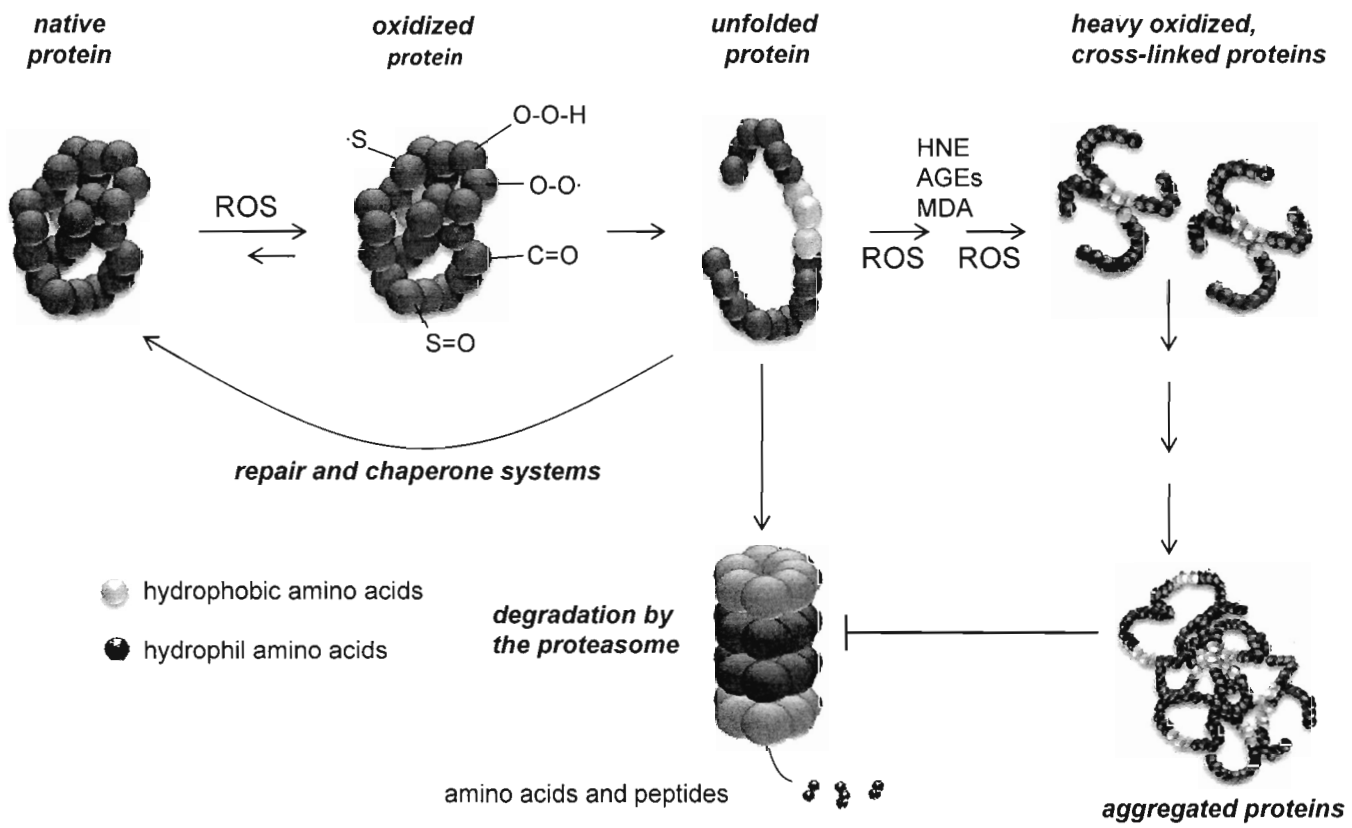

Fig. (2). Protein oxidation and the proteasomal system

Protein oxidation and the possibilities of the protein quality control systems are demonstrated here. Native proteins can be oxidized by various ROS. As a result the protein is getting partly unfolded. At this stage the unfolded protein can either be repaired and refolded into its native state or degraded by the proteasome. In case of heavy oxidative stress and/or an overload of the repair and removal systems, proteins will be further oxidized and cross-linked by other oxidation products like HNE, AGEs and MDA. Such cross-linked and heavy proteins cannot be degraded by the proteasome and tend therefore to accumulation and aggregation. In turn, these deposited and aggregated proteins can inhibit the proteasome and trigger the aggregation of further proteins, which finally promotes the aging process and a multitude of diseases.

ration of amino acids or errors in post-translational modifications like glycosylation, phosphorylation, ubiquitinylation and erroneous proteolytic cleavage are among the reasons for protein misfolding and aggregation [97; 98].

Despite these reasons, protein aggregation is mainly caused by chronic stress exposure, like extensive heat stress, UV irradiation or oxidative stress. Since protein aggregates, resulting of heat stress can be disaggregated by Hsps 104/70/40 [99; 100], it seems that UV irradiation and ROS are more important stress stimuli, resulting in insoluble protein aggregates.

Talking about oxidative triggered protein aggregation it has first to be explained, that protein oxidation is a step-wise process. First, a moderate oxidative modification occurs, like the mentioned alterations on SH- groups or carbonylation reactions [4; 101]. Depending on the location, the number of modified amino acids and the kind of oxidant such proteins show reduced activity, but might not be fully inactive. In the case of cysteine- and methionine modifications, it is possible to diminish or reverse the inflicted damage by the thioredoxin - MsrA/B – system [73].

Advanced oxidation which is associated with an unfolding process is the result of further modification. The unfolded protein structure is accompanied with a loss in protein functionality and activity [4; 101]. Depending on the unfolding status of the protein, there are different possibilities to handle these proteins. It has to be decided by the protein quality control, consisting of several members of the heat shock proteins (Hsps) and the proteolytic system, whether unfolded proteins undergo proteolysis or whether they can be rescued and refolded by Hsps. Both ways have advantages and disadvantages for the cell. According to Pacifici *et al.* proteolysis of oxidized proteins in an ATP-independent manner has three different advantages: (i) oxidized proteins are removed quickly, (ii) due to the fast degradation of oxidized proteins, the danger of aggregation and accumulation is reduced and (iii) intact amino acids can be recycled for new protein biosynthesis [102]. On the other side, after proteolysis it is necessary to re-synthetize the degraded proteins. However, new biosynthesis of proteins is a highly energy-costly process and from an energetic point of view there should be a favor for the attempt of protein repair by Hsps [103].

In the cytosol, unfolded proteins were detected by two members of the heat shock family - Hsp70 and 40. It is suggested that Hsp40 is necessary to provide the basis for the Hsp70 substrate specificity, like it was seen for bacterial Hsp40. There, Hsp40 (DnaJ) scans hydrophobic protein surfaces by recognizing aliphatic and aromatic side chains and marking them as potent substrates for Hsp70 [104]. Together, Hsp70 and 90 are responsible for protein refolding, whereas Hsp90 functions more as a stabilizer-chaperone and Hsp70 is responsible for the refolding process [105]. Both Hsps require ATP for protein maintenance. Besides Hsp70 and 90 there is an additional member of the heat shock family, which offers a more protected folding environment. There is talk of the TRIC/CCT (TCP1-ring complex or chaperonin containing TCP1) system [103; 106].

If the protein is not degraded or rescued and protein oxidation goes on, proteins become more heavily oxidized and form cross-links with other (unfolded) proteins. Cross-linking to other proteins is often mediated by the lipid oxidation products HNE, MDA, by carbohydrates and the hydrophobic surfaces of oxidized proteins after the unfolding process [107]. Especially the influence of oxidative stress results in a higher hydrophobicity of proteins, like it has been shown in several publications (Fig. **2**) [102; 108-110]. Avoiding further oxidation and protein aggregation of oxidized proteins, it is the major function of the proteasome to degrade these unfolded proteins. Due to overwhelming, chronic oxidative stress or a defect in the proteolytic system, it is possible that the amount of damaged proteins exceeds the proteolytic capacity and results, therefore, in protein aggregation.

In addition it has to be mentioned that protein aggregation mediated by ROS and UV- irradiation is not the result of one lonely event, but rather the result of chronic exposure, sometimes over years [111]. Therefore, protein aggregation caused by oxidative stress and UV- irradiation is closely connected to the aging process [112-115], whereas it is described by a vicious cycle: the phenomenon of aging seems partly to be initiated and promoted by the accumulation of oxidized, aggregated and cross linked proteins [112; 116] and the other way around it could be demonstrated in several publications that there is an augmentation of aggregated proteins with increasing life time [111-115; 117].

Lipofuscin

The yellow-brown material called lipofuscin is an extensively oxidized and cross-linked aggregate consisting of oxidized proteins (30-58%), lipids (19-25%) and metals like iron, copper, zinc, manganese and calcium (~ 2%) [118]. Two formation ways of lipofuscin are under discussion. One hypothesis connects lipofuscin formation to the mitochondrial-lysosomal axis theory of postmitotic cellular aging [119]. Oxidative production of ATP in the mitochondria is also accompanied by the inadvertent genesis of free radicals. Because mitochondria are also susceptible for membrane oxidation during aging and the ongoing ROS production, mitochondrial functionality is decreasing and the formation of aggregates is increasing. For this reason, it can be observed that impaired, oversized mitochondria are present in aged cells [120]. It is suggested that these enlarged mitochondria and other cross-linked proteins are taken up by macroautophagy. Due to the heavily cross-linked aggregates in the mitochondria, lysosomes are not capable to degrade the mitochondria completely. Moreover the enlarged mitochondria possesses a high amount of reactive iron leading to further oxidation within the lysosome [117]. Lipid peroxidation of the lysosomal membrane might result in its rupture and the release of redox active iron and cytotoxic aggregates (among other things lipofuscin) in the cytosol [118]. Free lipofuscin can then again be taken up by lysosomal compartments or accumulates in the cytosol.

Furthermore, the inhibition of the proteasome during oxidative stress and the formation of cross-linked proteins are also reasons for the generation of lipofuscin [121-123]. Decreasing proteasomal proteolysis augmented the pool of oxidized proteins, especially in aged cells. Accumulation of oxidized proteins facilitates again the inclusion of reactive metal ions [124] and the formation of cross-linked aggregates like lipofuscin. Ironically, already existing lipofuscin is also involved in the catalysis of this reaction by inhibition of the proteasome [117; 125]. Recent finding of our group, published by Höhn *et al.*, demonstrated that protein aggregates, like lipofuscin has the ability to grow in the cytosol [125]. Furthermore, 20S proteasomes bind to the hydrophobic peptide patches of lipofuscin. Due to sterical and mechanical reasons of the cross-linked material the inner core structure of the proteasome is unable to degrade the lipofuscin aggregate. Lipofuscin-bound 20S proteasomes remain functionless and lacking for further degradation of oxidized proteins[125]. Höhn *et al.* could also show, that a pre-treatment with proteinase K, degrades the hydrophobic surface residues, preventing (i) lipofuscin-mediated proteasome binding, (ii) depletion of protein turnover and (iii) accumulation of protein aggregates [125].

Lipofuscin is also known as ceroid, ceroid-like pigment or age pigment. The latter expression let suggest that the aging process is accompanied with the formation of lipofuscin. Thus, lipofuscin increases during the life time of the cell and is negatively correlated with the life expectancy of postmitotic cells [126; 127] which means: the higher the rate of intracellular lipofuscin formation, the shorter is the lifetime of the cell [113]. This fact indicates the high cytotoxic potential of lipofuscin. Lipofuscin is not an inert material, with its actively growing surface and the ability to bind metals it possesses an aggressive reactivity. Already in 1997, Elleder *et al.* showed in several tissues (e.g. brain or heart muscles) that a large part of the protein content of some lipofuscin are leftovers of the subunit c of the mitochondrial ATP synthase [128], resulting in a potential redox-active surface which keeps protein oxidation going on. As it was mentioned, lipofuscin is able to prevent proteasome mediated proteolysis, a fact that is clearly correlated with the aging process [129-131]. An additional point, which has to be considered, is the ongoing extension of lipofuscin material and the associated filling of cytoplasmatic volume over time, reducing the cellular functional space and leading finally to apoptosis [115; 132; 133].

Notably, lipofuscin occurrence is a phenomenon of the cytoplasm of aging cells, whereas the nucleus seems not to be affected [118]. This might be an explanation for the relative resistance of the nuclear proteasome to an age-dependent decline [134].

Glycation, advanced glycated end products (AGEs) and protein aggregation

Non-enzymatic protein glycation in cells is a multi-step reaction between carbonyl containing carbohydrates and amino groups. In a first step reducing sugars and other ketones/aldehydes react with amino groups of proteins to form Schiff bases and Amadori products. Finally, this reaction leads to irreversible modifications called advanced glycated end products. Especially lysine and arginine, N-terminal amino acids and the thiol groups of cysteine are vulnerable for protein glycation [135]. Presence of transitional metals catalyses the glycation reaction, whereas reducing compounds like ascorbate inhibit the formation of AGEs. During the Amadori rearrangement, several reactive α-dicarbonyl-intermediates arise, such as 3-deoxyglucosone and methylglyoxal. The latter is a most reactive component, which is suspected to elevate oxidative stress in the aging process [136]. Glycation continues over a period of several weeks, months or even years. Therefore, AGE occurrence affect mostly long living proteins, as structural components of the connective tissue matrix (collagen, myelin) or the complement C3, tubulin, plasminogen activator and fibrinogen [137; 138]. The most common reason for AGE formation is (age-dependent) diabetes or the metabolic syndrome in combination with oxidative stress [139; 140], but also food or tobacco smoking can be an exogenous source for AGEs [135]. Interestingly, not only the protein half-life is important for the protein modification by AGEs, but also certain structural features of the protein itself [141].

Due to their structure and chemical surface, AGEs are often involved in the formation of cross-linked and aggregated proteins. As it was already mentioned, long living proteins such as collagen are the main targets for these modifications. It is suggested, that this not fully understood reaction involves binding of lysine residues of the target proteins [142; 143]. Protein cross-linking, mediated by AGEs, results in decreased protein mobility and flexibility. AGE-containing protein aggregates 'harden' the protein structure and lead to an increased stiffness of the protein matrix. This affects not only the functionality of the proteins [144], but makes it also more difficult to remove these proteins *via* the proteolytic system and exchange it by new synthesized proteins. This restricted or failed

tissue remodeling is always a side effect of the aging process and mostly promoted by diabetes. AGE-dependent tissue stiffness is also connected to different sclerotic diseases such as sclerosis of the renal glomeruli and atherosclerosis [142; 145].

Cellular damage by AGEs is not only mediated by the formation of toxic protein aggregates. AGEs can also bind to receptors for AGEs (e.g. RAGE), which are formally expressed on macrophage surfaces. Binding to these scavenger receptors has a multitude of functions, exceeding the topic of this review. Generally, the activation of RAGE by AGEs leads to increased oxidative stress and an elevated expression of inflammatory signaling compounds like NF-κB [146].

PROTEIN AGGREGATES IN POSTMITOTIC TISSUES

Especially postmitotic cells are affected by accumulated protein aggregates. Because postmitotic cells have lost their ability to divide, such as neurons, cardiac myocytes or senescent fibroblasts, there is no possibility for dilution of accumulated oxidized proteins during cell division [116]. Therefore, it could be observed, that many age-related diseases occur in postmitotic tissues or tissues with a reduced cell turnover, as the brain [147], the heart [148], the lens [149] or the muscles [150]. Interestingly, non-postmitotic cells have lower rates of aggregated proteins. There are two reasons for this phenomenon: First, non-postmitotic cells have the ability to dilute aggregated proteins of a given cell by cell division. This means the defined amount of accumulated and aggregated proteins of one cell (x) becomes separated into x/2 by cell division into two cells. The second reason is that it has been observed in several organisms that a majority of aggregated proteins remains in the older "mother" cell after cell division, which prevents the new "daughter" cell of toxic effects by already existing protein aggregates [151-153]. Rujano *et al.* demonstrated, investigating the crypts of the small intestine from patients with spinocerebellar ataxia type 3, a directed transport of aggregated proteins, which were incorporated in aggresomes, to the short-lived differentiated cells and sparing the long-lived intestinal stem cells [153]. Additionally, the same group confirms these results on a *Drosophila melanogaster* model [153].

ROLE OF THE 20S PROTEASOME AND THE UPS IN OXIDATIVE STRESS

Oxidized proteins (depending on the degree of oxidation) are a good substrate for the proteasome. Additionally, it seems accepted that the bulk of oxidized proteins (70-80%) are degraded by the proteasome [31]. Nevertheless, there is still an ongoing debate whether oxidized proteins are preferentially degraded by the 20S proteasome in an ubiquitin- and ATP-independent manner or by the 26S proteasome, involving ATP-dependent ubiquitination of the oxidized proteins. Whereas several groups have convincing evidence for the degradation of oxidized proteins in an ubiquitin independent manner by the 20S proteasome [5; 31; 154-159], there are some limited experiments showing the participation of ubiquitin and the 26S proteasome in proteolysis of oxidized proteins [160-163]. However, ubiquitination and the following degradation by the 26S proteasome play a crucial part in stress signaling, e.g. as a control of p53, the MAP kinase pathway and induction of NF-κB. The array of these effects, maintaining cell cycle and cell functionality, may explain the augmentation of ubiquitination during (oxidative) stress [16; 164].

Furthermore, it has been shown that degradation of many oxidized model proteins, such as superoxide dismutase (SOD) or hemoglobin, is ubiquitin independent and conducted by the 20S proteasome [110]. Moreover there is some additional evidence for the role of the 20S proteasome in degradation of oxidized proteins. Some oxidative modifications on proteins prevent their ubiquitination. Thus, modifications on lysine residues to α-aminoadipic semialdehydes impair protein ubiquitination [84]. Also the group of Davies could show in the 1990s that recognition and degradation of oxidized proteins by the 20S proteasome is due to their unfolded and hydrophobic structure [102; 108]. In addition, Davies and colleges observed a reduction in proteasomal degradation of oxidized proteins of about 10-20% in cell-free lysates, after the addition of ATP [155; 156].

Another interesting point is the behavior of the two different kinds of proteasomes in the presence of oxidative components. The 26S proteasome is much more vulnerable for oxidative damages in comparison to the 20S proteasome. Reinheckel *et al.* have clearly demonstrated in K562 human hematopoietic cells that there is a loss of function of the 26S proteasome after exposure to increasing concentrations of hydrogen peroxide, whereas the functionality of the 20S proteasome remains unaffected [165]. Similar findings have also been made in plants [166]. Enhancement of oxidative stress leads to direct inhibition of the 26S proteasome. The loss of the 26S proteasome function is accompanied with an augmented biogenesis of the 20S proteasome in Arabidopsis. Due to this adaption step the plant cell increases the capacity to degrade oxidized proteins and makes it more resistant against oxidative stress [166].

THE PROTEASOMAL SYSTEM IN THE AGING PROCESS

In a previous chapter it has shortly be mentioned that the aging process is accompanied with a decline in the proteasomal function. Today the age-dependent decrease in proteasome function is an undoubted fact, which has been proven for many model systems. The majority of these experiments show a general decline in proteasome function, including the retina [167], brain [168], liver [169], lens [170], heart [171], kidney [169], T-lymphocytes [172] and epidermal cells [81]. The functional decline of the proteasomal activity in old cells various between the different tissues. Hayashi *et al.* observed in a comparison between young and old livers from F344 rats a 30% reduction of the chymotrypsin –like (CTL) activity of the 26S proteasome and a reduction of caspase like activity of both types of proteasome – 26S and 20S. Although the protein content of the proteasome remains unaffected and was similar to the amounts of young rats [173]. In contrast, Bulteau *et al.* demonstrated that the functional decline of the proteasome is accompanied or mediated by a simultaneous reduction of the 20S proteasome content in the heart of F344 rats [171]. However, in many cases the proteasomal decline is at least mediated by a decrease of the CTL activity [170; 173; 174]. Examining the unfunctional proteasomes in older cells revealed some post-translational modifications that may play a role in the declined proteasomal activity. Several groups suggested oxidative cysteine modifications of the β1 and β5 subunits that reduce the caspase-like and CTL activity [174-176]. Therefore, Kapphahn *et al.* treated retina cells with N-ethylmaleimide (NEM), a compound, which covalently modifies the sulfhydryl group of cysteine, as a way to mimic the aging process in retina cells. As a result the CTL-activity decreases to about 65% and the caspase-like activity to about 80% [174]. These reductions of proteasome activity were comparable to determined proteasome activity of old retina cells, indicating NEM as a suitable drug to mimic the aging process in retina cells. Further investigation of this group revealed the involvement of HNE in cysteine modification and aged dependent proteasome inhibition, respectively the CTL-activity inhibition [177]. In fact, also other groups reported that HNE is one of the compounds that is responsible for proteasome inhibition during aging [121; 171; 172].

Another reason which could play a role in age-dependent decline of the proteasomal function is the ongoing impairment of mitochondrial function during the aging process. This may affect the intracellular energy homeostasis and results in a decreased ATP level [178], which in turn affect the ATP dependent proteolytic machinery, including the ubiquitination reaction, Hsp interaction with the proteasome and the degradation of ubiquitinated proteins by the 26S proteasome.

Table 1. Summary of Proteasomal Decline Mediated Diseases

Disease	Protein Modification	Cellular Consequences	Phenotypical Symptoms
Age Related Diseases			
Skin aging	- HNE cross linked proteins [185] - Lipofuscin [188]	- Functional decline of mitochondria [187] - Inhibition of the proteasome [185] - Enhanced formation of lipofuscin [188]	- Wrinkle formation [189] - skin laxity [189] - dispigmentation [189] - rhytids [189] - erythema [189] - cancer [189]
Diseases of the aging eye Cataracts AMD	- Oxidation, glycation and aggregation of α, β and γ crystallins [191] - Mutated, non functional ubiquitin molecules [193]	- Inhibition of the proteasome [200] - Disruption of ubiquitination [193] - Neovascularization (exudative AMD) [196] - Accumulation of proteins/"drusen" [196; 198]	- lens opacification [191] - loss of acuity [191] - partial blindness
Atherosclerosis	- All kinds of protein modifications: oxidation, glycation (AGEs), HNE-cross linking [203-205] - Highly ubiquitinated aggregates [208]	- proteasome inhibition [209] - formation of foam cells [206; 207] - thickening of vascular walls [206; 207]	- Stroke - Death
Neurodegenerative diseases			
Alzheimer Disease	-Accumulation and deposition of fibrillar amyloid Aβ [214] - Hyper-phosphorylation of tau proteins [220] - Accumulation of helical tau filaments → Formation of neuritic plaques and neurofibrillary tangels [220]	- Dysregulation and inhibition of the UPS [215] - Apoptosis of neurons [223]	- confusion [224] - irritability [224] - hallucinations [224] - aggression [224] - language breakdown [224] - loss of long – term memory [224] - mood swings [224] - death [224]
Huntington Disease	- Mutation of the Huntingtin protein (polyglutamine strand) [227] -Accumulation of mutated Huntingtin protein/Huntingtin filaments→ Formation of Inclusion Bodies [227]	- Inhibition of the UPS [229] - Disruption of other quality control systems [227]	- Impaired muscle coordination [230] - Cognitive decline [230] - Dementia [230]
Parkinson Disease	- Mutation and aggregation of: - α-synuclein[232] - Parkin [233] - UCHL-1 [234]	- Functional decline of the UPS and the protein quality control system [233] - Apoptosis of dopaminergic neurons [235] - Presence of cytoplasmatic Lewy Bodies [231]	- Tremor [232] - Postural instability [232] - Slowness of movement [232] - Muscular rigidity [232]

Focusing on the ubiquitin system and its alterations during the aging process, there is only little and contradictory information available. On the one hand, there are some hints which indicate a down-regulation of some ubiquitin related enzymes, including the ubiquitin-ligase Nedd4 and the deubiquitinating enzyme Usp4 [179] in aging mice. On the other hand, contradicting the down regulation of ubiquitin related enzymes there are some observations which prove the accumulation of ubiquitinated proteins in older mice. This fact is promoted by two theses. One of it is based on the advanced breakdown of the 26S proteasome in older cells, which can lead to a depletion of the ubiquitin-dependent protein degradation [180]. Furthermore, the group of Taylor found also an augmentation of ubiquitinated proteins - partly aggregated- and ubiquitin mRNA in old Emory mice. In addition, these augmentation was accompanied by the activation of E1 and E2 enzymes [181; 182].

THE PROTEASOME IN AGE-DEPENDENT DISEASES

The age-induced reduction of proteolytic capacity of the proteasomal system is inevitably connected to an ongoing impairment of cell functionality and cell homeostasis. Depending on the degree of proteasome activity loss, this slight impairment could lead to sev-

eral typical known age related diseases, which will be considered more closely in the next chapter.

SKIN AGING

The skin is the largest tissue of the human body. Because of its more or less permanent exposure to daylight, skin aging is mainly mediated by UVA and UVB exposure. Taken together UVA and B irradiation have a multitude of effects in the skin – positive and negative. A positive effect of UV irradiation is the induction and activation of melanin synthesis, which could protect the epidermis from DNA damage [183]. On the other side, UV exposure has some negative effects. Despite the activation of matrix metalloproteinases, which is associated with collagen degradation and wrinkle formation, UV irradiation is responsible for DNA damages (thymine dimers and 8-OHdG) [184], oxidative stress and thereby emerged modifications of proteins and lipids [185]. Especially the formation of HNE is associated with UV induced reduction of proteasomal proteolysis. The group of Friguet could clearly show that HNE formation is increased after UV exposure and in old skin. Due to the poor property of HNE-modified proteins as substrates for the proteasome and their ability to inhibit the proteasome, the proteasome activity was significantly decreased in both cases [81; 185].

A recent work of Koziel *et al.* in human dermal fibroblast at different stages of ages (young, middle-aged and old) revealed another interesting point of proteasome inhibition during skin aging. Koziel *et al.* also showed an overall decline of the proteasome activity in middle old and old fibroblasts. Interestingly, inhibition of the proteasome affected the functionality and productivity of the mitochondria. In turn pharmacological inhibition of the mitochondrial function reduced the proteasomal activity, suggesting an interdependent co-regulation between this two systems [186].

In summary, declined proteasomal activity and declining energy levels due to impaired mitochondria trigger cell senescence [187] and the formation of lipofuscin [188] which in turn promotes again the inhibition of the proteasome inducing cell senescence. Phenotypical consequences for the skin of these processes are skin laxity, dispigmentation, rhytids, erythema / telangiectasia, solar elastosis keratoses, poor texture and thinner skin [189].

THE AGING EYE

Discussing the aging eye is primary the discussion of occurrence and formation of age- related macular degeneration (AMD) and cataract during aging. Cataracts are an age-related disease of the lens with a not unimportant hereditary factor [190]. From the beginning of eye development, the lens continues to produce new secondary fibers throughout the whole life time. The fiber cells containing the high soluble crystallins, which compose the majority of the lens proteins (90%) [191]. The 16 mammalian crystallins, which are categorized in three families (α-, β- and γ-crystallins), undergo extensive post-translational modification to maintaining the transparency of the lens. Disturbance, such as mutations of the crystallins leads to improper protein association, aggregation and insolubilization [191]. Aggregated proteins diffract the incoming light, resulting in lens opacification and loss of visual acuity, two main symptoms of cataract formation. In an impressive manner, Talla *et al.* visualized the intracellular aggregation of mutant cataract associated γ-crystallins. These γ-crystallins lacking their C-terminal 18 residues posses a decreased solubility, which is mostly based on an altered tertiary structure and a high hydrophobicity – a feature which is also achieved by oxidative stress or glycating compounds. Due to this higher hydrophobicity, γ-crystallins tend to denaturate and form aggregates, which was visualized in the paper of Talla *et al.* by an EGFP tag to the γ-crystallins in lens cells [192].

During the aging process the content of soluble proteins, first of all the crystallins, declines to about 20-25% at an age of 60 years [170]. It has been shown that the decline of soluble proteins is associated with elevated generation of insoluble, aggregated proteins, containing AGEs and polyubiquitin chains. In the same study, conducted by Viteri *et al.*, an overall decline of the proteasomal activity could be observed [170]. Due to the involvement of AGE-modified proteins or aggregates in the formation of cataracts it can be suggested that diabetes is a highly promoting factor of cataract diseases.

But not only mutations or modifications of crystallins are related to cataract formation. It has also been revealed, that there are ubiquitin modifications which impair the protein turnover by the UPS [193]. Thus, Stiuso *et al.* examined ubiquitinated crystallins from human cataractours lenses and found two different forms of ubiquitin. Apart from the normal and functional ubiquitin molecule from healthy lens cells, a second non-functional ubiquitin molecule was found which is not actively participating in the turnover. The non-functional ubiquitin lacking the C-terminal four amino acids 73-76, including the C-terminal glycine, which is essential for ubiquitin binding to proteins. It is suggested that Ca-dependent calpains in cataractours cells are responsible for the ubiquitin shortening, which in turn leads to an impairment of UPS and consequently to an enhanced accumulation of aggregates in the promotion of cataracts [193].

Besides the formation of cataracts, AMD is considered to be the most common age-related eye disease worldwide [194]. According to Fletcher around 2% of the population at the age of 40-49 suffering on lens opacities, 15% at the age 60-64 and 50% of those aged over 75 [195]. AMD is a chronic and progressive disease, affecting the vision by a loss of retinal cells. In most cases AMD does not end in total blindness, but prevents a sharp vision in the center of the field of view, which could make it nearly impossible to read or recognize faces. Often the peripheral vision is not affected equally, allowing a restricted participation in life.

There are two distinct forms of AMD, the "wet" form (exudative form) and the "dry" form (non- exudative form). The exudative form is clearly characterized / mediated by an abnormal growth of blood vessels (neovascularization) above or below the Bruchs's membrane, leading to a protein and blood leakage, which could cause irreversible damages to the photoreceptors [196]. The non-exudative form is accompanied by the formation and deposition of special protein aggregates between the retinal pigment and Bruch's membrane, without neovascularization [197]. These aggregates are called drusen, resulting of the accumulation of debris and waste products. Again, the accumulation and generation of these aggregates is also facilitated by the presence of oxidative stress, declining antioxidant levels and the formation of lipofuscin-like material [198]. But, particular in the eye the influence of light, especially the consequences of blue light, is a most important factor, leading to lipofuscin formation and thus to AMD [199]. Additionally, it is also suggested that the progression of AMD, including the formation of aggregates and the enhancement of oxidative proteins is connected to the age-related decrease of proteasome activity [200].

Contrarily, due to the inflammatory response against the deposited drusen aggregates during the progression of AMD an induction of the inducible β-subunits of the proteasome could be observed [201]. At stages of highly progressed AMD, Ethen *et al.* showed that there was a shift from ordinary β1 and β5-subunits to β_i1 and β_i5 subunits [201].

ATHEROSCLEROSIS

According to WHO, coronary heart disease/ atherosclerosis (AS) is the major risk for death. In high and middle income countries it is responsible for 14-16% of death. Even in low-income countries coronary heart disease take the second place of the 10 leading causes of death [202].

It seems totally accepted that AS is closely connected to the occurrence of oxidative stress [203-205]. Therefore, oxidative

stress, AGEs and metal ions are involved in the initiation and upholding the progression of atherosclerosis. By oxidation of normal LDL to ox-LDL, that migrates across the thin layer of endothelia into the artery wall, oxidative stress is implicated in the first hypothesized events of atherosclerosis [203-205]. Ox-LDL provokes the migration of leuko- and monocytes in the subendothelial space which results in phagocytosis and the massive production of ROS and pro-inflammatory cytokines [205]. Further oxidation of LDL and the ongoing migration of monocytes into the subendothelial space results first in the formation of foam cells and later, with the participation of smooth muscle cells, in the generation of fibrous plaques [206; 207]. Due to the high amounts of ROS and the enhanced inflammation status an increase of polyubiquitinated, unfolded and oxidized proteins could be observed with the progression of AS [208]. At early stages of the disease, there is an initial increase of the proteasomal activity, most likely to compensate the increasing amount of ubiquitinated and damaged proteins. However, with the ongoing development of the disease the proteasomal activity decreases progressively, inhibited by the growing bulk of aggregated and accumulated proteins in the artery walls [209]. As shown by Herrmann *et al.* in experimental hypercholesterolemia, there was an accumulation and debris of ubiquitinated and oxidized proteins in the vascular wall, especially when the compensatory increase of the proteasomal activity was suppressed [210]. Additionally, they observed a typical thickening of the intima. In particular, further reactions with the deposited proteins, such as glycation (particularly prominent in diabetes) or reactions with HNE, increasing their capability to impair or inhibit the proteasome [209].

Interestingly, in advanced AS can also be found deposits, exhibiting substantial quantities of amyloid fibrils (amyloid Aβ), which were also one of the main causes for Alzheimer disease [211]. Amyloid is a cleavage product of the amyloid precursor protein (APP). It is suggested that fibrillar amyloid- like proteins stimulate CD36-mediated signaling in atherosclerotic plaques and the resulting production of ROS and inflammational cytokines [212]. Thus, it seems obvious that amyloid Aβ is not only responsible for the pathophysiology of Alzheimer disease, but plays rather an important role in the endothelial injury and dysfunction in the progression of AS [213].

THE UPS IN NEURODEGENERATIVE DISEASES

Alzheimer disease (AD)

As it was mentioned previously, one reason for Alzheimer disease is the deposition and accumulation of fibrillar amyloid Aβ, a cleavage fragment of APP by the involvement of certain secretase enzymes [214]. These depositions result in the building of extracellular plaques. There is an ongoing controversy about the effects of Aβ aggregates to the proteasome. Gregori *et al.* and Tseng *et al.* clearly demonstrated that Aβ inhibits the proteolytic activity of the proteasome [215; 216]. On the contrary there are findings which define the $A\beta_{1\text{-}42}$ peptide as a good substrate for the 20S proteasome without an impairment of the proteasome activity [217; 218]. Maybe further oxidative modifications and cross linking reactions of the Aβ peptide are responsible for its ability to inhibit the proteasome. Shringarpure *et al.* revealed that Aβ- mediated proteasome inhibition only takes place if Aβ peptides possess additional modifications such as cross-links with HNE [219].

However, the aggregation and accumulation of Aβ is not the only factor for the progression of AD. There is another protein, which is closely associated with AD. The intracellular tau protein, is a component of the cytoskeleton (microtubuli) and can be found in every animal cell. In AD a hyperphosphorylated form of the tau protein accumulates and forms a paired helical tau filament, being the main component of the AD typical neuritic plaques and neurofibrillary tangels (NFTs) [220]. Immunostaining of neuritic plaques/NFTs reveals a high immunoreactivity for anti-ubiquitin antibodies, indicating a decreased proteolytic activity of the 26S proteasome in AD. Therefore, it appears that the dysregulation of the UPS is both a cause and a result for AD or in general for the neurodegenerative disease process. Indeed it has been shown, that the proteasome is significantly inhibited by hyperphosphorylated, proteolysis-resistant tau proteins in AD [221; 222]. Because non-modified tau proteins are normally good substrates for the proteasome, it seems convincing that the initial Aβ-mediated proteasome inhibition is responsible for the further formation of non-degraded, polyubiquitinated NFTs [215]. In progressed forms of AD the growing mass of neuritic plaques and NFTs progressively impair UPS which in turn facilitates the accelerated enrichment of further plaques and NFTs. Reaching a critical concentration of the deposits, neurons undergo apoptosis, leading to the typical AD symptoms [223] like confusion, irritability, hallucinations, aggression, language breakdown, long-term memory loss, mood swings and finally death [224].

In addition it has to be noticed here, that autophagy plays an important role in the development of AD. NFTs become not only K48-polyubiquitinated, they are also K63- ubiquitinated, targeting them for lysosomal degeneration. It could be shown that, K63-ubiquitination of NFTs is promoted and detected by p62. Knock-out of p62 leading to tau protein hyperphosphorylation, NFTs and AD typical symptoms in animal trials [225]. Thus, p62 binds to K63-linked polyubiquitinated tau to avoid its aggregation and deposition and deliver it to the lysosome [226].

Huntington Disease (HD)

Severe mutations of the Huntington gene and the Huntingtin proteins are responsible for the Huntington disease. Mutation of the Huntingtin protein includes an extention of a polyglutamine (polyQ) strand at the N-terminus of the protein [227]. While normal Huntingtin proteins possess a polyQ sequence of 9-36 glutamine residues, in Huntington disease the polyQ strand exceeds more than 40 glutamine residues. Today, the function of the Huntingtin protein is not absolutely clear. The fact that p53 binds a p53-responsive element in the Huntington gene and activates its transcription, indicates a role in the p53-mediated stress response and an anti-apoptotic function [228]. Due to the extended polyQ sequence, mutated Huntingtin proteins tend to build aggregates and accumulate in cytoplasmic inclusion bodies (IBs). Like in other protein aggregate-associated neurodegenerative diseases, a high rate of polyubiquitination of the aggregates can be observed. Moreover IBs contain proteasomal subunits, chaperons and autophagy proteins, indicating an IB-mediated inhibition of the UPS and may of other protein quality control systems [227]. However, it has been shown by Diaz-Hernandez *et al.* that the functional decline of the 26S proteasome is not mediated by large IBs, but rather through smaller Huntingtin filaments [229]. Because the 20S proteasome remains unaffected, it is supposed that the inhibition of the 26S proteasome is due to its ability to interact with the polyubiquitin moieties of the isolated filaments [229]. The loss of the proteolytic function of the proteasome promotes further protein aggregation (Huntingtin proteins and other proteins) and is responsible for the toxicity of HD, leading to impaired muscle coordination, cognitive decline and dementia [230].

Parkinson disease (PD)

Like AD and HD, PD is also closely connected to protein aggregation and a functional decline of the ubiquitin and proteasomal system. PD is characterized by a dramatic loss of dopaminergic neurons within the Substantia nigra pars compacta combined with the presence of intraneuronal, cytoplasmatic inclusions called Lewy bodies [231]. The dying of dopaminergic neurons is responsible of the typical symptoms of PD such as uncontrollable tremor, slowness of movement, muscular rigidity and postural instability [232].

There are three major proteins, which are involved in the formation of polyubiquitinated aggregates in PD: i) α-synuclein [232],ii) parkin [233] and iii) the ubiquitin-carboxy-terminal hydolase L1 (UCHL1) [234; 235]. Parkin and UCH-L1 are two important components of the ubiquitination pathway. UCH-L1 acts as a deubiquitinating enzyme in neurons and Parkin as an E3 ubiquitin ligase, interacting with the 19S regulator of the proteasome. UCH-L1 is an abundant protein in neurons and functions also as an E3 ubiquitin ligase when dimerized. Thus it is not surprising, that mice lacking UCH-L1 develop neurodegeneration and Lewy bodies found in most forms of Parkinson disease contain sequestered UCH-L1 [234; 235]. Mutations in Parkin are the most common reasons for familial PD. In general the mutations disrupt the ubiquitin ligase activity of Parkin and de-stabilize its ubiquitin-like domain [233]. Besides the ubiquitination of p38, α-tubulin, CDCrel-1/Sept5, glycosylated α-syn, Pael-R, Parkin ubiquitinates itself in the early step of the proteasome-mediated degradation process. Based on this it seems clear, that mutation and impairment of Parkin leads to considerable disruptions of the protein quality control mechanism in neurons and promotes the aggregation and functional loss of many proteins, including parkin itself [233].

Although PD is primarily a hereditary and genetic disease, the age-dependent loss of proteasomal functions is a considerable cause of PD, too. Stress stimuli like ROS or proteasomal inhibition has been shown to promote the accumulation of sequestered α-synuclein, Parkin and α-tubulin in insoluble fractions of neurons [233; 236]. In turn, the aggregated α-synuclein triggers oxidative stress and stimulates further aggregation of α-synuclein and Parkin - a process which is clearly promoted by the physiology of aging [233; 236].

Taken together, the dying of dopaminergic neurons in PD is mediated by a disruption of the ubiquitination system in neurons, the functional decline of some proteins and most importantly the aggregation and accumulation of a bulk of proteins in Lewy bodies and the accompanied generation of further oxidative stress.

THERAPIES IN AGE-DEPENDENT DISEASES AIMING THE UBIQUITIN-PROTEASOMAL SYSTEM

The proteasome was a primary target in anti-cancer therapies. Inhibition of the proteasome, for example by bortezomib, to achieve apoptosis is a promising treatment against cancer cells [237].

Focusing on age-related protein storage diseases it might be necessary to achieve a contrary effect by elevation of UPS activity. Among other things the idea for this strategy comes from investigations of centenarians. Proteasomal content and activity in cells of healthy centenarians were comparable with cells of much younger subjects [238]. To obtain an increase of proteasome content or activity several theoretical approaches are possible. One option is to enhance the three endogenous proteasome activators/regulators PA700 (19S), PA 200 and PA28 (11S). An up-regulation of the 11S regulator-containing immunoproteasome was observed in injured brains, as well as in AD and HD brains, suggesting that the immunoproteasome, respectively the 11S regulator, plays a role in neuronal protection [239]. Another effective approach would be to augment the 19S regulator, increasing the activity of the 26S proteasome, which degrades ubiquitinated proteins. However, so far an elevation of the expression levels of proteasomal regulators could only be achieved by genetic manipulation. At the moment there are no known compounds, increasing the content of proteasomal regulators.

Also an aim for genetic manipulations could be the proteolytic active ß-subunits of the 20S proteasome. This genetic up-regulation of the ß-subunits resulted in elevated resistance against oxidant-dependent cell death and a delay in senescence in human fibroblasts (WI38) and human leukemia cells (HL60) [158]. Further theoretical targets for genetic therapies could be different parts of the ubiquitination machinery, such as the carboxyl terminus of Hsp70-interacting protein (CHIP) [240] or E3 ubiquitin ligases like parkin [241]. Especially patients with loss-of-function mutations, such as in PD, would have a great benefit of gene therapies. Preliminary studies with monkeys, using an adeno-associated virus vector to deliver α- synuclein or α-synuclein+parkin into the striatum, resulted in an overexpression of parkin and a decreased accumulation of α-synuclein [241].

Although gene therapy is a promising instrument to cure age-dependent diseases, it is not established for humans yet.

Therefore, therapies targeting proteasome-dependent diseases have to rely on chemical compounds which have the ability to enhance proteasomal activity. Besides the detergent SDS and some synthetic compounds like peptidyl alcohols, esters, p-nitroanilidades and nitriles [239] the only known effective proteasome activity enhancer is an algae extract prepared from Phaeodactylum tricornutum [242]. This algae extract increased the proteasome peptidase activities of human keratinocytes and protected them from UVA/B induced protein damage [242].

CONCLUSION

There is growing evidence that a connection between oxidative protein modification, the age-dependent decline of the proteasomal function and the aging process exists. Accumulation and aggregation of proteins over decades leads to an impairment of the proteasomal system and cellular function, amplifying further protein modifications and accelerates the aging process. Especially postmitotic tissues are affected by long-time exposure to ROS and modified proteins, resulting in the typical development of age-dependent diseases like diverse neurodegenerative diseases. Thus, the free radical theory of aging and its variations are still the best approaches to give an explanation for all of the here discussed facts.

ABBREVIATIONS

AD	=	Alzheimer Disease
AMD	=	Age related macula degeneration
AS	=	Atherosclerosis
CDCrel-1	=	Cell division control-related protein-1
CHIP	=	Carboxyl terminus of Hsp70-interacting protein
CTL	=	Chymotrypsin-like
DSB	=	Double strand break
HNE	=	4-Hydroxy-nonenal
Hsp	=	Heat shock protein
LDL	=	Low density lipoprotein
MDA	=	Malondialdehyde
NER	=	Nucleotide excision repair
NFT	=	Neurofibrillary tangels
PD	=	Parkinson Disease
ROS	=	Reactive oxygen species
Sept5	=	Septin
sHsp	=	Small heat shock protein
SOD	=	Superoxide dismutase
UCHL-1	=	Ubiquitin-carboxy-terminal hydolase L1
WHO	=	World Health Organization

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3.2 Publication II: Proteins bearing oxidation-induced carbonyl groups are not preferentially ubiquitinated

Marc Kästle, Tilman Grune

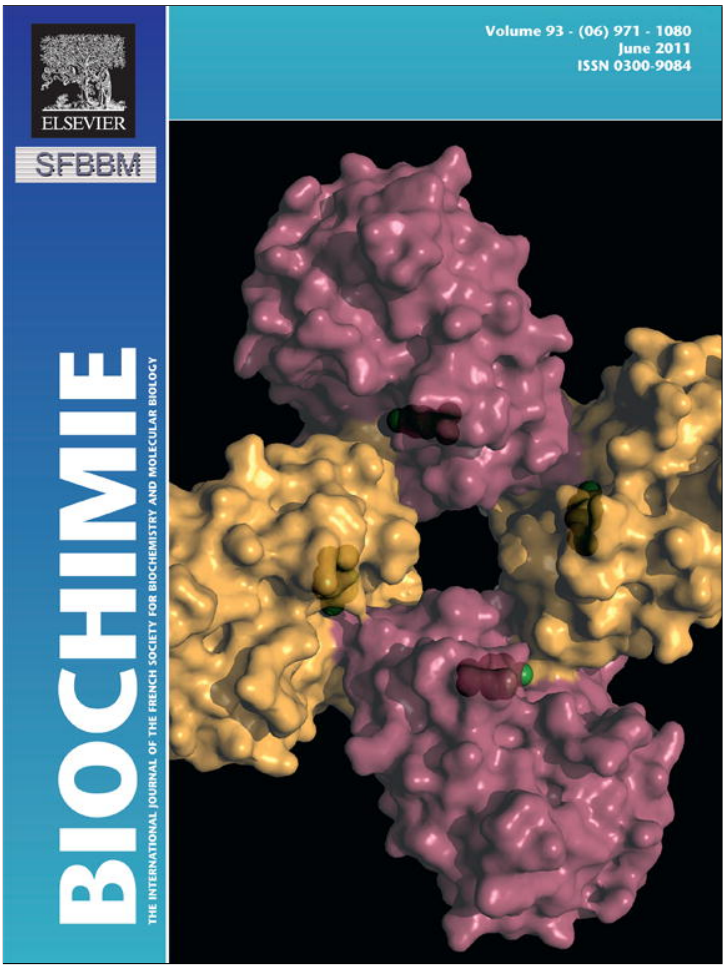
Biochimie, 2011, 93: 1076-1079

There is an ongoing discussion about the proteasomal degradation of oxidative modified proteins. The majority of publications show a connection between oxidized proteins and an ATP and ubiquitin-independent degradation by the 20S proteasome. However, there are also evidences that there is an increase of ubiquitinated proteins upon oxidative stress. This work should add clarity on the proteasomal degradation of oxidized proteins.

Thus, cells were treated with hydrogen peroxide and UV-A irradiation, causing cellular oxidative stress. Detection of carbonylated and ubiquitinated proteins by immunoblotting reveals that most oxidized proteins are not ubiquitinated. Isolation of ubiquitinated proteins by immunoprecipitation confirm that most protein carbonyls containing proteins are not ubiquitinated. The results of this study support the thesis that oxidized proteins are preferentially degraded by the 20S proteasome in an ubiquitin-independent manner and shows how the proteasome is involved in the cellular response to oxidative stress.

Own work:

- All practical experiments, including cell culture, western blots and immunoprecipitation
- Writing of the manuscript, creating the figures and preparation of the statistical analysis



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Short communication

Proteins bearing oxidation-induced carbonyl groups are not preferentially ubiquitinated

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ABSTRACT

A substantial part of soluble, oxidized proteins are degraded by the proteasome. However, it is still under debate whether these oxidized proteins are degraded by the 26S proteasome in an ubiquitin-dependent way or in an ubiquitin-independent way by the 20S proteasome. Therefore, we treated cells with H_2O_2 and UV-A irradiation and detected protein carbonyls and ubiquitination by immunoblotting. Separation of ubiquitinated proteins from non-ubiquitinated reveals that most oxidized proteins are not ubiquitinated.

1. Introduction

The bulk of soluble intracellular and nuclear proteins are degraded by the proteasome. The proteasome consists of a cylindrical 20S core unit, which is capable to degrade proteins in an ATP-independent way. The 26S proteasome, which degrades proteins in an ATP-dependent way, consists of the 20S core unit and up to two 19S regulator units [1]. For recognition and degradation of proteins by the 26S proteasome, it is necessary, that proteins are tagged with a polyubiquitin-chain. Ubiquitin is an intracellular peptide, which has a multitude of functions among which is also the label of proteins for degradation by the 26S proteasome. It seems to be accepted, that most abnormal, damaged or oxidized proteins are degraded by the proteasome, including 70–80% of oxidized proteins [2]. However, which form of the proteasome is involved in the degradation of oxidized proteins is still a matter of debate. Whereas numerous groups accumulated convincing evidence for the degradation of oxidized proteins in an ATP- and ubiquitin-independent fashion [2–8], a limited number of experiments seem to indicate some possible role of ATP or ubiquitin [9–12]. So the group of Taylor could show in several publications, that there is an increase of protein carbonyls and ubiquitination after exposure to hydrogen peroxide [9,13]. Medicherla and Goldberg found some evidence on a role of ubiquitination after oxidative stress in yeast [12]. Since due to the undoubted role of proteasomal ubiquitin-dependent degradation in stress response [14,15], and the array of effects of oxidative stress on cell cycle and cell functionality, it is highly expected that during oxidative stress an enhanced ubiquitination occurs.

However, it still remains unsolved whether oxidatively damaged proteins are preferentially/selectively ubiquitinated and degraded by the 26S proteasome. By inducing oxidative stress with UV-A and hydrogen peroxide and detecting oxidized and ubiquitinated proteins by immunoblotting and separation of ubiquitinated and non-ubiquitinated proteins we tried to resolve this issue.

2. Materials and methods

2.1. Materials

All chemicals were obtained from Sigma–Aldrich (Deisenhofen, Germany) or Carl Roth AG (Karlsruhe, Germany) unless otherwise indicated. Chemicals for cell culture were provided from Biochrom AG (Berlin, Germany).

2.2. Cell culture

All experiments were carried out, using the metastatic melanoma cell line WM451Lu. Cell cultivation was carried out like it is described by Kästle et al. [16].

Abbreviations: ATP, adenosine triphosphat; H_2O_2, hydrogen peroxide; UV-A, ultra violet A.

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2.3. Cell treatment

Cells were stressed with either hydrogen peroxide (H_2O_2) or UV-A irradiation. H_2O_2 treatment was performed using 1 mM, 2 mM and 5 mM H_2O_2 in PBS for 0.5 h. Cell exposure to UV-A was performed in a UV-A chamber (Dr. Gröbel UV-Elektronik GmbH, Ettlingen, Germany) using 60 J/cm^2. After cell exposure, PBS or H_2O_2 solutions were exchanged to normal growth medium and cells were incubated 16 h for recovery.

2.4. Cell isolation and immunoblotting

Cell isolation was conducted with standard protocol (lysis buffer: 20 mM TRIS base, 100 mM sodium chloride, 5 mM EDTA). Oxy-Blots were performed as described in Catalgol et al. [17]. After membrane stripping, ubiquitin was detected with an anti-ubiquitin antibody (BML-KW0150, ENZO Life Sciences, Lörach, Germany) (1:1000). The amounts were quantified by densitometry, considering background intensity, sample intensity and its area.

2.5. Capturing of ubiquitin-conjugated proteins

For isolation of ubiquitinated proteins, the UbiQapture™-Q Kit, containing a high-binding affinity matrix based on immobilized monoclonal anti-ubiquitin antibodies (UW8995-0001; ENZO Life Sciences, Lörach, Germany) was used and the procedure was performed as described in the manufacturer's instructions. After the separation there are three different fractions: the "Unbound Fraction" (UF), which did not bind to the ubiquitin capture matrix, the "Elution Fraction" (EF), which includes the proteins that were captured by the ubiquitin capture matrix and are eluted in a secondary step and a "Washing Fraction" (WF), which contains the proteins of the washing steps. In most experiments the concentration of proteins in the washing solution was below the detection limit.

3. Results

3.1. Protein oxidation and ubiquitination after H_2O_2 exposure

As a reliable marker for oxidized proteins in cells we measured protein carbonyls after exposure to different concentrations of H_2O_2. With an increasing H_2O_2-load of the cells there is a dose dependent increase of protein carbonyls (Fig. 1A). For 5 mM H_2O_2 there is a high significant augmentation (150%) of protein carbonyls compared to the control. As expected ubiquitinated proteins also increase after exposure to H_2O_2 (Fig. 1A, right blot). However, comparing the molecular weight distribution of the signals for oxidized and ubiquitinated proteins between the immunoblots, it becomes obvious that there are many oxidized proteins, which are not ubiquitinated (Fig. 1A, H_2O_2). Oxidized proteins can be observed

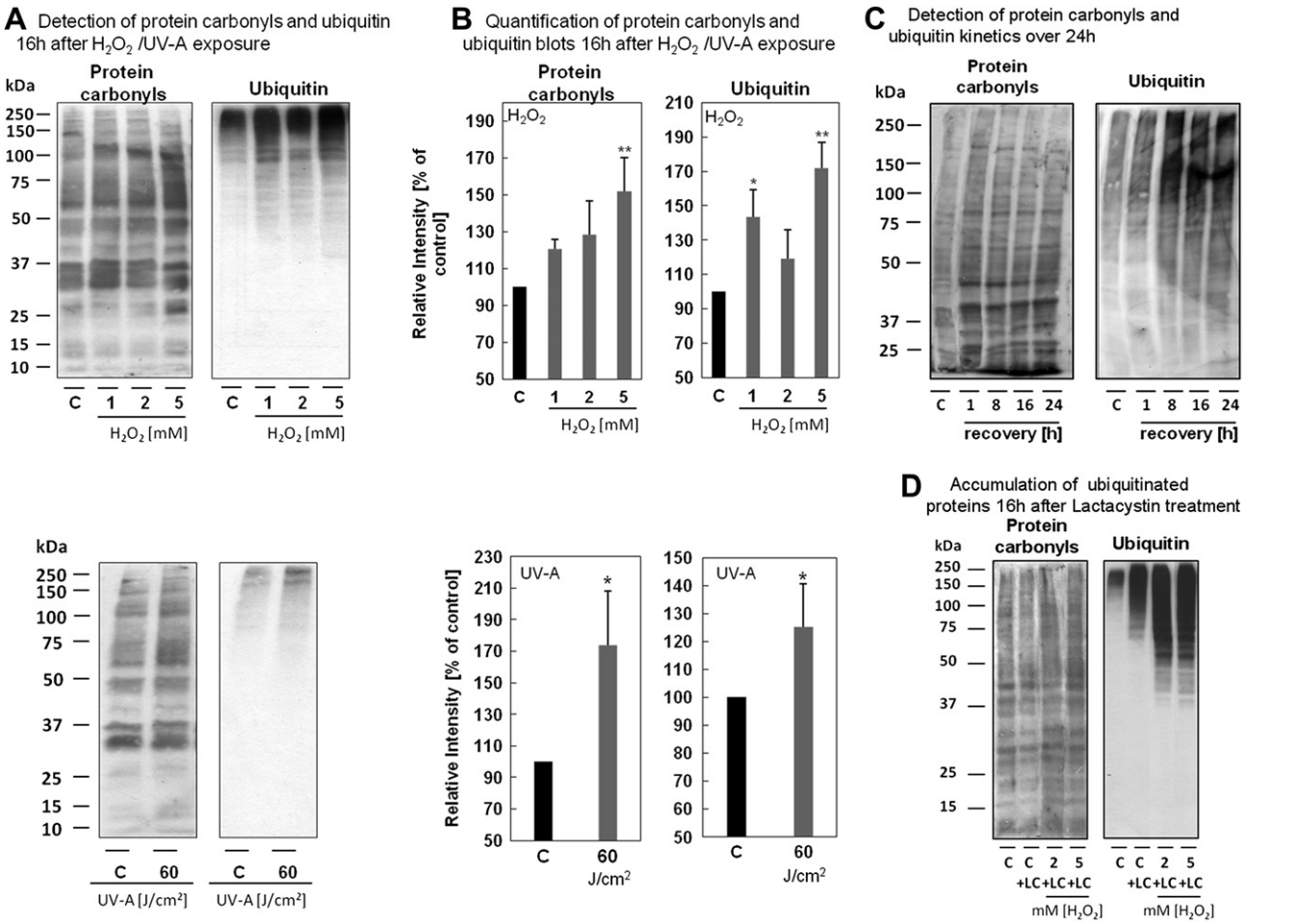

Fig. 1. Protein carbonyls and ubiquitination after H_2O_2/UV-A treatment. *Panel A* shows one representative immunoblot out of three independent experiments. In *panel B* the quantification results of the three independent immunoblots of protein carbonyls and ubiquitinated proteins are presented. *Panel C* and *D* represent protein carbonyls and ubiquitinated proteins at various time points after treatment and after lactacystin (20 μM) treatment. Shown are the Mean ± SD; $p < 0.05$ ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, statistical significance to control, Tukey post hoc test.

in a molecular weight range from 250 kDa to 10 kDa. Especially the regions from 100 to 50 kDa and 37 to 25 kDa contain a high amount of protein carbonyls (Fig. 1A, H_2O_2). On the other hand a signal, using an anti-ubiquitin antibody can only be detected in the high molecular weight range. Therefore, it is clearly visible, that the oxidative stress induced increase in signal intensity takes place in the whole molecular weight range, whereas the ubiquitination signal is increasing only in the high molecular weight range. Even during kinetic studies and after lactacystin treatment it is obvious that there are no changes in the molecular masses of carbonylated and ubiquitinated proteins with time or upon blocking of the degradation of ubiquitinated proteins (Fig. 1C and D).

3.2. Protein oxidation and ubiquitination after UV-A exposure

To test whether this phenomenon is also true for other oxidative stress models, cells were exposed to UV-A. Exposure to 60 J/cm^2 of UV-A leads to a 1.7 fold significant increase of protein carbonyls (Fig. 1). Also the amount of ubiquitinated proteins after UV-A irradiation is some 25% higher compared to the control. Additionally there is the same protein distribution of carbonylated and ubiquitinated proteins, as in the case of hydrogen peroxide treatment (Fig. 1A). Therefore, it can be concluded that this is not a special effect of H_2O_2 treatment, but rather a common effect after cellular oxidative stress. However, it seems treatment with UV-A irradiation produces lower amounts of ubiquitinated protein than treatment with H_2O_2.

3.3. Detection of protein carbonyls and ubiquitinated proteins after ubiquitin-capturing

Further on we separated the ubiquitinated proteins from the non-ubiquitinated by using an ubiquitin capture matrix. Fig. 2A shows the detection of protein carbonyls and ubiquitinated proteins of the two protein fractions gained by this method. The Unbound Fraction (UF), which contains the proteins, which were not captured by the ubiquitin capture matrix, contains most of the oxidized proteins after exposure to 5 mM H_2O_2 (Fig. 2). In the Elution Fraction (EF), which contains the proteins captured by the ubiquitin capture matrix – that means the ubiquitinated proteins, only some 40–60% of the protein carbonyl concentration of the unbound control could be detected. This indicates a clear negative selection by the ubiquitin capture matrix for oxidized proteins. The increase of protein carbonyls in the UF after exposure to 5 mM H_2O_2 is with 150% significant compared to control proteins of the UF and also significant compared to the proteins of the EF (Fig. 2).

Furthermore, the ubiquitin blots indicate that there is a significant increase of ubiquitination after 5 mM H_2O_2, as it was observed in the experiments before (Fig. 2). Ubiquitinated proteins are found predominantly in the EF fraction, which contains almost none of the oxidized proteins. Consequently separation of ubiquitinated and non-ubiquitinated confirms the findings, that most oxidized proteins are not preferentially ubiquitinated.

4. Discussion

In the current study, we could demonstrate that there is an augmentation of oxidized proteins and ubiquitinated proteins after exposure to several sources of oxidative stress. Furthermore, we could show that most of the formed oxidized proteins are not ubiquitinated. The bulk of oxidized proteins become degraded by the proteasome [2]. Recognition and degradation of proteins by the 20S and the 26S proteasomes are different. In most cases substrates of the 26S proteasome have to be ubiquitinated for degradation [5]. Pickart et al. have shown that it is mainly the hydrophobic effect of

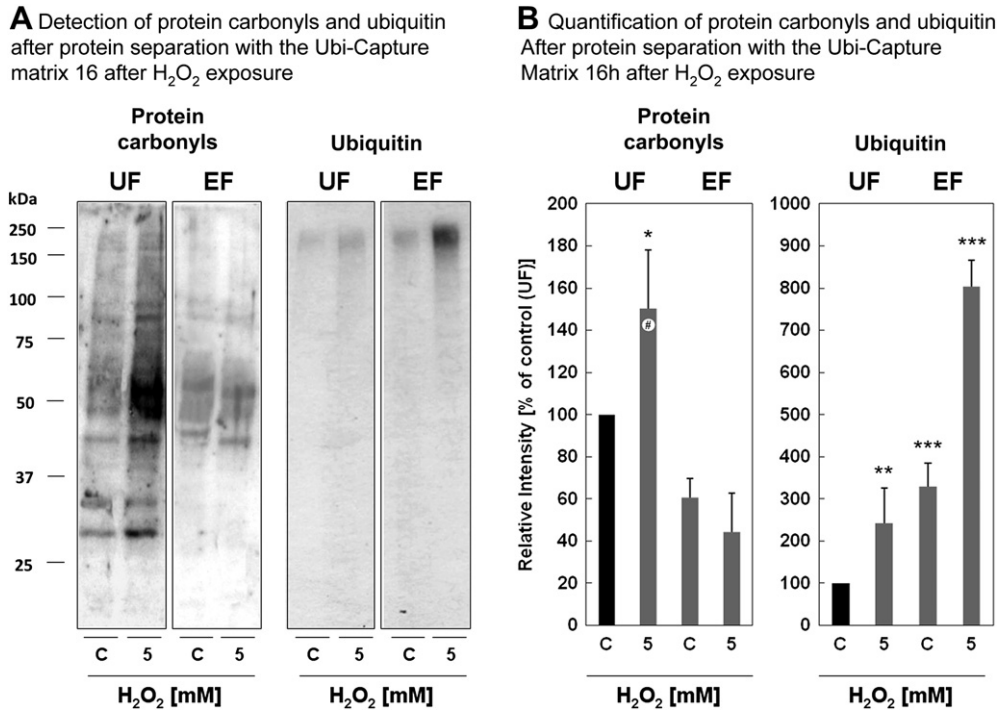

Fig. 2. Protein carbonyls and ubiquitination after H_2O_2 treatment and separation of ubiquitinated proteins using the ubiquitin capture matrix. Cells were treated and lysed as described above. Afterwards cells lysates were separated into the unbound fraction (UF, non-ubiquitinated proteins) and the bound and eluted fraction (EF, ubiquitinated proteins). Fractions were analyzed as described above. Three independent experiments were performed. In *panel A* one set of representative blots is demonstrated, whereas in *panel B* the quantification of all three experiments is shown. Data are the means of three different experiments. Mean ± SD; $p < 0.05$ ANOVA, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, statistical significance to control and $^{\#}p < 0.01$, statistical significant to both EFs, Tukey post hoc test.

the polyubiquitin chain that allows recognition and degradation of ubiquitinated proteins by the 26S proteasome [18]. In general the 26S proteasome is considered to be responsible for the degradation of short lived, regulatory proteins and unfolded proteins. Therefore, such proteins have to be tagged by a hydrophobic polyubiquitin chain for degradation [5]. On the other hand multiple studies demonstrated that oxidatively damaged proteins are good substrates for the 20S proteasome, due to oxidant-mediated unfolding and the increased surface hydrophobicity of the protein substrate [4]. No ubiquitin is required for this degradation. Therefore, moderately oxidized, unfolded and hydrophobic proteins seem to be ideal substrates for the 20S proteasome, which works ubiquitin independent [1,2]. Furthermore, it is likely, that oxidized proteins are not suitable for ubiquitin binding, because oxidative modification of lysine residues to α-aminoadipic semialdehyde [19] impairs proteins for ubiquitination. In addition to that, the group of Davies has shown that ATP plays no crucial role in proteolysis of oxidized proteins. Furthermore, it was observed that addition of ATP decreases proteasomal degradation of oxidized proteins of about 10–20% in cell free lysates [4,5,20].

The reported high level of ubiquitinated proteins after oxidative stress as found by the group of Taylor in rabbit epithelial lens cells shows the same molecular weight distribution as reported here [10,11,13]. Since a separation of ubiquitinated and non-ubiquitinated proteins was not performed in these studies we interpret the results differently. Most likely a set of regulatory proteins is ubiquitinated as a consequence of stress response. However, we could show in the here presented data that a vast majority of carbonylated proteins is not ubiquitinated. These results fit further in the role of the 20S proteasome in the degradation of oxidized proteins [2–8], the degradation of oxidized proteins in cells lacking an ubiquitination system [5], and the loss of 26S proteasomal activity during oxidative stress [21]. Reinheckel et al. have clearly demonstrated that there is a loss of function of the 26S proteasome after exposure to oxidative stress, whereas the 20S proteasome remains unaffected [21]. Besides enhanced ubiquitination as a consequence of the stress response, the loss of 26S proteasomal activity might contribute to the accumulation of ubiquitinated proteins after oxidative stress.

Additionally, in plants could be shown that a loss of 26S proteasome activity leads to an increased 20S proteasome biogenesis, which enhances the cellular capacity to degrade oxidized proteins [22].

In summary, it can be suggested that the majority of oxidized proteins are not preferentially ubiquitinated and, therefore, not degraded in an ubiquitin- and ATP-dependent fashion. Therefore, it is likely that other proteolytic pathways, including the 20S proteasome play a crucial role in the removal of oxidized proteins after oxidative stress.

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3.3. Publication III: Chaperones, but not oxidized proteins, are ubiquitinated after oxidative stress

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In **publication II** it was demonstrated that most oxidized proteins are not ubiquitinated, although there is also an increase of ubiquitinated proteins as part of oxidative stress. Due to this it is of special interest to identify the ubiquitinated proteins upon oxidative stress.

Therefore, ubiquitinated proteins were treated with hydrogen peroxide (H_2O_2) and/or lactacystin (LC) and isolated by immunoprecipitation. The isolated fraction of ubiquitinated proteins was separated by gel-electrophoresis, visualized by Coomassie-staining and identified by MALDI-TOF-TOF analysis.

As a result 24 proteins were identified and were dedicated to cytoskeleton/intermediate filament proteins, chaperones, energy metabolism proteins, transcription/translation regulators and clathrin. Just a few of them were uniquely identified when there was an involvement of H_2O_2. Only some chaperones presented increased ubiquitination. All in all the obtained results confirm that the majority of proteins found in the ubiquitin fraction upon oxidative stress are ubiquitinated due to regulatory reasons.

Own work:

- Tryptic protein digestion and preparation of the samples for MALDI-TOF-TOF analysis
- Scientific instruction and design of western blot experiments
- Literature research
- Writing of the manuscript and making the figures

Chaperones, but not oxidized proteins, are ubiquitinated after oxidative stress

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Abstract

After oxidative stress proteins which are oxidatively modified are degraded by the 20S proteasome. However, several studies documented an enhanced ubiquitination of yet unknown proteins. Since ubiqutination is a prerequisite for degradation by the 26S proteasome in an ATP-dependent manner this raises the question whether these proteins are also oxidized and, if not, what proteins need to be ubiquitinated and degraded after oxidative conditions.

By determination of oxidized- and ubiquitinated proteins we demonstrate here that most oxidized proteins are not preferentially ubiquitinated. However, we were able to confirm an increase of ubiquitinated proteins 16h upon oxidative stress. Therefore, we isolated ubiquitinated proteins from hydrogen peroxide treated cells, as well as from control and lactacystin, an irreversible proteasome inhibitor, treated cells, and identified some of these proteins by MALDI tandem mass spectrometry (MALDI MS/MS). As a result we obtained 24 different proteins which can be categorized into the following groups: chaperones, energy metabolism, cytoskeleton/intermediate filaments, and protein translation/ribosome biogenesis. The special set of identified, ubiquitinated proteins confirm the thesis that ubiquitination upon oxidative stress is no random process to degrade the mass of oxidized proteins, but concerns a special group of functional proteins.

Keywords: Oxidative stress, ubiquitin, proteasome, proteolysis, protein oxidation

Introduction

Oxidative stress is defined as the imbalance between reactive oxygen species (ROS) and the antioxidative response mechanism, whereas under stress conditions an overweight of ROS exists [1]. Besides lipids [2] and the DNA [3], proteins are the most abundant targets for oxidative modifications upon oxidative stress. The most familiar protein-/amino acid modification upon oxidative stress is the formation of protein carbonyls. The most sensitive amino acids for oxidative –dependent carbonylation are arginine, lysine, proline, threonine and glutamic acid. Due to the high occurrence of protein carbonyls after oxidative stress, they are a well established biomarker for oxidative stress and the degree of oxidative protein damage [4,5]. In contrast with other oxidative protein modifications, like the formation of methionine sulfoxide (MetO), protein carbonylation is irreversible. Thus, proteins containing protein carbonyls have to be degraded by the proteasome, in order to maintain the cellular protein homeostasis [6].

The proteasome is a cylindrically shaped, multicatalytical protease with a molecular mass of about 700 kDa [7]. The proteasome consist of a 20S core structure (also called the 20S proteasome), formed by two α-rings and two β-rings. The two β-rings possess the proteolytic activity of the proteasome, whereas the α-rings function as regulators of substrate access into the inner β rings. Additionally, the 20S proteasome is able to build complexes with different regulator subunits. If the 20S proteasome binds two 19S regulators –one on each side of the barrel- it forms the 26S proteasome [8]. The 26S proteasome binds and degrades, in an ATP-dependent manner, polyubiquitinated proteins. Therefore, the great majority of the 26S proteasome substrates have to be polyubiquitinated by the E1- (ubiquitin-activating enzyme), E2- (ubiquitin-conjugating enzyme) and E3- (ubiquitin ligase) ubiquitination enzymes. Then several different subunits within the 19S regulator arrange the deubiquitination, unfolding and transfer of the substrate to the 20S proteasome [7]. In general the 26S proteasome is responsible for the degradation of the large spectrum of short lived regulatory-, onco- and structural proteins [8]. Degradation by the 20S proteasome alone is ATP- and ubiquitin-independent, however, the proteins have to be in an already unfolded state, exposing hydrophobic surface structures [9].

It was a matter of debate, whether oxidized proteins are degraded ubiquitin-independent by the 20S proteasome or ubiquitin-dependent by the 26S proteasome. A bulk of publications groups accumulated convincing evidence for an ubiquitin-independent degradation of most oxidized proteins by the 20S proteasome [10-16]. However, there are also some confusing results, indicating a possible role of the ATP/ ubiquitin-dependent degradation by the 26S

proteasome [6,17,18]. So Shang et al. demonstrated, that there is a coincident increase of protein carbonyls and ubiquitinated proteins after exposure to hydrogen peroxide (H_2O_2) [17,18]. In a previous study, we could also observe an augmentation of protein carbonyls and ubiquitinated proteins after H_2O_2- or UV-treatment. However, we proposed that most oxidized proteins were not ubiquitinated by comparison of the molecular weight spectrum of oxidized proteins and the ubiquitinated ones [19]. This suggests that the majority of oxidized proteins are degraded in an ubiquitin-independent way and that ubiquitination upon oxidative stress affects only distinct proteins [19]. However, the question which proteins are ubiquitinated after oxidative stress remains unsolved.

Therefore, we decided in the here presented study to confirm that oxidized proteins are not preferentially ubiquitinated and to elucidate the identity of ubiquitinated proteins after exposure to hydrogen peroxide. By separation and MALDI MS/MS analysis we identified 24 ubiquitinated proteins after exposure to oxidative stress and tested their time dependent content in total and in an ubiquitin fraction.

Materials and Methods

Materials

All chemicals are obtained from Sigma-Aldrich (Hamburg, Germany) or Carl Roth AG (Karlsruhe, Germany), unless otherwise indicated. Cell culture material were provided from Biochrom AG (Berlin, Germany)

Cell Culture and oxidative stress

For all experiments WM-451-Lu melanoma cells were used. Cells were cultivated in a humidified 37°C incubator with 5% CO_2. Cell culture medium comprised MCDB 153 basal medium (with phenol red), 20% Leibovitz L15 medium, 2% fetal calf serum (FCS), 2 mM glutamine, 50 µg/ml gentamycin, and 5 µg/ml insulin. Medium was changed after 3 days. For hydrogen peroxide (H_2O_2) treatment cells were seeded on cell culture dishes (22 cm^2). Reaching 80-90% confluence, cells were treated for 0.5h with 5 mM H_2O_2 solution, prepared in PBS. After the incubation, 5mM H_2O_2 was discarded and cells were cultured for additional 16h in normal growth medium for recovery. In case of proteasome inhibition, normal growth medium comprised additional 20µM lactacystin (LC) for 16h.

MTT viability assay

To determine the cell viability after H_2O_2 and UV-A exposure the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid (MTT) – viability assay (Carl Roth, Germany) was used. Following 16h recovery time, medium was exchanged by MTT solution (5 mg/ml). Following the incubation time of 1h, MTT-solution was discarded and cells were solubilized with DMSO containing 10 % SDS and 0.6 % acetic acid. Viability was determined on a 96 well plate reader at a wavelength of 580 nm (reference wavelength: 660 nm).

Cell lysate preparation and isolation of ubiquitinated proteins

For cell lysate preparation, cells were washed twice with PBS and treated with lysis buffer (20mM TRIS base, 100mM sodium chloride, 5mM EDTA). After homogenization and incubation on ice, cells were treated with ultrasound and subsequently centrifuged at 12500 rpm for 10 min. The supernatant was used for further experiments. Protein content of the sample was determined by Bradford protein assay [20]. Cell lysates were either directly analyzed by immunoblot or the ubiquitinated proteins were isolated. For isolation of ubiquitinated proteins, the UbiQapture™-Q Kit, containing a high-binding affinity matrix

based on immobilized monoclonal anti-ubiquitin antibodies was used and the procedure was performed as described in the manufacturer's instructions (UW8995-0001; ENZO Life Sciences, Lörach, Germany). After the separation there are two different fractions: the "Unbound Fraction" (UF), containing ubiquitinated and non ubiquitinated proteins, the "Elution Fraction" (EF), which includes the proteins that were captured by the ubiquitin-capture matrix (solely ubiquitinated proteins) and are eluted in a secondary step. Elution of ubiquitinated proteins was conducted with the ubiquitin hydrolase USP2 (BML-UW9850; ENZO Life Sciences, Lörach, Germany). Elution with USP2 results in a deubiquitination of the isolated ubiquitinated proteins.

Gel electrophoresis and Immunoblotting

30-50 µg protein of each sample was separated by SDS-page (gel: 7.5% acrylamide) and transferred onto nitrocellulose membrane. Derivatization and detection of protein carbonyls was conducted as described before in Catalgol et al. [21]. For detection of all other proteins the following antibodies were used: ms anti ubiquitin (BML-KW0150, ENZO Life Sciences, Lörach, Germany), ms anti Hsp70 (ab6535), ms anti Hsp90 (ab13492), rb anti β-tubulin (ab6046) (abcam, Cambridge, UK), ms anti β-actin (A5441, Sigma-Aldrich, Germany). Detection of the membranes was conducted with the Odyssey infrared detection system from LI-COR (LI-COR Biosciences, Bad Homburg). Blot evaluation was conducted with Odyssey Application Software Version 3.0 from Li-Cor.

Coomassie staining, in-gel digestion, mass spectrometry protein identification and database searches

Cell lysates for mass spectrometry were pooled and concentrated by Amicon Ultra centrifugal filters for protein purification and concentration (Milipore, Germany). Samples were separated by gel electrophoresis and stained with Coomassie for 0.5h. Following several washing steps with 10% acetic acid, all visible protein bands were excised and used for mass spectrometry protein identification. Sample preparation methods for mass spectrometry were modified from Shevchenko et al. [22]. Gel bands were washed with deionized water followed by two washes with 100% acetonitrile for 15min and 2 min respectivelly. Subsequently, the proteins in the gel plugs were reduced with 10 mM DTT for 0.5h and alkylated with 55 mM iodoacetamide for additional 0.5h. Following a washing step with 50% acetonitrile, samples were treated with 100% acetonitrile for 15min and the gel plugs were dehydrated in a vaccum centrifuge. In gel protein digestion was carried out using 2% trypsin (Promega Inc., Madison

WI, USA) in 50 mM NH_4HCO_3 at 4°C. After 20 min trypsin incubation, the excess trypsin solution was exchanged by 50μM NH_4HCO_3 and digestion proceeded at 37°C overnight, followed by storage at -20°C until use. Peptide purification and concentrating was handled using in house prepared reverse-phase microcolumns as described before Ahmed et al. [23]. Bound peptides were eluted with 0.8 μL of matrix solution (5 μg/μL a-cyano-4-hydrocynnamic acid in 70% acetonitrile and 0.1% TFA) directly onto the matrix assisted laser desorption ionization (MALDI) target plate. Peptide mass spectra were acquired in positive reflector mode on a 4800 Plus MALDI-TOF/TOF™ Analyzer (Applied Biosystems, Foster City, CA, USA). Each spectrum was generated with a total of 1000 laser shots and was externally calibrated using standard peptides from tryptic digested β-lactoglobulin. From the raw data output, peak lists were generated using Data Explorer (Applied Biosystems, Foster City, CA, USA). MS and MS/MS peak lists were combined into search files and used to search NCBInr protein databases using Mascot program (Mascot server version 2.3.0,
Matrix Science, UK). The following search parameters were used: Database — NCBInr (October 2011), Taxonomy — Metazoa (Animals), maximum number of missed cleavages 1, cysteine carbamidomethylation as fixed and methionine oxidation as variable modification,mass tolerance of 70 ppm in MS mode and 0.6 Da for MSMS.

Results

Protein oxidation and ubiquitination upon exposure to H_2O_2

To confirm the results from our preliminary study [19] and to clarify the distribution of protein carbonyls and ubiquitinated proteins, we treated cells with H_2O_2 and/or LC and tested the samples for protein carbonylation and ubiquitination. In **Fig.1A** it is indicated that there is an augmentation of protein carbonyls with increased stress stimuli. Treatment with LC alone shows only a slight or no increase of protein carbonyls compared with the control, indicating that LC mediates no severe oxidative stress. Exposure to H_2O_2 leads to a visible elevation of protein carbonyls compared to the control. By inhibiting the proteasome and additional treatment of the cells with H_2O_2 we detected an accumulated mass of protein carbonyls, observing the greatest amount of oxidized proteins compared to the other conditions **(Fig. 1A)**. With regard to ubiquitination, the treatment with LC results in an expected raise of ubiquitinated proteins. Treatment with H_2O_2 leads also to a further increase of ubiquitination. By comparing the molecular weights of oxidized and ubiquitinated proteins it can be observed that a majority of oxidized proteins are at a mass range between 15 kDa to 50 kDa, whereas the majority of ubiquitinated proteins are located above 70 kDa **(Fig. 1A)**. To confirm that we investigate viable cells after the various treatments, we determined viability by an MTT assay. Cell treatment with LC, H_2O_2 or both reduces cell viability not more than 35% compared to the control (data not shown).

Moreover, we decided to test whether there is the same time dependency of protein oxidation and ubiquitination. As it is shown in **Fig.1B,** protein carbonylation increases expectably very fast after H_2O_2 exposure, resulting in the highest concentration 1 h after start of the treatment **(Fig. 1B)**. Contrarily the increase of ubiquitinated proteins is more moderate after H_2O_2 treatment. Although, there is a slight increase directly after hydrogen peroxide treatment (0.5h) the long term accumulation of ubiquitinated proteins is much more dramatic. Again, throughout all time points the majority of ubiquitinated proteins are located in the high mass protein fraction **(Fig. 1B)**. Since our original aim was to identify some of the preferentially ubiquitinated proteins after oxidative stress we choose the 16h incubation time after H_2O_2 treatment for analysis.

Isolation and determination of ubiquitinated proteins

Thus we isolated the ubiquitinated proteins using the UbiQapture™-Q Kit (a method, based on immunoprecipitation) after exposure to H_2O_2 and/or LC and separated the proteins by gel electrophoresis. 24 different protein bands were analyzed by MALDI MS/MS **(Fig. 2)**. All of

the 24 bands were successfully identified. In Table 1 the protein names, accession number, Mascot score, theoretical protein mass (Da), sequence coverage (%), number of peptide matched and sequenced peptides are listed **(Tab. 1)**. Among the isolated ubiquitinated proteins we found a large group of cytosolic proteins as well as proteins from cell organelles, such as mitochondria and the endoplasmatic reticulum. The identified ubiquitinated proteins can be categorized into the following groups: (i) chaperones, (ii) energy metabolism enzymes, (iii) cytoskeleton/ intermediate filaments, (iv) proteins participating in protein translation/ ribosome biogenesis/mRNA stabilization and (v) the clathrin heavy chain protein 1 **(Fig. 3A)**. We identified eight proteins ubiquitinated in the control and after oxidative stress. This are the three chaperones Hsp90, Hsp70 and Hsp60 as well as the cytoskeleton and intermediate filament proteins β-tubulin, actin, vimentin and myosin, as well as the elongation factor eEF1a **(Table 1, Fig. 3A)**.

Additionally, we found also peptides belonging to ubiquitin, respectively ubiquitin C splicing variant which are indicated in Table 1 by the number 19 and 20. This is a further proof for the ubiquitination of the isolated proteins and the efficiency of separation of ubiquitinated proteins. The theoretical mass of this two ubiquitin samples are about 8.5 kDa and 17.0 kDa **(Tab. 1)**. Interestingly the detected bands were cut out at a protein size of about 250 kDa, indicating the formation of polyubiquitin-chains with higher masses. We detected no additional proteins after LC treatment, so we proceeded only with control- and H_2O_2 treated cells for the next experiments.

Comparison of ubiquitinated proteins between the control and H_2O_2 treated cells

If some proteins are ubiquitinated in control cells and also after the treatment with H_2O_2 it is of special interest whether under stress conditions the amount of the given protein ubiquitinated is changing in comparison to control levels. Thus, we determined the blot density of the scanned coomassie gels (control and H_2O_2 treated) with the Odyssey Application Software and built the ratios of all these proteins between the control and H_2O_2 treated cells. The value of the ratio reflects the relative difference between the two groups; a ratio of one indicates no change in protein levels between the two groups, whereas a ratio of two stands for the two fold increase in the amount of ubiquitinated proteins after H_2O_2 treatment. Interestingly, after examining these eight mentioned proteins we found three proteins whose protein content increases in the ubiquitin fraction after oxidative stress: Hsp90, Hsp70 and Hsp60. Whereas after H_2O_2 treatment the content of ubiquitinated Hsp90 doubled to a ratio of two, the Hsp70 and Hsp60 reached a 1.5 fold increase of ubiquitination

compared to the control **(Fig. 3B)**. In contrast, there is no remarkable difference between the control and H_2O_2 treated cells of the other 5 proteins. Ubiquitination of all the cytoskeleton and intermediate filament proteins seems to remain largely unchanged after oxidative stress. Surprisingly, the content of eEF1a in the ubiquitin fraction is even a little bit lower after H_2O_2 than in the control (ratio: 0.9).

As it was already shown in Fig.1A, that there is an increase of ubiquitination after H_2O_2 treatment compared to the control. The total summarized band intensity of ubiquitinated proteins after H_2O_2 treatment was twice as high compared to the control (data not shown), confirming the results from Fig.1. We decided, therefore, to test whether the absolute amount and the share of chaperones, as the proteins preferentially ubiquitinated after oxidative stress is really increasing within the pool of total ubiquitinated proteins. As demonstrated in **Fig.4** the absolute level of ubiquitinated chaperones is more than twice as high compared to the control (Fig.4A) and the proportion of chaperones is increasing by almost one third, whereas the share of cytoskeletal proteins as the major ubiquitinated protein fraction under control conditions is rather declining. Proteins related to translation/RNA interaction do not change their share and a collection of further proteins is ubiquitinated, too. This underlines the preferential ubiquitination of chaperones after hydrogen treatment.

Time dependent ubiquitination of identified proteins

In a next step it would be interesting on the one hand to verify the densitometric results of the coomassie gels of the ubiquitination fraction and on the other hand examining the expression of these proteins in total cell lysates and the ubiquitin fraction. Hence, we performed an immunoblot of the interesting chaperones HSP70 and HSP90 and for comparison of the cytoskeleton protein actin. Here, we either took the whole cell lysate for immunoblotting or isolated first the ubiquitinated proteins and used the ubiquitin fraction for immunoblotting. In Fig.5 the immunoblots of whole cell lysates and the isolated ubiquitin fraction of Hsp70, Hsp90 and actin are shown at the indicated molecular weights of each of the proteins. In total cell lysate the content of functional HSP90, HSP70 and actin remained unchanged **(Fig. 5)**. However, the ubiquitination of HSP70 and HSP90 was drastically increases (more than 50%), as indicated by the content of the protein in the ubiquitinated fraction. Interestingly, also the content of actin was somewhat higher (20 %) in the ubiquitinated fraction of hydrogen peroxide treated cells. This confirms the prominent detection of actin as a ubiquitinated protein by the mass spectroscopic determination. On the other hand the pure quantification by analyzing the coomassie gel band seems to be less sensitive to such minimal changes.

Discussion

In the current study we detected protein carbonyls and ubiquitinated proteins after oxidative stress. We were able to confirm results from a preliminary study of our group, where it was demonstrated that oxidized proteins are not preferentially ubiquitinated [19]. Due to the fact that there is also an augmentation of ubiquitinated proteins during oxidative stress, we isolated the ubiquitinated proteins, subjected them to MALDI MS/MS analysis and identified 24 different proteins among the ubiquitin fractions.

There is a wealth of literature demonstrating that that oxidized proteins are preferentially degraded by the 20S proteasome in an ubiquitin and ATP-independent manner [10-16]. In addition to that there is increasing evidence showing an instability and inhibition of the 26S proteasome during oxidative stress. It was suggested that under oxidizing conditions a disassembly of 26S proteasomes takes place, enhancing the 20S proteasome content, giving the possibility for a more effective removal of damaged, irreparable proteins [10,24-26]. In a recent published work of the group of Huang it is demonstrated the disassembly of the 26S proteasome into the two 19S regulators and the 20S proteasome by mass spectrometry upon treatment with H_2O_2 [26]. Further on, the loss of the 26S proteasome activity was accompanied by an accumulation of ubiquitinated proteins. However, although there was an accumulation of ubiquitinated proteins, it was additionally demonstrated that oxidized proteins were degraded by the remaining 20S proteasome. The H_2O_2 triggered disassembly of the 26S proteasome seems to be dependent on the proteasome interacting protein Ecm29. It was shown that Ecm29 is substantially recruited to the 19S regulator in response to oxidative stress, resulting in its dissociation from the 20S core particle [26]. Our group was already earlier able to show a decline of the 26S proteasome activity and content after oxidative stress [27,28] and later an involvement of HSP70 in the 26S proteasome dissociation [24]. By using temperature-sensitive ubiquitination deficient cells we could demonstrate, that cells under restrictive conditions – no ubiquitination possible – were still able to degrade oxidized proteins [29].

Although, it seems to be a clear fact, that ubiquitination is not a required prerequisite for the degradation of oxidized proteins, it is also documented by several groups that after oxidative stress an enhanced ubiquitination takes place [17-19,30]. However, it already by a pure comparison of the molecular weight distribution of the oxidized/carbonylated proteins and the ubiquitinated proteins it is obvious that this is a different protein pool. So it was demonstrated by us, that not the oxidized proteins were preferentially ubiquitinated after oxidative stress [19]. This again raises the question on the nature of the proteins ubiquitinated after oxidative

stress. Here we identified overall 24 different ubiquitinated proteins, which can be allocated to several groups: chaperones, energy metabolism, cytoskeleton/intermediate filaments, protein translation/ribosome biogenesis and a clathrin protein **(Fig. 3, Fig. 4)**. Compared with other studies conducting a proteomic approach to identify ubiquitinated proteins in untreated cells we find some similarities. Vasilescu et al. determined ubiquitinated proteins in human cancer cells (MCF-7) by mass spectrometry [31]. Similar to our results they also identified the chaperones Hsp60, Hsp70, Hsp90, the eEF1A and filaments like actin, β tubulin or filamin A in untreated cells. In a second study Vasilescu et al. could confirm the ubiquitination of Hsps and some cytoskeleton proteins like actin [32]. The results of Vasilescu et al. and our results are also in accordance with the findings of Matsumoto et al, which did a large scale proteome analysis of ubiquitinated proteins in HEK239T cells [33]. After protein categorization they also demonstrated the ubiquitination of Hsps, ribosomal and translation related proteins, proteins participating in the energy metabolism and proteins of the cytoskeleton [33]. In addition to these studies we also made a proteomic characterization of ubiquitination after oxidative stress. Upon the treatment with H_2O_2 we identified proteins of the untreated control and some new, additional proteins. Interestingly, some of the proteins that were already identified in the control increase in the ubiquitin fraction upon oxidative stress. Especially Hsps are more ubiquitinated after H_2O_2 treatment, whereas the cytoskeleton proteins remain largely unchanged. Whether all these ubiquitinated chaperones are destined for proteasomal degradation or whether some of them are ubiquitinated to change their functionality is not yet clear.

In summary we could demonstrate for the first time that in response to oxidative stress HSP proteins are preferentially ubiquitinated, whereas the carbonylated/oxidized proteins are not. Interestingly, all of the identified ubiquitinated proteins are also ubiquitinated under control conditions, however to a lesser extent. Interestingly, the increase in the ubiquitination is not accompanied by changes of the functional protein level in the cell lysate and seems to be selective for some proteins. The ubiquitination of chaperones is a late event after oxidative stress and takes place at a time point when most of the oxidized proteins are already removed, indicating that the chaperone activity of the proteins is not longer required. In conclusion this makes the enhanced ubiquitination after oxidative stress a regulatory phenomenon, reflecting the response of the cellular metabolism and not the removal of oxidized proteins.

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Abbreviations

CHIP	C terminus of Hsc70-interacting protein
DTT	dithiothreitol
eEf1α	eukaryotic elongation factor 1 α
GRP	glucose regulated protein
hnRNP U protein	heterogeneous nuclear ribonucleoprotein U
LC	lactacystin
USP2	ubiquitin specific protease 2

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Figure Legends

Figure 1 Protein oxidation and ubiquitination in WM-451 melanoma cells

Cells were treated and harvested as described in the Methods section. In **panel A)** one of three representative immunoblots of protein carbonyls (left part) and ubiquitinated proteins (right part) 16h after H_2O_2 and/or LC treatment is shown. Panel **B)** demonstrates the time dependency of protein carbonylation (left part) and ubiquitination (right part) after H_2O_2 treatment. One of three representative immunoblots is show.

Figure 2 Coomassie staining of ubiquitinated proteins

Cells were treated and harvested as described in the Methods section Furhteron the fraction of ubiquitinated proteins was isolated using a UbiQapture™-Q Kit. After the isolation of ubiquitinated proteins two fractions were analyzed: unbound fraction (UF) and elution fraction (EF), resulting from the deubiquitination of the captured proteins. Electrophoresis was peroformed in an 7.5% acrylamide gel, followed by Coomassie staining. The black arrows indicate the excised proteins from the UF fraction of the Coomassie gels, which were selected for determination by MALDI MS/MS analysis. The results of the identification are listed under the indicated numbers in **Table 1**.

Figure 3 Ubiquitination of proteins after oxidative stress

A) Summary and categorization of the identified ubiquitinated proteins. The identified ubiquitinated proteins from Table 1 were categorized into 5 different functional groups (light gray boxes). In the dark gray boxes below, all proteins are listed which were found in the control and after H_2O_2 treatment. **B)** Comparison between the quantified Coomassie bands of the control and the H_2O_2 samples. A ratio of one means that there are no differences between the protein levels of the control and the H_2O_2 treated cells.

Figure 4 Chaperones are preferentially ubiquitinated after H_2O_2 treatment

This graph visualizes the absolute amoung (panel A) and the share (panel B) of the ubiquitinated proteins of a given protein group under control and oxidative conditions. The values were calculated by summarizing the band intensity of the grouped protein bands (panel A) and by dividing the level achieved in panel A by the sum of all identified protein band densities. This result is given in percent.

Figure 5 **Expression of Hsp70, Hsp90 and actin in whole cell lysates and the isolated ubiquitin fraction.**

Cell lysates were obtained like it described in “Materials and Methods”. Ubiquitin fraction was isolated using the UbiQapture™-Q Kit as described above. “C” indicates the control, which was not incubated with H_2O_2 (black columns). The samples were treated H_2O_2, cells were harvested and protein analyzed according to the above described procedures 16h after hydrogen peroxide treatmentValues are the mean ± SD of 3 independent experiments.

Table(s)

Table 1

Ubiquitinated proteins in human WM451Lu melanoma cells upon oxidative stress and/or proteasome inhibition

Sample	Identification	Accession no.	Mascot score %	Theoretical mass (Da)	Sequence coverage %	No. of matched peptides	No. of matched MS/MS	Treatment
1	nucleophosmin isoform 2	gi 40353734	152	29617	23	5	3	H_2O_2
2	Chain A, Ca2+-Binding Mimicry In The Crystal Structure Of The Eu3+- Bound Mutant Human Macrophage Capping Protein Cap G	gi 21730367	329	38786	24	8	6	C, H_2O_2 , H_2O_2+LC, LC
3	actin, cytoplasmic 1	gi 4501885	602	42052	44	13	6	C, H_2O_2 , H_2O_2+LC, LC
4	Eukaryotic translation elongation factor 1 alpha 1	gi 48734966	484	50433	25	12	4	C, H_2O_2 , H_2O_2+LC, LC
5	Tubulin, beta	gi 18088719	565	50096	43	14	7	C, H_2O_2 , H_2O_2+LC, LC
6	Tubulin, alpha	gi 340021	858	50804	43		9	H_2O_2 , H_2O_2+LC
7	vimentin	gi 62414289	972	53676	41	18	13	C, H_2O_2 , H_2O_2+LC, LC
8	60 kDa heat shock protein, mitochondrial	gi 31542947	686	61187	32	14	8	C, H_2O_2 , H_2O_2+LC, LC
9	cytoplasmic chaperonin hTRiC5	gi 609308	128	22059	28	4	2	H_2O_2+LC
10	heat shock 70kDa protein 8 isoform 2 variant	gi 62896815	631	53580	38	10	5	C, H_2O_2 , H_2O_2+LC, LC
11	heat shock cognate 71 kDa protein isoform 1	gi 5729877	251	71082	22	7	5	H_2O_2+LC, LC
12	78 kDa glucose-regulated protein precursor	gi 16507237	288	72402	17	12	6	H_2O_2 , H_2O_2+LC, LC
13	heat shock protein HSP 90-alpha isoform 2	gi 154146191	782	85006	33	13	5	C, H_2O_2 , H_2O_2+LC, LC
14	heat shock protein gp96 precursor	gi 15010550	213	90309	14	9	2	H_2O_2
15	nucleolin, isoform CRA_c	gi 119591368	255	58576	15	5	3	H_2O_2
16	hnRNP U protein	gi 32358	156	89631	13	7	2	H_2O_2
17	CLTC protein	gi 30353925	179	189538	16	14	1	H_2O_2
18	myosin-9	gi 12667788	329	227646	14	25	7	C, H_2O_2
19	ubiquitin C splice variant	gi 54300702	100	17142	38	3	3	H_2O_2+LC, LC
20	ubiquitin	gi 229532	81	8446	25	3	1	H_2O_2+LC
21	FASN variant protein	gi 68533031	261	279819	11	22	3	H_2O_2
22	nonmuscle myosin heavy chain (NMHC)	gi 189036	129	145623	15	11	1	LC
23	filamin A, alpha	gi 119593150	303	250859	13	16	7	H_2O_2
24	fatty acid synthase	gi 915392	79	275542	7	8	2	H_2O_2+LC

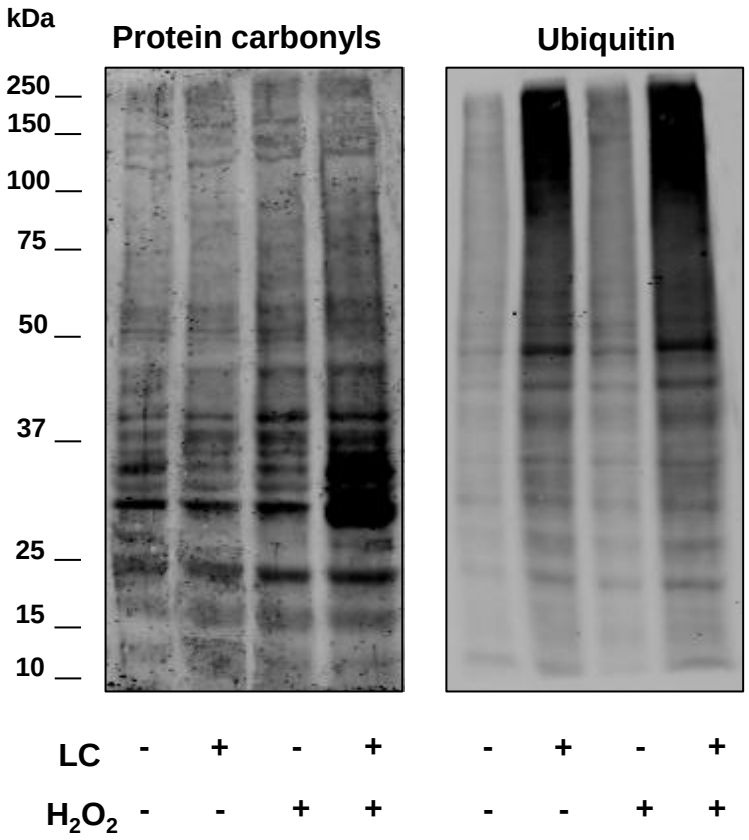
Spots of interest were identified by MALDI-TOF/TOF-MS as described under Material and methods

[a] Protein spot refers to numbered spots in Fig. 2

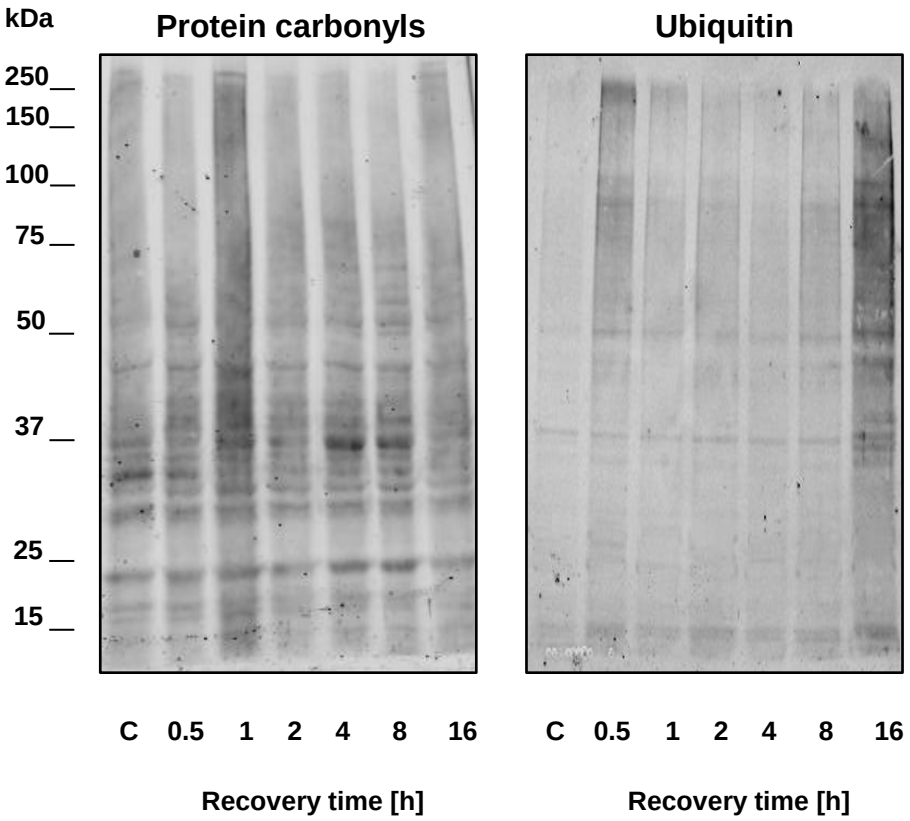
[b] Mascot protein scores greater than 55 are significant ($p<0,05$)

Figure(s)

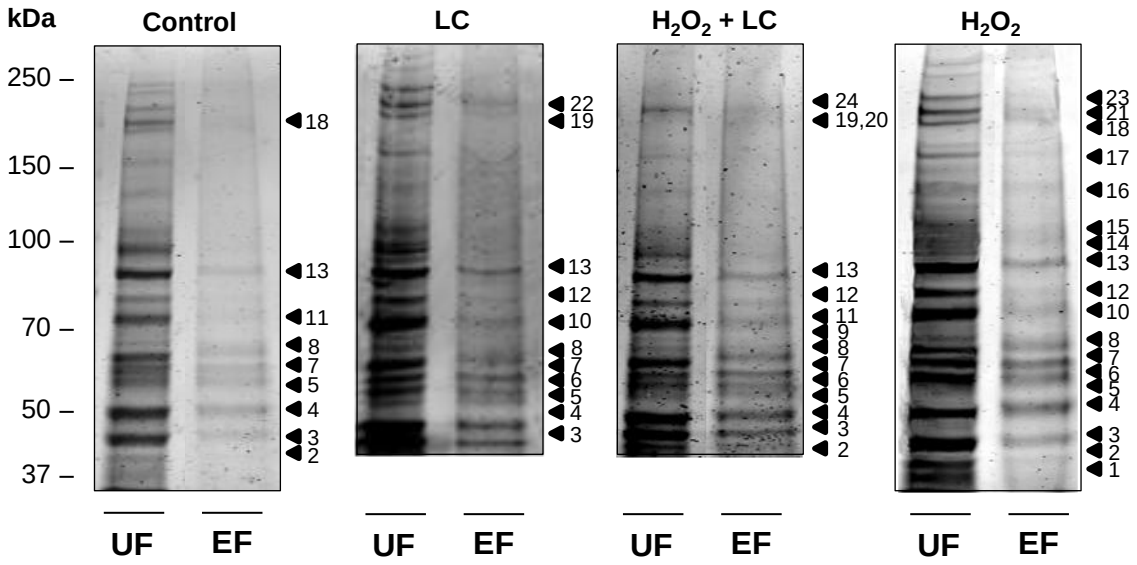
A) Protein carbonyls and ubiquitination after treatment with H_2O_2 and/or LC and 16h recovery time



B) Time dependent content of protein carbonyls and ubiquitination after H_2O_2

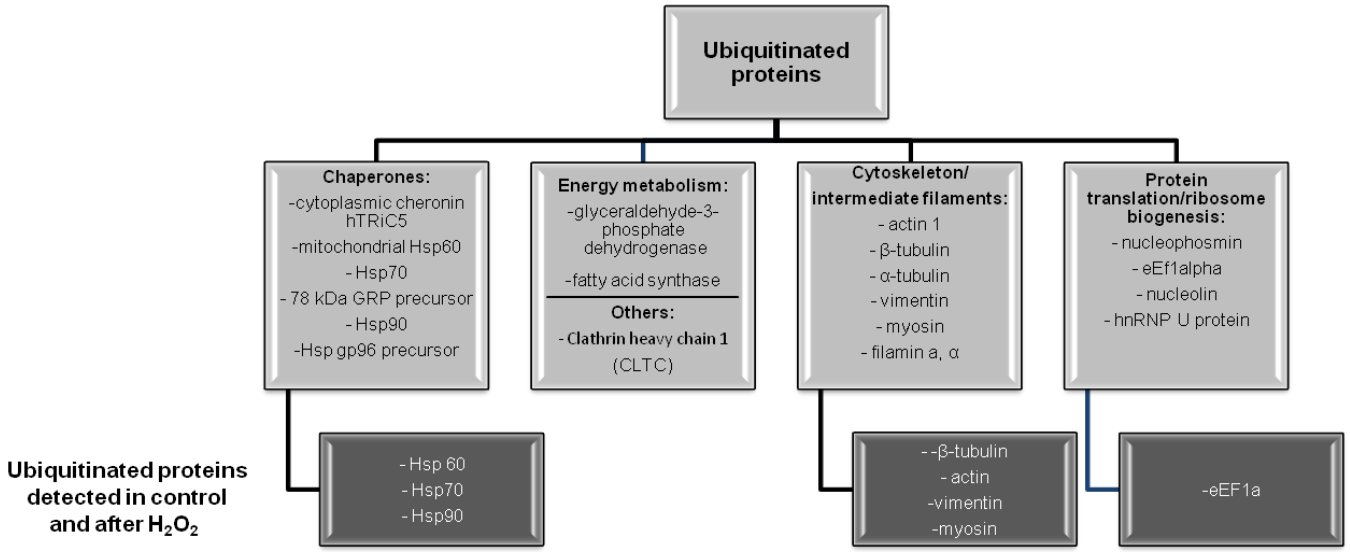


Kästle et al.: Fig.1



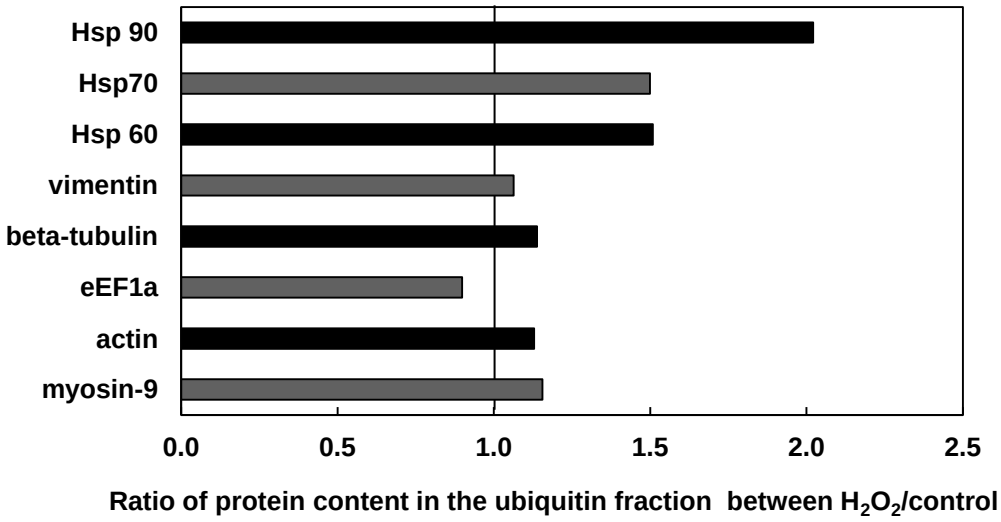
Kästle et al.: Fig.2

A) Classification of the identified ubiquitinated proteins



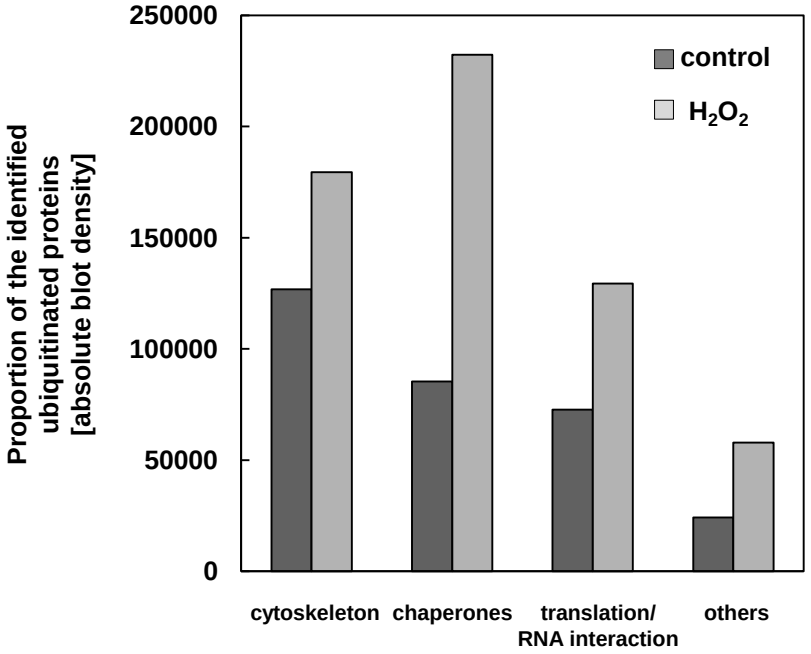
Kästle et al.: Fig.3A

B) Comparison of ubiquitination levels in control and H_2O_2 treated cells

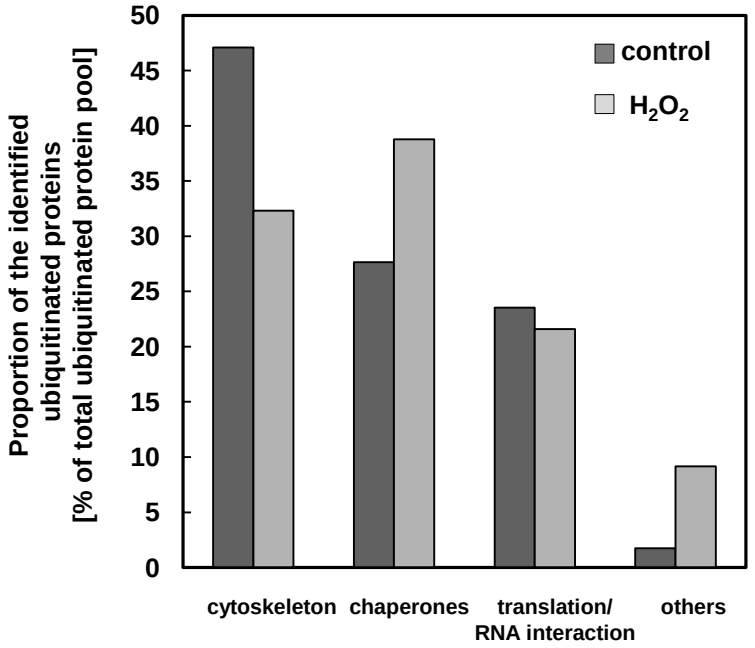


Kästle et al.: Fig.3B

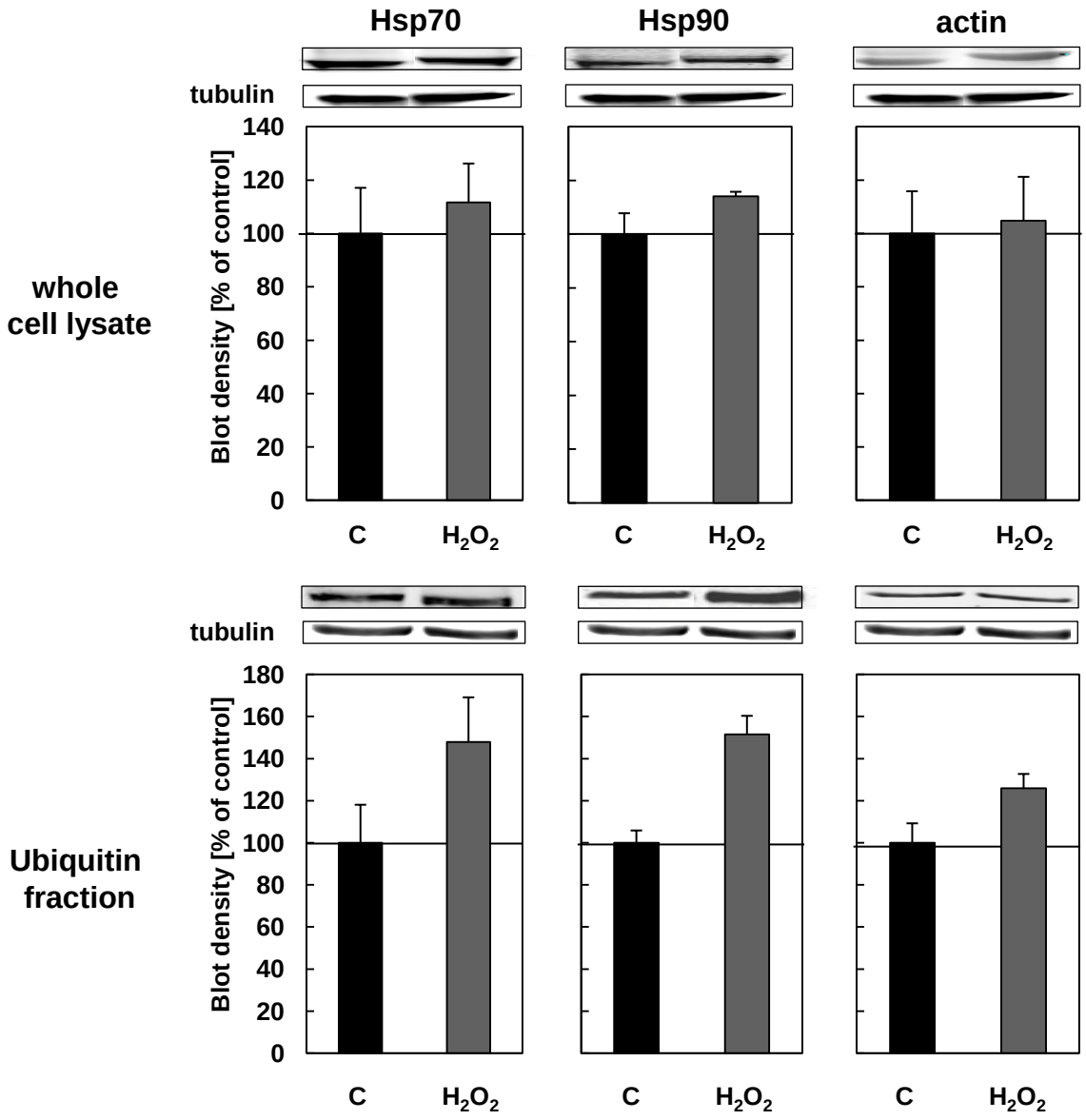
A) Absolute amount of ubiqutinated protein groups



B) Percentual contribution of selected protein groups



Kästle et al.: Fig.4



Kästle et al.: Fig.5

3.4 Publication IV: Histone deacetylase 6 (HDAC6) plays a crucial role in p38MAPK-dependent induction of heme oxygenase-1 (HO-1) in response to proteasome inhibition

Marc Kästle, Esther Woschee, Tilman Grune

The Journal of Biological Chemistry, 2012, in revision

The proteasome plays a crucial part in the network of protein homeostasis by degrading unfunctional, polyubiquitinated proteins. Inhibition of the proteasome results in an accumulation of polyubiquitinated proteins and urges the cellular system to deal with the increasing amount of polyubiquitinated proteins. In previous studies, it was described that there is an HDAC6-dependent up-regulation of some classical heat shock proteins, i.e. Hsp27and Hsp70, functioning as chaperones to prevent protein aggregation. It was also observed that there is an induction of heme-oxygenase 1 (HO-1) alias Hsp32 upon proteasome inhibition.

It could be verified in this study that there is an up regulation of classical heat shock proteins at gene and protein levels. As it was postulated, this up-regulation was dependent on the involvement of HDAC6. Moreover, it could be shown by HDAC6 repression, using RNAi technology, that the induction of HO-1 upon proteasome inhibition is also HDAC6 dependent. Thereby, HDAC6 is involved in a signal pathway, mediated by p38MAPK activation and Nrf-2 translocation into the nucleus, where HO-1 gene expression is started.

The results of these experiments give new insights of the role of HDAC6 in proteostasis upon proteasome inhibition and help to understand the limited efficiency of proteasome inhibitor based cancer therapy.

Own work:

- Cell culture and cell treatment
- siRNA transfection
- Western blots and verification of gene expression by RT-qPCR
- Separation of nucleus and cytoplasm
- Immunocytochemistry, Immunoprecipitation
- All enzymatic activity assays
- Writing of the manuscript, preparing the figures and preparation of statistical analysis

Histone deacetylase 6 (HDAC6) plays a crucial role in p38MAPK-dependent induction of heme oxygenase-1 (HO-1) in response to proteasome inhibition

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Running title: *Role of HDAC6 in HO-1 induction after proteasome inhibition*

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Keywords: proteasome inhibition, HDAC6, heme oxygenase-1, Nrf-2, Hsp

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Background: Proteasome inhibition leads to an up-regulation of several heat shock proteins, including HO-1.
Results: Deacetylation of p38 by HDAC6 enables the induction of HO-1 upon proteasome inhibition.
Conclusions: HDAC6 plays a crucial role in cell homeostasis upon proteasome inhibition.
Significance: This work shows the mechanistic processes after proteasome inhibition, which is important for cancer therapy.

Summary

The proteasome is responsible of the degradation of polyubiquitinated proteins. Inhibition of the proteasome leads to an accumulation of polyubiquitinated proteins and thus to an impairment of the cellular protein homeostasis. To prevent cellular damage upon proteasome inhibition there is an up-regulation of several heat shock proteins (Hsps), including Hsp27, Hsp70 and Heme Oxygenase-1 (HO-1). It was demonstrated that the induction of classical Hsps, such as Hsp27 and Hsp70, is dependent on a HDAC6-dependent mechanism which releases HSF-1 and induce the expression of new synthesized Hsps. In this study we demonstrate that the up-regulation of HO-1 upon proteasome inhibition is mediated by p38MAPK and Nrf-2. Interestingly we found additional evidence, proving the involvement of HDAC6 in the up-regulation of HO-1. By using RNAi technologies against HDAC6 we demonstrate that there is a lack of the expected induction of HO-1, Nrf-2 and phosphorylated p38 (pp38) after proteasome inhibition. Furthermore, we can show, that p38 is acetylated in unstressed cells and is a good substrate for HDAC6 mediated deacetylation. Therefore we propose that upon proteasome inhibition HDAC6 deacetylates p38, allowing the following phosphorylation of p38 and the consequently activation of NRF-2. NRF-2 enters the nucleus and functions as a transcription factor for HO-1.

Maintenance of cellular protein homeostasis is mainly mediated by autophagy, heat shock proteins and the proteasome. The proteasome is a cylindrical shaped, multicatalytic protease consisting of a 20S “core” structure and some regulation units, including the 11S and the 19S proteasome subunit. The complex of two 19S regulators with one 20S core structure builds the 26S proteasome, which is responsible for the ATP-dependent degradation of ubiquitinated proteins (1).
Ubiquitin is a highly conserved, small protein consisting of 76 amino acids (2). Polyubiquitination of proteins, which comprises at least 4 ubiquitin molecules, is mediated by three different sets of distinct enzymes in an ATP-dependent manner. The ubiquitin activating enzyme (E1) binds in a first step one molecule of ubiquitin at its C-terminal glycine. In a second step the bound ubiquitin molecule is transferred to an ubiquitin conjugating enzyme (E2). The third step comprises complex formation of the substrate protein, the E2 enzyme with the bound ubiquitin and one of the several E3 ubiquitin ligases. Depending on the type of the participating E3 ubiquitin ligase, ubiquitin is either tagged directly to the substrate or indirectly in a two-step mechanism (3).

Ubiquitin-depended degradation by the proteasomal system concerns mainly the large spectrum of short-lived-, regulatory-, onco- and structural proteins (4).
Inhibition of the proteasome leads to an accumulation of undegraded, ubiquitinated proteins (5). Trying to compensate the proteolysis of ubiquitinated proteins by the proteasome, there is an up-regulation of the heat shock response system, including heat shock factor 1 (HSF1), Hsp27 and Hsp70 (6;7). In 2007, Boyault et al. demonstrated that the class two histone deacetylase HDAC6 functions as a linker molecule between accumulating ubiquitinated proteins and the induction of heat shock proteins after proteasome inhibition (6). Moreover, it was observed in several studies that heme oxygenase 1 (HO-1), which is also known as Hsp32, is also up-regulated after proteasome inhibition (8;9). Interestingly, HO-1 does not participate in protein binding or stabilization, but rather functions as a potent antioxidant and anit-inflammatory protein, promoting general cell survival upon cellular stress (10). Induction of hmox1 gene transcription is mainly connected to the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2), which binds to several stress response elements (StREs) at the hmox1 promotor region (10). In unstressed cells NRF-2 is suppressed by Kelch-like ECH-associated protein 1 (Keap-1) and trapped in the cytosol (10). Upon cellular stress NRF-2 dissociates from Keap-1 and translocates into the nucleus. Dissociation and activation of NRF-2 is mediated by phosphorylation, which involves different kinases, such as protein kinase C (PKC), phosphoinositol-3-kinase (PI3K) or MAPK (11).
In the present study we show that induction of HO-1 upon proteasome inhibition is mediated by p38-MAPK-dependent activation of NRF-2. Furthermore, we demonstrate that HDAC6 is not only necessary to activate the “classical” chaperone response, but rather HDAC6 is also involved in the p38-dependent induction of HO-1 after proteasome inhibition.

Experimental procedure

Cell Culture and cell treatment - Human foreskin fibroblast (a kind gift from Dr. Wlaschek, University of Ulm), WM451Lu melanoma cells, HT29 colon carcinoma cells were cultured in Dulbecco’s modified Eagel’s medium, fortified with penicillin (100units/ml)/streptomycin (100µg/ml), 10% fetal calf serum and 1% Glutamax and K562 chronic myelogenous leukemia cells were cultured in RPMI medium fortified with 10% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C. Experiments were conducted with cells of passage 14-22. For siRNA transfection it was used either medium without penicillin/streptomycin or serum-free medium without penicillin/streptomycin. For inhibition of the proteasome it was used 20µM Lactacystin (LC) (Sigma-Aldrich, Germany) with 0.5h or 16h pre-incubation. The MAPK/p38 inhibitor SB203580 (Merck, Calbiochem) was diluted in DMSO and added to the culture medium 0.5h before LC application. Final concentration of SB203580 was 5µM (0.19% DMSO).

siRNA-Transfection - RNAi experiments were conducted with ON-TARGET plus smart pool siRNAs (Thermo Scientific/Dharmacon). ON-Target plus smart pool siRNA is a pool of four different siRNAs against one target with reduced off-target effects. For siRNA transfection, cells were seeded with antibiotic-free medium. Preparation and dilution of the used siRNA were carried out according to the manufactures instructions. Final siRNA concentration was 25nM. siRNA delivery was mediated by DharmaFECT-reagent 1 (Thermo Scientific/Dharmacon). siRNA were used for the knock-down of HDAC6 mRNA and ubiquitin activating enzyme E1 mRNA. Sequences of smart pool siRNA for HDAC6 are: (i) GGG AGG UUC UUG UGA GAUC, (ii) GGA GGG UCC UUA UCG UAGA, (iii) GCA GUU AAA UGA AUU CCAU and (iv) GUU CAC AGC CUA GAA UAUA. Sequences for E1 siRNA are: (i) GAA GUC AAA UCU GAA UCGA, (ii) CCA CAU AUC CGG GUG ACAA, (iii) CCU UAU ACC UUU AGC AUCA and (iv) GCG UGG AGA UCG UAA GAA. Following siRNA sequences were used for p38 (MAPK14): (i) GGA AUU CAA UGA UGU GUAU, (ii) UCU CCG AGG UCU AAA GUAU, (iii) GUA AUC UAG CUG UGA AUGA, (iv) GUC CAU CAU UCA UGC GAAA. siRNA sequences for Nrf-2 are: (i) UAA AGU GGC UGC UCA GAAU, (ii) GAG UUA CAG UGU CUU AAUA, (iii) UGG AGU AAG UCG AGA AGUA, (iv) CAC CUU AUA UCU CGA AGUU. As a control for unspecific siRNA effects we used scrambled siRNA (non targeting siRNA pool; D-001810-10-05) as a negative control.

Quantitative PCR analysis - After treatment with 20µM LC, total mRNA was isolated with the Qiagen RNeasy Mini Kit (QIAGEN, Hilden). Only mRNA with RNA integrity > 9 was used for further experiments.

Concentration and purity of mRNA was determined by a Nanodrop ND-1000 Spectrometer (peq LAB Biosciences, Erlangen). cDNA synthesis was performed with Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was conducted using a Bio-Rad IQ-iCycler and the Bio-Rad IQ SYBR Green Super-Mix. Primer sequences used in this work were as follows: human HSP27 forward, CTG CAA AAT CCG ATG AGA CT; human HSP27 reverse: ACA GGT GGT TGC TTT GAA CTT; human HMOX1 forward: GGT GAT AGA AGA GGC CAA GAC; human HMOX1 reverse, CCA CCA GAA AGC TGA GTG TAA; human HSPA1A forward: CCA TGA CGA AAG ACA ACA ATC; human HSPA1A reverse, GTC CTC CGC TTT GTA CTT CTC. As housekeeping genes for relative mRNA quantification beta actin and GAPDH were chosen; human beta actin forward, AGG ATG CAG AAG GAG ATC ACT; human beta actin reverse, AAG AAA GGG TGT AAC GCA ACT; human GAPDH forward, GGG TGT GAA CCA TGA GAA GTA; human GAPDH reverse, GTC CTT CCA CGA TAC CAA AGT.

Immunoblot Analysis - Cells were lysed at 4°C with SDS-Lysisbuffer containing 10mM TRIS-HCl (pH7.5), 1mM Pefabloc, 0.9% Nonidet P40 and 0.1% SDS. Protein concentrations of the samples were determined according to Lowry (12). 20µg of total protein were denatured at 95°C for 5min in reducing Laemmli Buffer (0.25M TRIS (pH6.8), 8% SDS, 40% glycerol and 0.03% Orange G). Samples were separated with 10% (w/v) acrylamide, followed by blotting on nitrocellulose according to standard procedures. Detection of the membranes was conducted with the Odyssey infrared detection system from LI-COR (LI-COR Biosciences, Bad Homburg). Blot evaluation was conducted with Odyssey Application Software Version 3.0 from Li-Cor. Following primary antibodies were used: ms anti Hsp70 (ab6535), ms anti Hsp27 (ab2790), ms anti HO-1 (ab13248), rb anti NRF-2 (ab62352), ms anti HDAC6 (ab56926), rb anti E1 ubiquitin activating enzyme (ab96737), ms anti GAPDH (ab8245), ms anti p38 (ab31828), rb anti phosphorylated p38 (ab32557), goat anti aspartate amino transferase (ab85857) (abcam, Cambridge, UK) and rb anti PARP(9542L) and rb anti acetyl-lysine (9441S) (Cell Signalling, Boston)

Determination of ROS with 2,7-dichlorofluorescin diacetate (DCF) – Cells were incubated 45 min with 5µM DCF, diluted in 99% ethanol, before they were harvested with 1x trypsin. For immediate treatment with 1mM H_2O_2, DCF was given 45 min before H_2O_2 application. Detection of DCF fluorescence was carried out, using black 96-well plates (Nunc, Denmark) and a 96-well plate reader (ex 485nm, em 528nm)

MTT Viability assay – Cell viability after proteasome inhibition and/or treatment with E1/HDAC6 siRNA was determined with a MTT-viability assay (Carl Roth). After cell treatment normal growth media was exchanged by MTT solution (10mg/ml) and incubated for 1.5 h at 37°C and 5% CO_2. Thereafter MTT solution was discarded and cells were lysed using DMSO containing 10% SDS and 0.6% acetic acid. Viability was determined photometric at a wavelength of 580nm (reference: 660nm)

Confocal laser scanning microscopy - Cells were seeded into small glass bottom dishes (diameter 35mm). After the indicated treatment cells were washed twice with PBS, fixed with ethanol/diethyl ether (1/1) and blocked with 1% FCS. Following the blocking step, cells were treated with 0.1% Triton X-100 for 5 min and incubated with primary antibodies (rb anti NRF-2, ab31163, abcam) at 4°C over night. As secondary antibodies we used “goat anti rabbit Alexa Fluor 546nm-conjugated”.Immediately before cells were used for microscopy, cell nuclei were stained with 10µM DAPI. Pictures were made with a LSM710 from Carl Zeiss (Carl Zeiss, Germany).

Isolation of nucleus fraction- Cells were washed twice with PBS and harvested with trypsine. After centrifugation, the supernatant was discarded and cell pellets were diluted in buffer A, containing 10mM Hepes (pH7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 µM DTT, 1 µM sodium orthovandate and 1x protease inhibitor cocktail “complete Mini” (Roche Diagnostics, Mannheim). After 45 min incubation on ice, samples were treated with Nonidet P40, vortexed and centrifuged. The supernatant was isolated as cytosolic fraction and pellet was diluted in buffer B, which consists of 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 µM DTT, 1 µM sodium orthovandate and 1x protease inhibitor cocktail “complete Mini” (Roche Diagnostics, Mannheim). Incubation time of 15 min was disrupted by vortexing and followed by centrifugation. The supernatant was used as nuclear fraction.

HDAC6 activity assay –Isolated HDAC6 was purchased from Calbiochem (Merck-

Calbiochem, Germany) and its activity was measured with a colorimetric HDAC Assay Kit from Millipore (Merck-Millipore, Germany). The assay was executed according to the manufacturer's protocol. HDAC6 activity was measured with 77.1U, 154.2U, .308.4U, 616.8U and 1233.6U. HDAC activity of cell culture samples was conducted with whole cell lysate.

p38 deacetylation assay and immunoprecipitation - Cell lysates were harvested as it is described in the "immunoblot analysis" -section. Immunoprecipitation was conducted with the "Immunoprecipitation Kit-Dynabeads Protein G" from invitrogen (Invitrogen Dynal, Norway). The samples were either incubated with HDAC6 (771 units) before or after immunoprecipitation with ms anti p38 (abcam, ab31828) antibody. HDAC6 incubation was conducted for 60 min at 37°C.

p38 activity assay – To determine the activity of p38MAPK of treated and untreated cells, p38 was first isolated by immunoprecipitation like it was mentioned before. The activity of immunoprecipitated p38 was then detected by the "Z´-Lyte Kinase Assay Kit-Ser/Thr 4 Peptide" from invitrogen (invitrogen, Carlsbad, CA). The assay was conducted according to the manufacturer's manual.

Results

Expression of heat shock proteins after proteasome inhibition – To confirm the recent findings of the literature, we verified the augmentation of Hsps following proteasome inhibition via RT-qPCR analysis and western blot detection. The samples of figure 1 show protein (A) and RNA (B) expression of HO-1, Hsp27 and Hsp70 after 0.5h or 16h LC incubation. Treatment with LC for 0.5h does not lead to a significant increase of all three observed Hsp proteins, neither at the state of mRNA-expression nor at protein levels. In contrast, incubation for 16h with LC results in a high significant augmentation of HO-1 gene expression compared to the control (Fig.1B). The significant increase of HO-1 with 16h LC treatment is also detectable at protein levels, where the LC treated sample has an 8-fold higher HO-1 protein expression than the control (Fig.1A). The observations for Hsp70 expression are similar. 16h LC incubation results in a drastic and high significant increase of Hsp70 mRNA expression and Hsp70 protein content compared to the control (Fig.1). For Hsp27 it was also observed that mRNA levels enhance significantly after 16h LC treatment, certainly the augmentation of Hsp27 protein content is not significant.

Gene silencing of HDAC6 and E1 ubiquitin activating enzyme –Before we tested the effects of polyubiquitination and HDAC6 on Hsp induction, we proved whether the used siRNAs lead to a significant knockdown of the target genes. Cells were treated respectively with siRNA for HDAC6, E1 or a scrambled siRNA as negative control to exclude side effects by the transfection procedure or incorporation of RNA into the cell. Gene silencing with siRNA against HDAC6 and E1-ubiquitin activating enzyme resulted in a significant down-regulation of the particular protein (Fig.S1A, Supplemented Data). Incorporation of a scrambled siRNA, functioning as a negative control, has no effect on HDAC6 or E1 protein content/regulation (Fig.S1A, Supplemented Data).

Influence of HDAC6/E1 on Hsp response and cell death following proteasome inhibition – Previously it was demonstrated that induction of Hsps upon proteasome inhibition is mediated by the accumulation of ubiquitinated proteins and the activation of HDAC6 (6). To verify whether this is also true for our cell system, we treated cells either with siRNA against HDAC6 or E1 ubiquitin activating enzyme and inhibited the proteasome by treatment with LC for 16h. As it is shown in figure 3, induction of Hsp70 after proteasome inhibition is indeed significantly reduced, when E1 or HDAC6 is knocked down (Fig.2A).

Due to the fact that HO-1 is also up-regulated when the proteasome is impaired, we wondered if HDAC6 may also play a role in HO-1 induction and if yes, where in the HO-1 induction cascade does HDAC6 interfere. Thus, we treated cells again with HDAC6/E1 siRNA and LC for 16h. Detection of HO-1 protein expression demonstrates a high significant augmentation of HO-1 after proteasome inhibition (Fig. 1 and 2A). Interestingly, pre-treatment with HDAC6 or E1 siRNA leads to a strong decrease in HO-1 expression upon proteasome inhibition (Fig. 2A). In addition we also detected Nrf-2, p38 and phosphorylated p38 (pp38) after LC treatment and siRNA transfection. Consistent to the results of HO-1, Nrf-2 increased also significantly after LC treatment. This effect is reduced by about the half when cells were transfected with HDAC6 siRNA. Transfection with E1 siRNA even reduces Nrf-2 to one third of single LC treatment. (Fig. 2A)

pp38 is also enhanced after proteasome inhibition, whereas p38 content remains largely unaffected. When cells are coincidently transfected with either HDAC6- or E1 siRNA to LC treatment, pp38 reaches protein levels of untreated control cells (Fig. 2A). Treatment with scrambled siRNA and coincident inhibition of the proteasome results nearly in same protein levels than LC alone, indicating again that the transfection and incorporation of siRNA evoke no random effects (Fig 2A).

To verify if this effect is not a cell typical phenomenon and due to the fact that proteasome inhibition is a potent treatment in anti-cancer therapies we also tested three different cancer cell lines (WM451Lu, K562 and HT29) for Hsp70, Ho-1, Nrf-2, p38 and pp38 after proteasome inhibition and/or silencing of HDAC6 via siRNA (Fig. 2B). In accordance with the results of the tested fibroblasts, there is also an induction of Hsp70, Ho-1, Nrf-2 and pp38 16h after LC treatment in all three cell lines. Knockdown of HDAC6 diminishes this effect drastically, whereas incorporation of a scrambled siRNA has no effects (Fig. 2B).

To verify the effect of HDAC6 and E1 on cell death, we measured cell viability via a MTT-viability assay. Compared to an untreated control gene silencing of E1 ubiquitin activating enzyme or HDAC6 reduce cell viability by about 40%. The coincident inhibition of the proteasome with LC reduces cell viability again by about 60% compared to the control, which means a very significant reduction of viable cells compared to siRNA alone (Fig. 2C).

In regard to the western blotting experiments, we concluded that HDAC6 is involved in HO-1 induction upon proteasome inhibition. Further on, this seems to be mediated by Nrf-2 and the involvement of the p38MAPK pathway.

Influence of HDAC6 on Nrf-2 after proteasome inhibition – When Nrf-2 is activated, it translocates from the cytosol into the nucleus and binds to StREs of target genes. Due to the observed augmentation of Nrf-2 upon proteasome inhibition, we wanted to verify if Nrf-2 is located in the nucleus after 16h LC treatment and if this can be interrupted by siHDAC6 treatment. Therefore we incubated normal fibroblasts and siHDAC6 transfected fibroblasts with LC for 16h. Following the incubation time we separated the nucleic fraction from the cytosol and used the two fractions for western blot detection. As expected, there is a strong accumulation of Nrf-2 in the nucleus fraction after proteasome inhibition (Fig. 3A), leading to a high significant increase compared to the control. When cells were additionally treated with HDAC6 siRNA, Nrf-2 content diminishes more than a half in the nucleus fraction and is comparable to the protein levels of the untreated control (Fig. 3A). The amount of Nrf-2 in the cytosolic fraction remains consistent at low levels and shows no clear differences between the different treatments (Fig. 3A). Further evidence for Nrf-2 translocation into the nucleus upon LC treatment and its attenuation by HDAC6 inhibition is given by Figure 4. There it is visualized by confocal laser scan microscopy that Nrf-2 clearly accumulates in the nucleus after proteasome inhibition and exceeds in total amount of Nrf-2 compared to the control and to the cells treated with HDAC6 siRNA (Fig. 4).

To exclude that Nrf-2 is mainly activated by oxidative stress after proteasome inhibition, we detected ROS levels after LC treatment and as a comparison hydrogen peroxide (H_2O_2). In Figure 3B it is shown that the generation of ROS after proteasome inhibition is nearly at the same levels than the controls. Moreover, treatment with 1mM H_2O_2 for 0.5 h and 16h recovery time has also only low effects in ROS generation and reaches the same levels than the control or treated with LC. Contrarily, generation of ROS increases eight fold immediately after the incubation for 0.5 h with 1 mM H_2O_2 (Fig. 3B).

The role of p38 in Nrf-2/HO-1 activation after proteasome inhibition – In Figure 2 it was demonstrated that p38MAPK is also activated upon proteasome inhibition, resulting in the phosphorylation of p38 (Fig. 2A/B). To prove whether p38 is involved in Nrf-2 and HO-1 signaling, we treated cells with the well known p38MAPK inhibitor SB203580 in combination with proteasome inhibition. Due to the fact that SB203580 is diluted in DMSO, the control group was treated with same volumes of DMSO or DMSO and LC. Compared to the DMSO control, treatment with 5µM SB203580 leads to a significant decrease of Nrf-2 expression (Fig 5A). In consistence with this down regulation of Nrf-2, HO-1 expression is also drastically attenuated after p38MAPK inhibition, resulting in a 4-fold reduction of HO-1 in SB203580 samples compared to the samples with DMSO + LC. Treatment with DMSO or SB203580 alone has no remarkable effects on NRF-2 or HO-1 expression. As it is demonstrated, application of 5 µM SB203580 or the corresponding volume of DMSO has no effects on protein expression of total p38 (Fig. 5A). Although, SB203580 is a

specific inhibitor of the kinase activity of p38 (8;13;14) , it seems to have also some influence on the phosphorylation level of p38, even if this effect is not significant (Fig 5). Possibly this is the result of the inhibition of p38 auto-phosphorylation.

To control the results with the p38 inhibitor SB203580, we also silenced p38 and Nrf-2 with RNAi technology. The knockdown of p38 and Nrf-2 with siRNA is clearly demonstrated in Figure 5B. As it is depicted, silencing of p38 retards the induction of Nrf-2 and HO-1 after LC treatment (Fig. 5B), which confirms the results of the pharmacological inhibition of p38. Silencing of Nrf-2 with a siRNA also inhibits the induction of HO-1 after proteasome inhibition with LC, demonstrating its involvement in the induction of HO-1 (Fig. 5B).
Summarizing the results of p38MAPK inhibition upon LC treatment, it seems evident that p38 plays a crucial role in NRF-2 activation and HO-1 induction in response to proteasome inhibition.
In regard to this results and the influence of HDAC6 to Nrf-2, HO-1 and phosphorylated p38, we hypothesized that HDAC6 interacts directly with p38 after proteasome inhibition.

Interaction of HDAC6 with p38 upon proteasome inhibition – There are two possibilities how HDAC6 influencing the activation of p38 upon proteasome inhibition: Either HDAC6 interferes with an up-stream p38 activator or HDAC6 deacetylates p38 directly, facilitating its phosphorylation and activation. Therefore p38 of untreated cells was isolated by immunoprecipitation (IP) and detected for p38 and acetylation status by western blotting. As it is depicted in Figure 6, p38 is clearly acetylated under unstressed conditions (Fig. 6A). Interestingly, when the proteasome is inhibited by LC, p38 shows no acetylation pattern in the IP-fraction. Further on, this effect can be abolished if LC treatment is accompanied with the HDAC6 siRNA (Fig. 6A). To clarify if acetylated p38 is really a substrate of HDAC6, we incubated either the whole fibroblast cell lysate with pure HDAC6, followed by an IP of p38 or we incubated isolated p38 with HDAC6 following an IP of p38. By detection of the acetylation status of the IP-fractions, it becomes obvious that acetylated p38 can be deacetylated by HDAC6. Incubation of isolated p38 with HDAC6 results in a total loss of acetyl at p38, whereas the unbound fraction of this IP shows still acetylated proteins (Fig. 6A). Previous incubation of the fibroblast cell lysates with HDAC6 before the IP results also in a drastic decrease of p38 acetylation. The unbound fraction of this approach still contains some acetylated proteins, indicating that not all acetylated proteins are substrates of HDAC6 (Fig 6A). We also incubated the whole cell lysate and the immunoprecipitated p38 with pure HDAC1, to check whether deacetylation of p38 is a specific feature of HDAC6 or an unspecific effect, which can be achieved by any HDAC. As it is depicted in Figure 6, incubation with HDAC1 has only weak to no effects regarding deacetylation of p38 (Fig 6A), showing a certain specificity of p38 as a substrate of HDAC6 dependent deacetylation.

To guarantee the specific deacetylation activity of the used HDAC6, increasing contents of the used HDAC6 were tested in a standardized HDAC activity assay. Thereby, resulted increasing amounts of HDAC6 in an increasing amount of deacetylated substrate, proving the functionality of the isolated HDAC6 (Supplemented data, Fig. S1B).

In regard to our theory there must be a measurable effect of HDAC activity and p38 activity after the inhibition of the proteasome and/or the involvement of HDAC6 siRNA and isolated HDAC6. Therefore we detected the HDAC activity and p38 activity after proteasome inhibition. Cell treatment with LC results in an increase of HDAC activity and a coincident augmentation of p38 activity by about 10% compared to the control (Fig 6B/C). Although HDAC activity was measured in whole cell lysates where it is difficult to measure drastic effects of HDAC activity due to the presence of all other HDACs, it is all the more impressive that silencing of HDAC6 via siRNA with coincident LC treatment results in a 25% reduction of HDAC activity compared to the control and a 37% reduction compared to the single LC treatment (Fig.6B).

As it was mentioned inhibition of the proteasome results also in an increase (~10%) of the activity of immunoprecipitated p38 compared to the untreated control (Fig. 6C). Nearly same effects are achieved when the immunoprecipitated p38 was incubated for one hour with pure HDAC6 (Fig. 6C).

Discussion

In the present study we demonstrate that inhibition of the proteasome by LC causes an induction of “classical” heat shock proteins and HO-1 (Fig.1). Moreover, we show that HDAC6 plays a central role in the coordination of the

induction of these two systems, which follows two different activation pathways.

Up-regulation of classical Hsps upon proteasome inhibition has been observed in several other studies before (6;15-17). Due to the impairment of the proteasome it is crucial to compensate the disrupted proteolysis of (ubiquitinated) proteins by binding and stabilizing these proteins, preventing protein aggregation and apoptosis (6;15-17). We also could show that elevation of Hsps upon proteasome inhibition is mediated by an enhancement of Hsp mRNA (Fig. 1), indicating a *de novo* synthesis of heat shock proteins and the preceded liberation and activation of HSF1, which is absolutely necessary for Hsp gene transcription (18). This is consistent with the results of Boyault et al. (6) and Wu et al. (8) which also demonstrated that there is a *de novo* synthesis of heat shock proteins after proteasome inhibitor treatment. It was also shown in the work of Boyault et al. that activation of HSF1 and induction of the chaperone response is initiated by HDAC6 after proteasome inhibition. Thereby, in unstressed cells, HDAC6 is involved in a complex with Hsp90, p97/VCP and HSF1, keeping HSF1 in an inactive, monomeric state. Upon proteasome inhibition HDAC6 binds accumulated, ubiquitinated proteins, leading to a dissociation of the complex and liberation of HSF1(6). Free HSF1 builds homotrimers in the cytosol and translocates into the nucleus, where HSF1 binds to heat shock elements (HSEs) of distinct target genes, like Hsp27 or Hsp70 (19) (Fig. 7). By gene silencing of either HDAC6 or E1 ubiquitin activating enzyme, we observed drastic decrease of the Hsp70 response after LC treatment (Fig 2), confirming the essential role of HDAC6 in Hsp activation upon proteasome inhibition.

Besides, the classical Hsps we also find enhanced levels of HO-1 mRNA and proteins after 16h LC treatment. Evidence from several studies has indicated that HO-1 elevation during proteasome inhibition is mediated by Nrf-2 and the activation of p38-MAPK pathway (8;20;21). Nrf-2 is a unique stress response transcription factor, involved in the induction of a variety of genes encoding for proteins, participating in the detoxification and metabolism of xenobiotics and oxidative stress response (22). Nrf-2 binds to StREs of target genes, which are also frequently found in the promotor region of HO-1 (10). Activation of Nrf-2 is mediated by a large variety of different kinases, such as protein kinase C (PKC), phosphoinositol-3-kinase (PI3K) or MAPK (11). Especially the p38-MAPK pathway seems to be an important activator of Nrf-2 in case of proteasome inhibition. Moreover the effect is independent from the used proteasome inhibitor. Studies with MG132 (8;20) or bortezomib (20;23) show similar results than we observed with LC. In all these studies it was suggested that p38-related activation of Nrf-2 is integrated in an anti-apoptotic response upon proteasome inhibition. Moreover, it can also be excluded that Nrf-2 is induced by oxidative stress after proteasome inhibition, due to the fact that 16h incubation with LC results in no remarkable elevation of oxidative stress (Fig. 3).

This fits very well with the anti-apoptotic properties of up-regulated HO-1. HO-1 cleaves its substrate heme into Fe^{2+}, biliverdin and carbon monoxide (CO) (24). Fe^{2+}, which is itself a high inducer of oxidative stress by employing the Fenton reaction (25), results in a compensating induction of ferritin and Fe-ATPase, functioning as an anti-oxidant and diminishing the amount of intracellular iron (24). The second reaction product, biliverdin, is immediately metabolized to bilirubin, which is also a strong anti-oxidant, scavenging peroxyl-radicals and diminishing membrane peroxidation (26). The anti-inflammatory and anti-apoptotic effects of HO-1 are mainly mediated by CO, acting as a second messenger. CO eases the expression of pro-inflammatory cytokines like TNF-α, IL-1β and MIP-1β and promotes the up-regulation of anti-inflammatory cytokines such as IL-10 (27).Further on, it was demonstrated that the increase of HO-1-related CO could suppress TNF-α mediated apoptotic cell death in endothelial cells after actinomycine D treatment (28). In addition it was observed that enhanced HO-1/CO expression lowering apoptotic tumor cell death by inhibition of p53 expression and blockage of mitochondrial cytochrome c release, during anti-tumor therapies (29).

Disruption of p38-MAPK activity by SB203580 or RNAi leads to a coincident breakdown of Nrf-2 and HO-1 expression (Fig. 5), which is another evidence for their dependency on the p38-MAPK pathway and in accordance with other groups, showing also a p38MAPK-dependency of Nrf-2 and HO-1 induction (8;14;20). Interestingly we found similar effects when we decreased the accumulation of ubiquitinated proteins or the expression of HDAC6 by RNAi technologies. siRNA against HDAC6 resulted in a drastic attenuation of p38 phosphorylation after proteasome inhibition. Further on we

demonstrated that p38, which is acetylated in unstressed cells, is a proper substrate for HDAC6-related deacetylation (Fig. 7B). Due to this we suggest a direct interaction of HDAC6 and p38 during accumulation of polyubiquitinated proteins, where HDAC6 deacetylates p38 and facilitates therefore the phosphorylation of p38. Moreover, phosphorylated p38 leads to the phosphorylation of Nrf-2, which dissociates consequently from Keap-1 and translocates into the nucleus, resulting in the transcription of Nrf-2 target genes, such as HO-1 (Fig. 7).

Because the proteasomal system is an integral part for the cellular protein homeostasis, it became a promising target in cancer therapy. The efficiency of single bortezomib application as a specific proteasome inhibitor was tested in many clinical trials, such as the SUMMIT-, CREST- and APEX trial (30-32). Nevertheless, success of this therapy is attenuated since cancer cells exhibit a resistance to bortezomib treatment (33). So far, it has been suggested that the compensational elevation of Hsps (6) and aggresome formation (34) in cancer cells are responsible for the diminished efficiency of proteasome inhibition in anti-tumor therapies (35). Both, the Hsp induction and aggresome formation, are coordinated by the activation of HDAC6 upon proteasome inhibition (6;34). In regard to our findings, the elevation of Nrf-2 and HO-1 is also a HDAC6 coordinated survival mechanism during proteasome inhibition, which could impair the efficiency of bortezomib treatment. To overcome the resistance to bortezomib, there are some recent trials, where proteasome inhibition is combined with HDAC class I and II inhibitors, resulting in an elevation of ROS, mitochondrial injury, caspase activation and apoptosis (36-38). Due to the fact that the three cancer cell lines tested in our study also show equal protein expression pattern after proteasome inhibition and/or HDAC6 knock-down than the tested fibroblasts (Fig. 2B), it should be promising to combine proteasome and HDAC6 inhibition.

Taken together this study demonstrates the involvement of HDAC6 in proteasome inhibition related induction of Nrf-2 and HO-1 and elucidates the crucial function of HDAC6 for protein homeostasis.

Abbreviations

DAPI	4′,6-diamidino-2-phenylindole
DCF	2′,7′-dichlorofluorescein
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HO-1	heme oxygenase-1
Hsp	heat shock protein
Keap-1	Kelch-like ECH-associated protein 1
NRF-2	nuclear factor (erythroid-derived 2)-like 2

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FIGURE LEGENDS

FIGURE 1. Induction of Hsps 0.5h and 16h after proteasome inhibition. Cells were cultured as described in "Experimental procedure" and treated with LC for 0.5h or 16h. Following the indicated incubation times total cell lysates or RNA were used for western blot- or RT-qPCR analysis. **A)** Representative western blots of HO-1, Hsp27 and Hsp70 and the related GAPDH blots of three independent experiments are depicted. Panel **B)** are the results of RT-qPCR. mRNA expression of target genes is adjusted to house-keeping genes and referred to the particular controls. **A)** and **B)** are means ± SD; n=3;*p<0.05,**p<0.01 versus untreated control; unpaired t-test.

FIGURE 2 Role of ubiquitination/HDAC6 in HO-1 induction during proteasome inhibition. Cells were cultivated, transfected and blotted as described in "Experimental procedure". Proteasome was inhibited with 20µM lactacystin (LC). **A)** Depicted western blots are an example of three independent experiments with human dermal FF95 fibroblasts. **B)** The shown western blots are examples of two independent experiments of three different cancer cell lines: WM451L melanoma cells, K562 chronic myelogenous leukemia cells and HT29 colon cancer cells. **C)** MTT-viability assay of LC and siRNA treated cells. Relative values are compared to the control. Columns are means ± SD, n=3, $^{\#}$p<0.001 versus control, *p<0.05, **p<0.01 versus siRNA treated cells, ANOVA, Tukey's post test.

FIGURE 3 **A)** Translocation of Nrf-2 into the nucleus after proteasome inhibition. Cells were incubated with LC for 16h. Separation of cytosolic and nucleic fraction was conducted like it is mentioned in "Experimental procedure". To verify the two fractions, poly(ADP-ribose)polymerase (PARP) was used to identify the nucleic fraction and aspartat-amino-transferase (ASAT) for cytosolic fraction. Columns are means ± SD of 4 independent experiments. $^{\#}$<0.01 versus control, *<0.05 versus siHDAC6+LC, ANOVA, Tukey's post test. **B)** Detection of ROS levels after LC treatment with DCF. Cells were incubated for 16h with LC and then used for DCF assay. Cells treated with 1mM H_2O_2 were either taken for DCF assay immediately after 0.5h incubation with H_2O_2 or they recovered 16h in normal growth medium before they were harvested for DCF assay. Columns are means ± SD of 3 independent experiments.

FIGURE 4 Visualization of Nrf-2 translocation into the nucleus after proteasome inhibition and HDAC6 siRNA treatment. Nucleus is depicted in red (DAPI) and Nrf-2 is presented in green (AlexaFluor546). Yellow areas represent the co-localization of Nrf-2 with the nucleus.

FIGURE 5 Influence of p38MAPK activity on HO-1 induction after proteasome inhibition. **A)** Cells were pre-incubated with 5µM SB203580 for 30min. After that, 20µM LC was added for additional 16h. Depicted are representative Western blots of four individual experiments. Columns are means ±

SD; n=4; *p<0.05; ***p<0.001, versus SB203580 treatment; unpaired students t-test. **B)** Verification of the role of p38 and Nrf-2 in HO-1 induction after proteasome inhibition. Cell treatment and siRNA transfection were conducted like it is mentioned in “Experimental Procedures”.

FIGURE 6 Influence of HDAC6 on immunoprecipitated p38. **A)** Cell lysates were either pre-incubated before the IP with LC / LC+siRNA HDAC6 / HDAC6 / HDAC1 (column 2/3/5/7) or first immunoprecipitated with p38 and then incubated with HDAC6/HDAC1 (column 4/6). The control (column 1) remained untreated and was also immunoprecipitated with p38 like it is described in “Experimental procedure”. UF = unbound fraction; IP = immunoprecipitation fraction. **B)** Detection of HDAC activity after proteasome inhibition and the influence of HDAC6 siRNA. Cell treatment and HDAC activity assay was conducted like it is explained in “Experimental Procedures”. Columns are means ± SD **C)** Measurement of p38 activity after proteasome inhibition and the role of HDAC6. After cell treatment, cells were immunoprecipitated with a p38 antibody. Isolated p38 was then used for the kinase activity assay. Incubation with pure HDAC6 lasted 1h. Columns are means ± SD

FIGURE 7 Schematic depiction of the role of HDAC6 on Hsp-regulation upon proteasome inhibition. As a consequence of proteasome inhibition there is an accumulation of polyubiquitinated proteins. HDAC& detects these ubiquitinated proteins by its ubiquitin-binding domain, leading to the dissociation of a complex which composes p97/VCP, Hsp90, HSF-1 and HDAC6 (6).Before HSF-1 enters the nucleus, the HSF-1 monomers trimerize and become phosphorylated. In the nucleus HSF-1 enables the transduction of “classical” Hsp-genes. Following deacetylation by HDAC6, p38 becomes phosphorylated and activates NRF-2, dissociating from Keap-1. Phosphorylated Nrf-2 enters the nucleus and induces the transduction of HO-1.

FIGURE S1 **A)** Validation of HDAC6- and E1 ubiquitin activating enzyme siRNA transfection. Transfection of fibroblasts was conducted like it is mentioned in “Experimental Procedure”. One representative example of HDAC6 and E1 western blots with corresponding GAPDH blots are depicted after siRNA transfection. Columns are means ± SD of three independent experiments. *p<0.05, **p<0.01,one way ANOVA, Tukey’s post test. **B)** HDAC6 activity was determined by deacetylation of a colorimetric acetylated HDAC class II substrate. Deacetylation of the substrate results in an augmentation of the colorimetric molecule

Figures

Figure 1

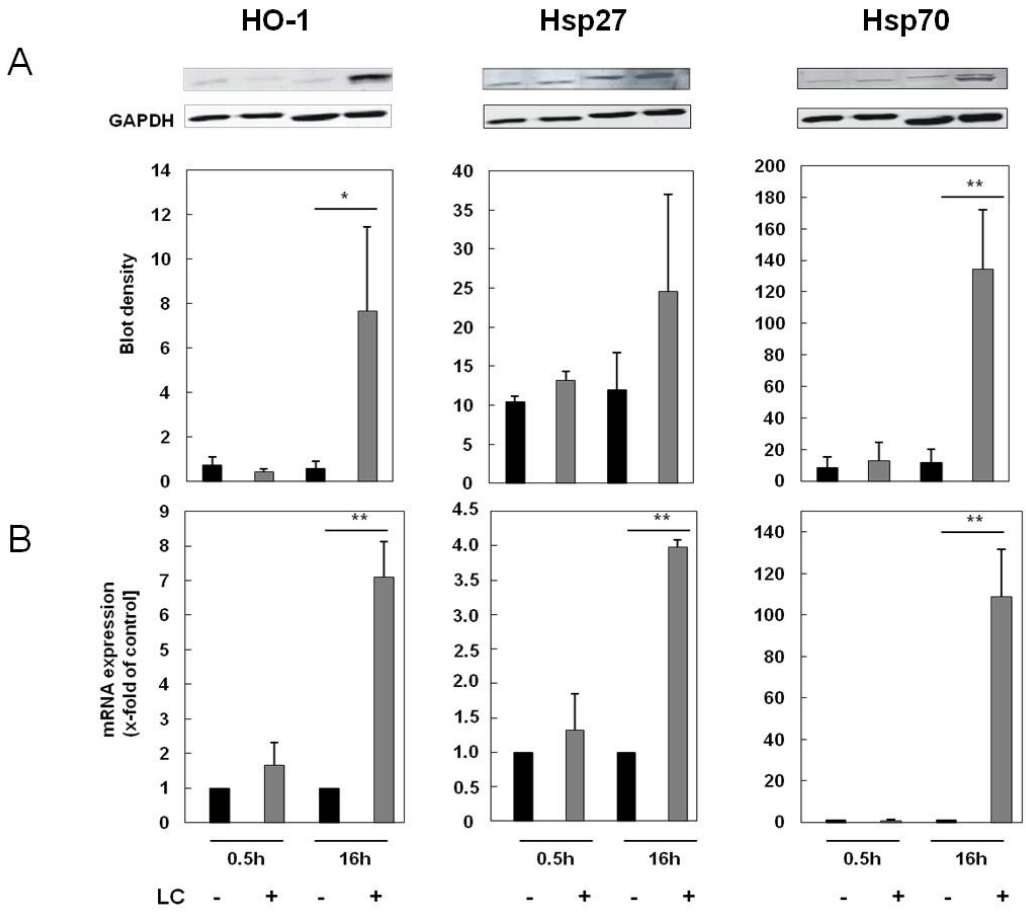


Figure 2

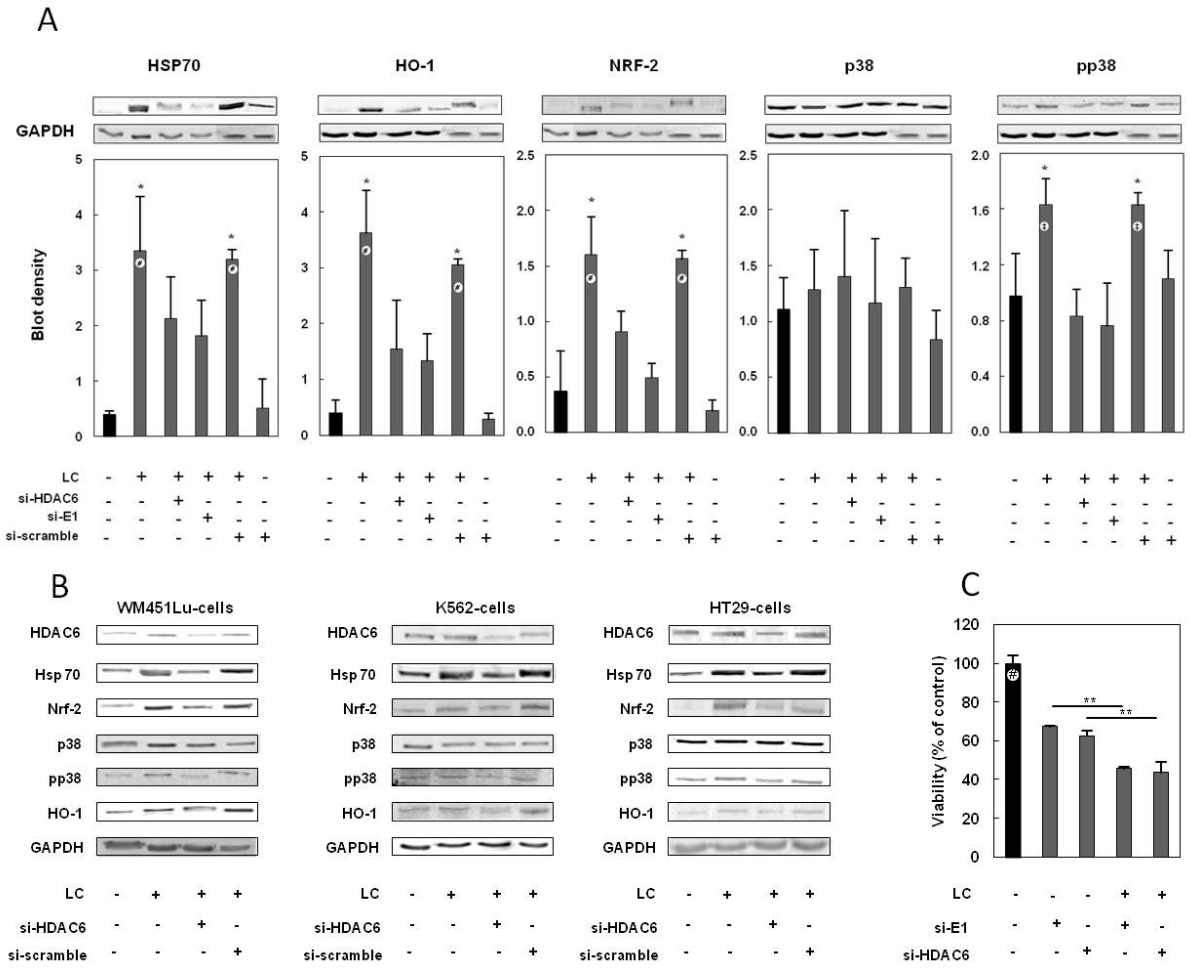

A
HSP70
HO-1
NRF-2
p38
pp38
GAPDH
Blot density
LC
si-HDAC6
si-E1
si-scramble
B
WM451Lu-cells
K562-cells
HT29-cells
HDAC6
Hsp 70
Nrf-2
p38
pp38
HO-1
GAPDH
LC
si-HDAC6
si-scramble
C
Viability (% of control)
LC
si-E1
si-HDAC6

Figure 3

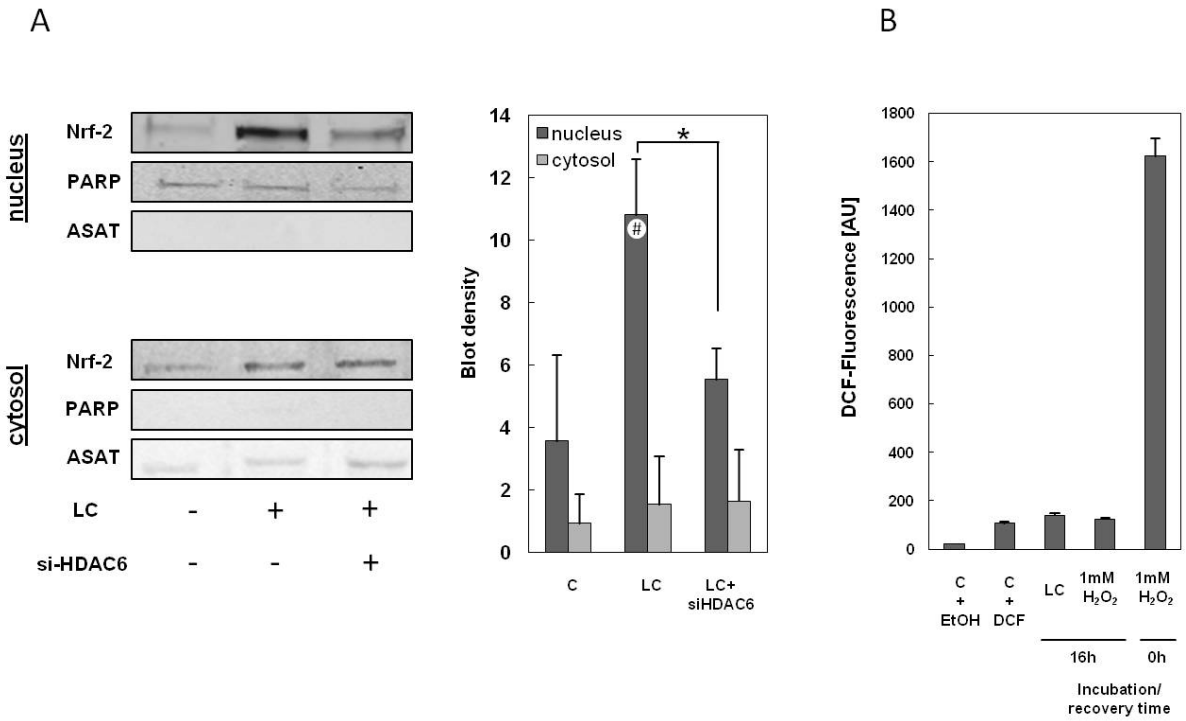

A
nucleus
Nrf-2
PARP
ASAT
cytosol
Nrf-2
PARP
ASAT
LC - + +
si-HDAC6 - - +
nucleus
cytosol
Blot density
*
#
C
LC
LC+ siHDAC6
B
DCF-Fluorescence [AU]
C + EtOH
C + DCF
LC
1mM H_2O_2
1mM H_2O_2
16h
0h
Incubation/ recovery time

Figure 4

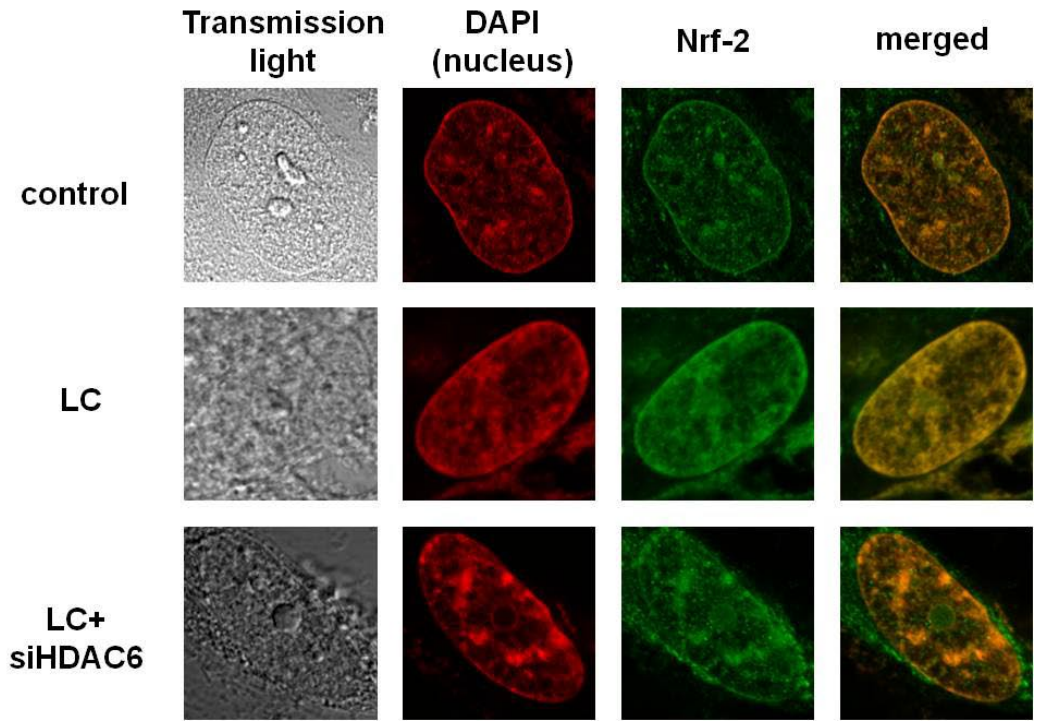

Transmission light
DAPI (nucleus)
Nrf-2
merged
control
LC
LC+ siHDAC6

Figure 5

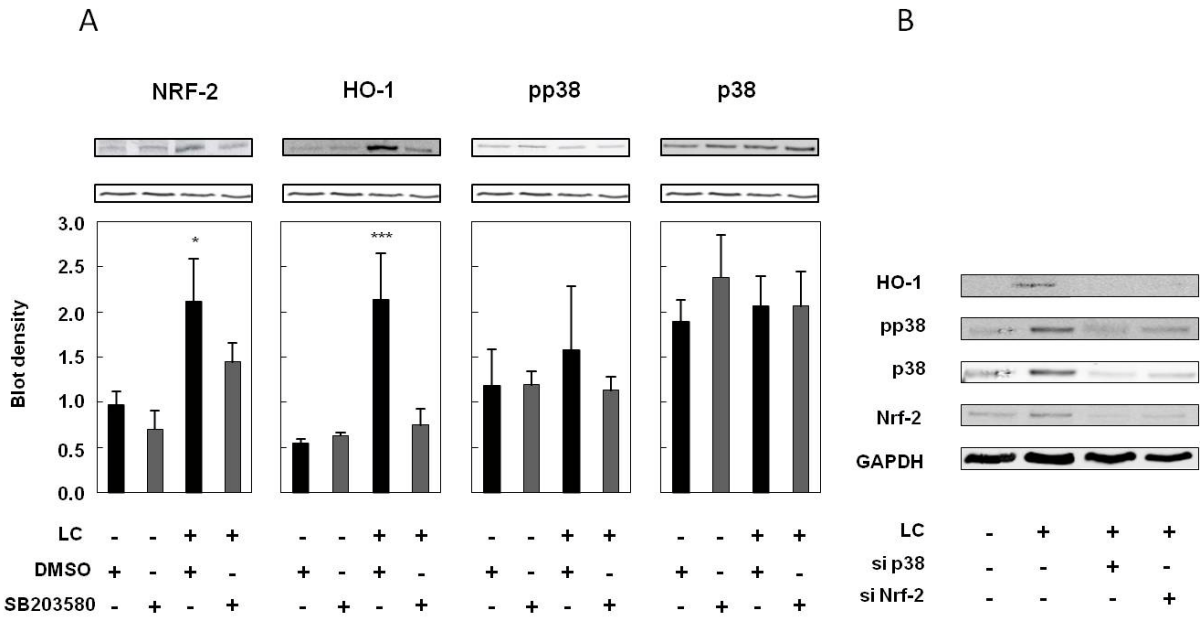

A
B
NRF-2
HO-1
pp38
p38
Blot density
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
***
LC
DMSO
SB203580
HO-1
pp38
p38
Nrf-2
GAPDH
LC
si p38
si Nrf-2

Figure 6

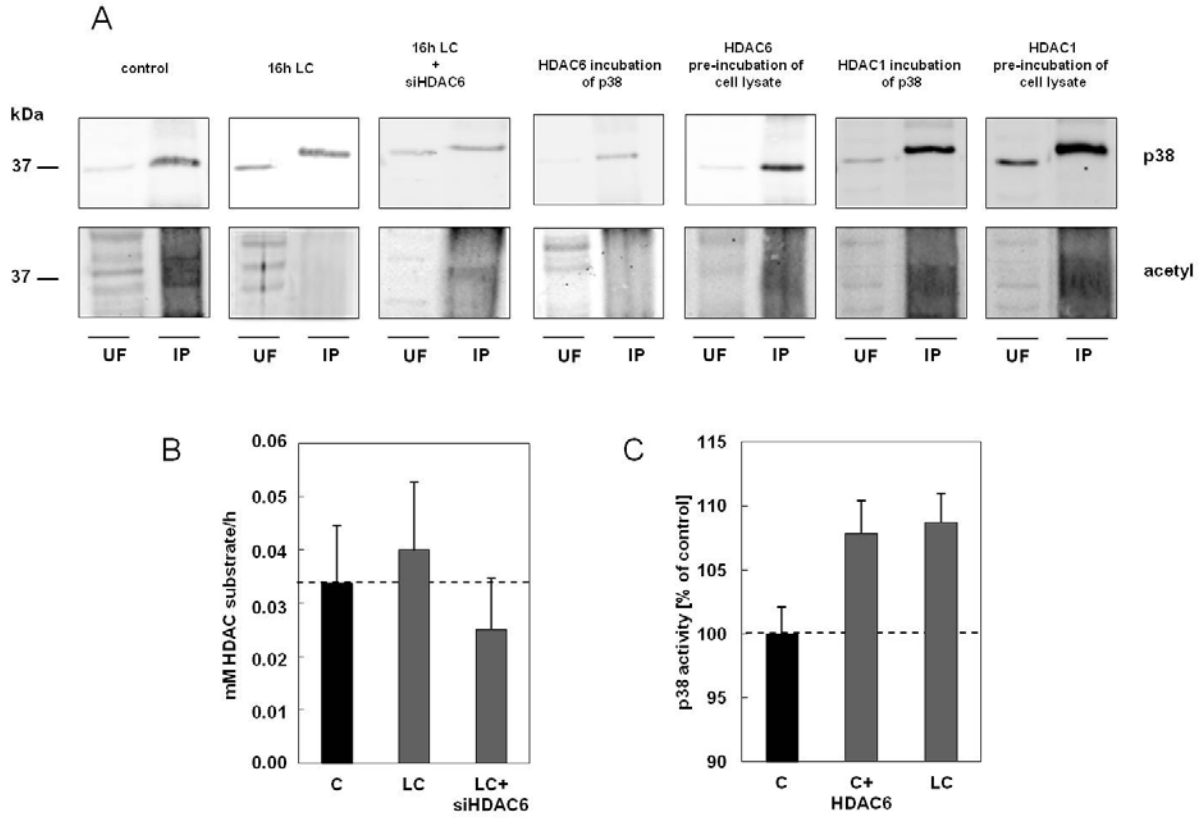

A
control
16h LC
16h LC + siHDAC6
HDAC6 incubation of p38
HDAC6 pre-incubation of cell lysate
HDAC1 incubation of p38
HDAC1 pre-incubation of cell lysate
kDa
37
37
p38
acetyl
UF
IP
B
mM HDAC substrate/h
0.06
0.05
0.04
0.03
0.02
0.01
0.00
C
LC
LC+ siHDAC6
C
p38 activity [% of control]
115
110
105
100
95
90
C
C+ HDAC6
LC

Figure 7

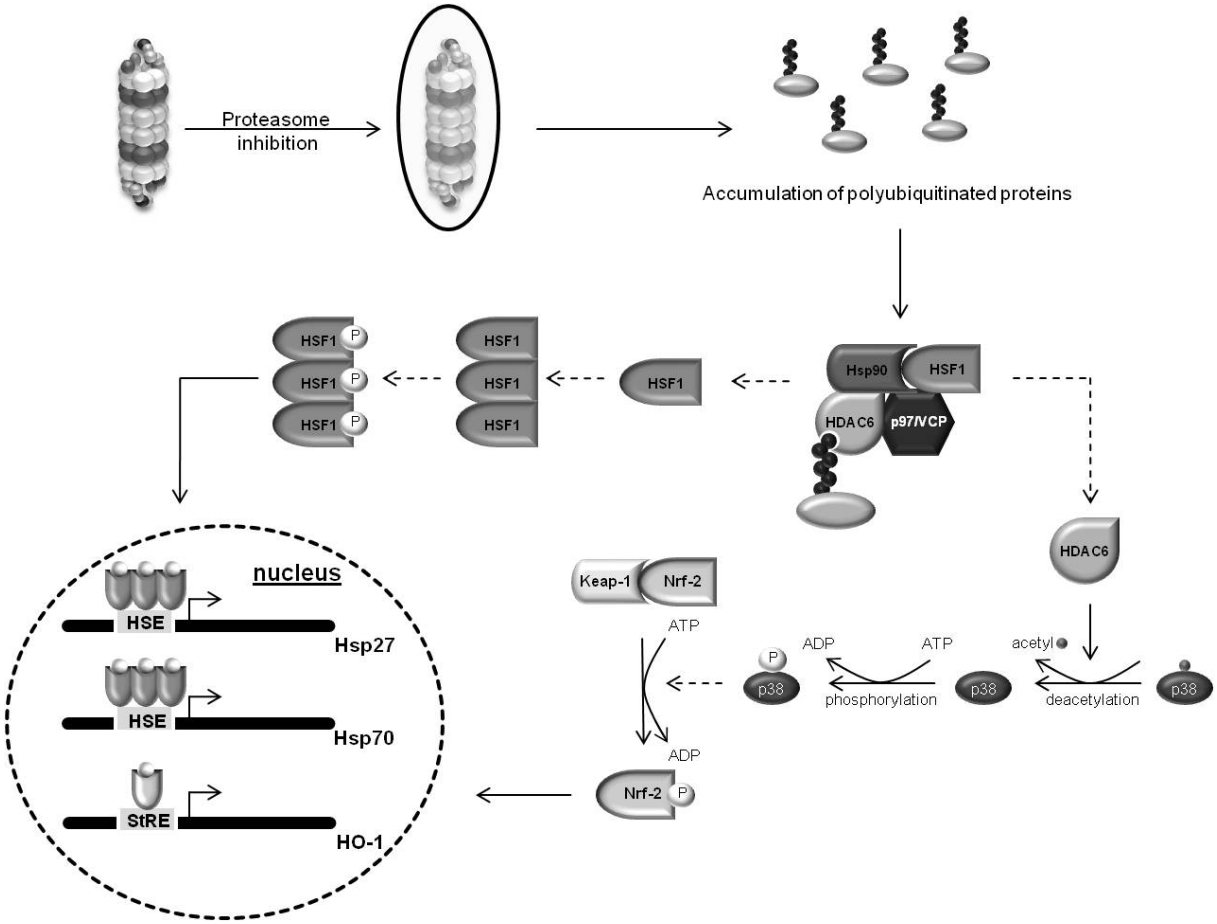
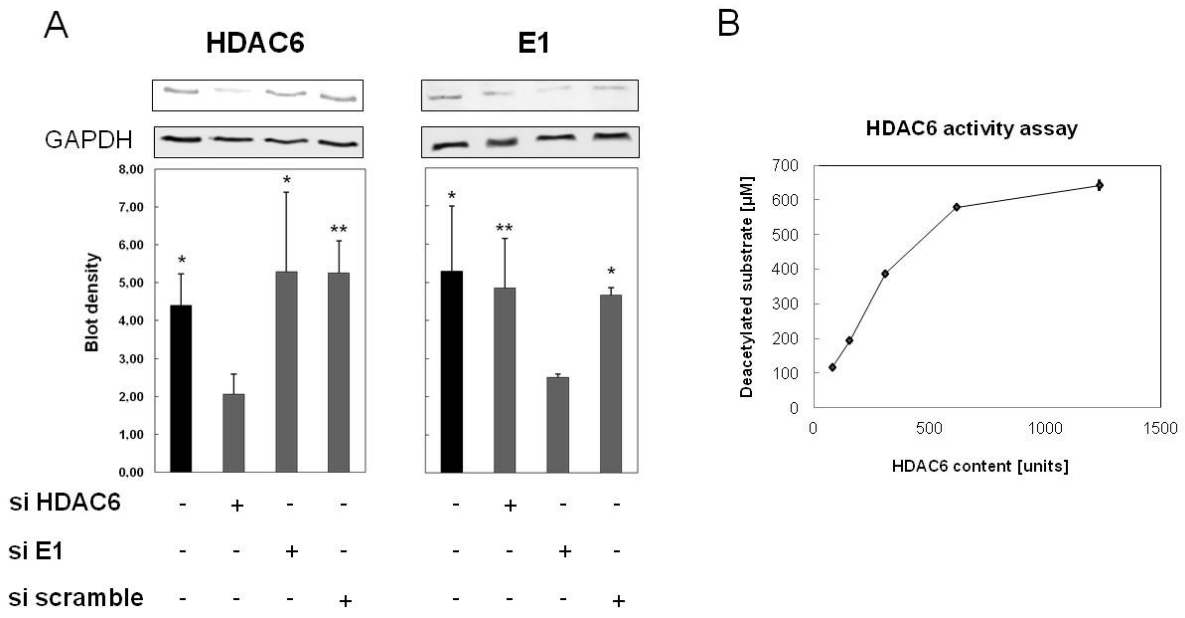

Proteasome inhibition
Accumulation of polyubiquitinated proteins
Hsp90
HSF1
HDAC6
p97/VCP
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HSF1
HDAC6
nucleus
HSE
Hsp27
HSE
Hsp70
StRE
HO-1
Keap-1
Nrf-2
ATP
ADP
Nrf-2
P
p38
ADP
ATP
phosphorylation
p38
acetyl
deacetylation
p38

Figure S1


A
HDAC6
E1
GAPDH
Blot density
8,00
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
si HDAC6 - + - - - + - -
si E1 - - + - - - + -
si scramble - - - + - - - +
B
HDAC6 activity assay
Deacetylated substrate [µM]
700
600
500
400
300
200
100
0
0
500
1000
1500
HDAC6 content [units]

4. Discussion

4.1 Assessment of protein carbonyls as a marker for oxidative stress

Since proteins are the most abundant molecules in the cellular system for oxidative modifications, there is the interest to quantify and qualify the oxidative damage of proteins. The very heterogeneous molecular structure of amino acids and proteins as well as different forms of oxidants, the environment, relative location and the availability of antioxidative systems, result in a large variety of oxidative protein modifications [149]. Many of these oxidative modifications are very special and affects only single amino acids, such as the reaction from tryptophan to kynurenine , tyrosine residues to dihydroxy derivates or methionine to methionine sulfoxide (MetO) [137]. Some of these modifications can easily be repaired, like it was shown for MetO, which is repaired by the thioredoxin $TR(SH)^2$-dependent methionine sulfoxide reductase [150]. Thus, such oxidative modifications are improper markers for protein oxidation, because they will decrease over time, sophisticating the result.

In contrast to the mentioned oxidative modifications, protein carbonyls affect more than one special amino acid residue. In Table 1 all amino acids contributing to carbonyl modification are listed **(Tab. 1)**. Additionally, protein carbonyls also emerge after oxidative cleavage of peptide backbones via the α-amidation pathway [137].

Table 1 Amino acids prone to oxidative carbonyl formation

(modified from [151])

Amino acid residues	Carbonyl derivative upon oxidation
Alanine	Acetone
Valine	Formaldehyde
Leucine	Isobutyraldehyde
Aspartate	Glyoxylic acid
Proline	2-pyrolidone, glutamic semialdehydes
Arginine	Glutamic semialdehydes
Lysine	Aminoadipic semialdehydes
Histidine	Asparagines
Tryptophan	N-formylkynurenine

Several reactive oxygen species like $^{\bullet}O_2^-$, $^1O_2$, H_2O_2 (reacting with metals, i.e. Fe^{2+}),$HO^{\bullet}$, $ROO^{\bullet-}$, hypochlorus acid, $ONOO^-$ and ozone can directly contribute to the

formation of carbonyl groups [152]. But not only primary reactions with oxidants lead to protein carbonyls. Secondary reactions of some residues with lipid oxidation products (HNE, MDA) or reducing sugars and their reaction products are also reasons for protein carbonyl formation [153-155].

Due to the fact that protein carbonylation affect nearly 50% of all proteinogenic amino acids (Tab.1) and also occurs after oxidative cleavage of peptide backbones it seems to be a highly suitable marker for all proteins upon oxidative stress. Moreover, protein carbonyls function as markers for primary and secondary oxidation reactions. As it was mentioned, protein carbonyls can be measured by different methods. By derivatization with 2,4-dinitrophenylhydrazine, protein carbonyls can be detected via western blotting, ELISA technique, ICC and IHC, allowing to make a point about the quantity of protein carbonyls, the affected proteins and the cellular/histological localization of protein carbonyls [151,156]. In regard to this reasons protein carbonyls are a well established, frequently used and trustworthy marker for oxidative stress and oxidative modified proteins [157-161]. Induction of oxidative stress in WM451Lu melanoma cells by application of UV-A irradiation or H_2O_2 results also in a dose-dependent and time-dependent increase of protein carbonyls **(Publication II and III).**

Since D. Harman postulated the free radical theory of ageing in 1956, it is accepted that ROS plays also a important role in the ageing process [162]. Thus, protein carbonyls are as well a widely used marker for age dependent protein damage. It was demonstrated that there is also an accumulation or protein carbonyls during the ageing process. This was shown for rats [163], aging yeast [164], aging nematodes [165] and several human tissues [166]. In humans, especially until the age of about 45, protein carbonyls accumulate exponentially with increasing life span [167].

4.2 The usage of siRNA for gene silencing and protein repression

To examine the role of HDAC6 and ubiquitin in the regulation of proteostasis upon proteasome inhibition, translation of these proteins was suppressed via the application of siRNA against HDAC6 and ubiquitin activating enzyme E1 (**Publication IV**). Due to the fact that these proteins/genes has already successfully silenced by the application of siRNA [168,169] as well as the transfection with shRNA [170,171], it was necessary to ponder which the most convenient method is. Due to the usage of vectors and their incorporation into the genome, shRNA has the great advantage that the gene-silencing-

responsible-sequence is stable expressed and suppress the target genes for several month. Moreover, the shRNA sequence is passed to daughter cells during cell division, guaranteeing an ongoing down regulation of the target genes [172]. Apart from that, shRNA transfection, which is normally done by lentiviral transfection, has to be conducted in laboratories with biosafety level 2-3. Due to the incorporation of a vector into the genome there are also some more difficulties. First, transfection of cells with viral vectors takes more time and effort than normal transfection with conventional siRNA and needs an additional selection of successfully transfected cells by a puromycin assay. Second, integration of viral vectors is a random mechanism. Therefore, the efficiency of gene silencing varies significantly depending on the integration site into the target genome, which can result in damages of important cellular genes [144].

Conventional siRNA remains in the cytoplasm after transfection, which precludes any interactions with the host genome. However, cells have to be transfected anew for each experiment with siRNA. After successful transfection with siRNA, gene silencing should be stable for at least 4-5 days. After that period siRNAs degrade over time and dilutes by cell division. Thus, stability and silencing efficiency is applicably dependent on the proliferation velocity. The experiments of publication IV were done with human dermal fibroblast at passage 14-22 **(Publication IV)**. Before transfection with siRNA against HDAC6 or E1, cells were seeded out in a way that they reach 80% confluency after 2 days, guaranteeing that dilution effects of transfected siRNA via cell proliferation is decreased to a minimum. Moreover, due to “contact- and cell density inhibition of growth”, human dermal fibroblasts are counted among slow proliferating cell lines, which also facilitate the usage of siRNA [173]. Another critical point of gene silencing, using siRNA is the occurrence of off-target effects, usually evoked by limited target sequence complementation to the siRNA guide strand [174], usage of very high concentrations of siRNA [175] or the induction of interferon response [176]. Lin et al. demonstrated in a widespread knockdown experiment, examining the involvement of kinases in the induction of HIF-1, that all of the “top candidates” resulted from off-target gene silencing [176]. Interestingly, they also found that some of these administered kinase siRNAs contribute directly to the degradation of the *Hif-1* RNA. In accordance to this, Fedorov et al. investigate the overall consequence of off-target effects on cell-physiology [177]. Fedorov et al. conducted as well as Lin et al. [176] a large-scale knockdown experiment using 176 randomly selected siRNAs. As a result

they demonstrated that off target effects lead to “false positives in broad RNAi-based phenotypic screens” and the occurrence of siRNA induced toxicity, resulting in apoptosis [178]. The observed siRNA induced off-target effects of this work were sequence-dependent, target-independent and relied on the ability to enter the RNAi pathway. Interestingly, they could show that chemical modification (2’-O-methyl-substitution) on position two of the guide strand of the siRNA limit the occurrence of off target effects [179] – an effect which was also confirmed by Jackson et al. [180]. An additional mechanism concerning the restriction of off-target effects is the use of a pool of different siRNA sequences against one target. By the coincident application of different siRNA sequences against the same target, the total amount of one specific siRNA is decreased on the one side and forms on the other side multiple target regions at the mRNA of interest. Avoiding the occurrence of off-target effects, the employed siRNA in **Publication IV** was purchased from Dharmacon RNAi Technologies. They offer “ON-TARGETplus SMARTpool” siRNA solutions, combining all the mentioned possibilities to decline off-target effects. The used siRNAs for gene silencing of HDAC6 and E1 were transfected as a pool made out of 4 different and preselected sequences. In addition they possess chemical modifications on both strands (antisense and sense), resulting in a blockage of sense strand uptake into the RISC and reduce the antisense strand induced off-target effects.

In a study from Rey et al. repression of HDAC6 in breast cancer cells was also conducted with “ON-TARGETplus SMART pool” siRNA from Dharmacon, resulting in a significant reduction in HDAC6 expression compared to the control and siRNA control [181]. This is absolutely in accordance to the results of Publication IV, using the same pool of siRNA sequences **(Publications IV)**.

4.3 The role of the proteasome in oxidative stress – Degradation of oxidative modified proteins

Since the identification and definition of the ubiquitin-proteasomal system, there is an ongoing debate about the degradation of oxidative modified proteins. Nevertheless, it is undisputed, that the proteasome is responsible for the degradation of the bulk (70-80%) of cellular oxidized proteins [182]. The essential question still is whether the majority of oxidized proteins become degraded by the 26S proteasome in an ubiquitin- and ATP dependent manner or by the 20S proteasome, which is ubiquitin and ATP independent

[183]. In Publication II and III it was demonstrated that oxidation of proteins with H_2O_2 or UV-irradiation affects nearly the whole content of cellular proteins, whereas ubiquitination of proteins occurs only between 250 kDa and 60 kDa **(Publication II, Publication III, Fig 1A)**. These results indicate that most proteins are not ubiquitinated upon oxidative stress. Therefore, proteins bearing oxidative derived carbonyl groups seem not to be preferentially degraded by the 26S proteasome. However, the increase of protein carbonyls after H_2O_2 + inhibition of the proteasome with LC compared with single H_2O_2 treatment point out that these oxidized proteins are degraded by the proteasome, which then has to be the ubiquitin independent 20S proteasome **(Publication III, Fig. 1A)**.

There are a multitude of publications supporting the proteasomal degradation of oxidative modified proteins in an ubiquitin-/ATP independent way. Already in 1989 the group of Davies could demonstrated with isolated 26S and 20S proteasomes that there is a preferential degradation of oxidized proteins by the 20S proteasome [184]. Hence, the isolated proteasome fractions were incubated with radio labeled hemoglobin (Hb)/ oxidized Hb or SOD/oxidized SOD in the absence or presence of ATP/ubiquitin and detected for their degradation. As a result they could show that the oxidized forms of the proteins were mainly degraded by the 20S proteasome. Degradation of oxidized proteins by the 20S proteasome remains nearly unchanged compared to the non-oxidized proteins. Interestingly addition of ATP and ubiquitin to the 20S fraction decrease the relative amount of degraded oxidized proteins [184].A specific degradation of isolated oxidized proteins without the involvement of ubiquitin had been shown in the following years for several other proteins, such as calmodulin, ornithine decarboxylase, catalase, serum albumin, myoglobin and aconitase [185-187]. In another study from the Davies group with E1 mutant ts20 cells, it was shown that despite the lack of a functional ubiquitin activating enzyme E1 oxidized proteins had been successfully eliminated by the proteasome [188]. Ubiquitin independent degradation of oxidized proteins is further supported by the observation of reversibly disabled ubiquitin activating E1 enzymes and ubiquitin conjugating E2 enzymes during oxidative stress [189,190]. This fits very well with the observations that have been made in publication II, where 1h after treatment with H_2O_2 protein carbonyls reach a drastic increase, but protein ubiquitination remain at basal levels, indicating a low activity of the ubiquitination machinery **(Publication II, Fig. 1C)**. Similar findings have been made for the 26S proteasome. After exposition to increasing concentrations of H_2O_2 it was observed that there is an overall decline in

detectable 26S proteasome activity, whereas the 20S proteasome remains largely unaffected [191,192]. In a recent published study from Grune et al. [193] it was shown, that there is no decline of 19S proteasome regulators during oxidative stress, but a dissociation of the 19S regulator from the 20S proteasome, captured and stabilized by Hsp70. Therefore it is suggested that the 19S regulator dissociates from the 20S proteasome to enhance the cellular capability to degrade oxidized proteins immediately after oxidative stress [194]. Moreover, lysine residues, which are the ubiquitin conjugation sites of proteins, are also prone for oxidative modifications, forming 2-aminoadipic semialdehydes. 2-aminoadipic semialdehydes belong also to the group of protein carbonyls and are inadequate for ubiquitin conjugation, reducing the amount of free available ubiquitin conjugation sites [195]. Due to the inactivation of the 26S proteasome and the ubiquitin conjugating system during oxidative stress it seems unlikely that the ubiquitin-26S proteasome pathway deals with the degradation of the bulk of oxidized modified proteins.

Due to an ubiquitin independent degradation of oxidized proteins by the proteasome, there must be another selection to enter the inner proteolytic core of the 20S proteasome. According to this, it was shown that there is a substrate degradation by isolated 20S proteasome upon incubation with hydrophobic tetra-peptides [196]. This was introduced by a peptide- induced opening of the α-ring due to the hydrophobicity of the peptides. Focusing on oxidized proteins, there is also an reported increase of hydrophobic moieties on protein surfaces after oxidative stress [197,198] (Fig.4.).

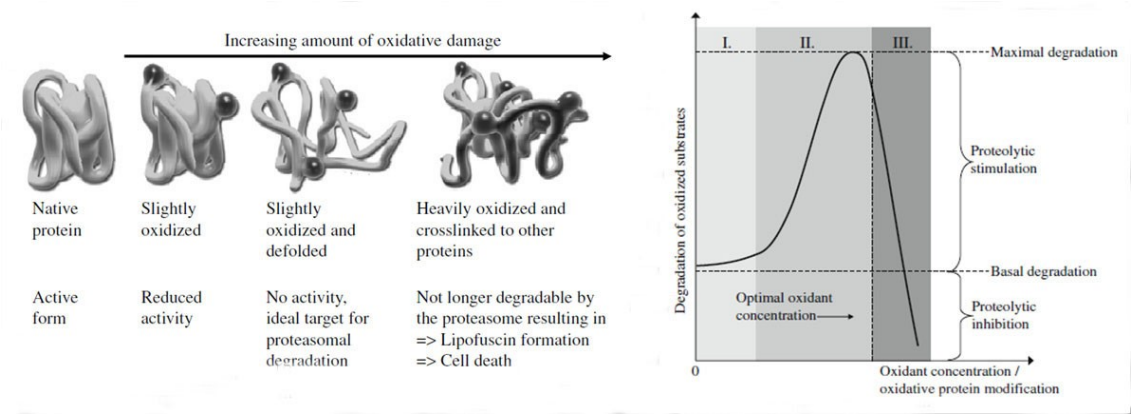


Figure 4 Different stages of protein oxidation and proteasomal degradation
Exposure of native proteins to an increasing amount of oxidative stress results in an ongoing unfolding process of the protein. Due to the protein unfolding the inner hydrophobic patches expose to the protein surface, functioning as signal for proteasomal degradation. Progressed unfolding of the protein leads to the formation of protein aggregates [199,200].

In consequence of oxidative modifications of some functional groups of the amino acid residues, like disruption of disulfide bonds or intermolecular interactions because of protein carbonylation, proteins are a subject of an ongoing unfolding process. In regard to the oxidation related unfolding process, Jung et al. has summarized a theory where proteins can be categorized into three different stages, according to the progression of their oxidation- and unfolding status (Fig. 4) [201]. Exposure to very low concentrations of ROS/RNS results only in a slight oxidation of the protein. At this stage the protein possesses a reduced activity, but is fully intact. It should also be possible to reduce the protein damage at this stage by the effort of the mentioned thioredoxin $TR(SH)^2$-dependent methionine sulfoxide reductase. Further oxidation of the protein can induce a critical damage of the protein, resulting in a partial unfolding. Due to this unfolding process, hydrophobic amino acids that usually are hidden in the inner side of soluble, globular proteins expose to the outer protein surface. According to Jung et al. and many others this is an ideal state for recognition and degradation by the 20S proteasome [202-205]. Heavy oxidation and unfolding of the protein increase its hydrophobicity and the probability for protein aggregation. Protein aggregates, such as lipofuscin, are no longer proper substrates for the proteasome and can even inhibit the proteasomal machinery [206]. Therefore, the proteolytic capability of the 20S proteasome to degrade oxidized proteins can be described like the curve of Figure 4. Slight oxidation of stage one does not require proteasomal degradation, but then with increasing oxidative modification, hydrophobicity and proteolytic degradation increase coincidently. At a critical point protein damage exceeds the proteolytic capability of the 20S proteasome, due to the formation of aggregates inhibiting the proteasome (Fig.4).

In contrast, there is also limited evidence for the involvement of ubiquitin and the 26S proteasome in oxidized stress [207-209]. Most of this work was conducted by the group of A. Taylor with human lens cells. They also detected protein carbonyls and ubiquitinated proteins upon exposure to H_2O_2 [210,211]. Interestingly, they observed an augmentation of ubiquitinated proteins upon increasing concentrations of H_2O_2 and/or the application of LC. In further accordance with the results of **Publication II/III** they found the vast majority of ubiquitinated proteins at the high molecular weight region. Surprisingly, they determined only the total amount of protein carbonyls, ignoring the distribution/molecular weight of the carbonylated proteins [212] or if they show the molecular weight of the protein carbonyls, they only depict the high molecular weight region [213]. Hence, the interpretation of the results is difference. The group of Taylor

suggested a “common” participation of the 26S proteasome pathway, involving the preferential ubiquitination of oxidized proteins [214,215]. In contrast, interpreting the data from Publication II and III it has to be suggested that only a minority of proteins are ubiquitinated upon oxidative stress and most of these proteins are also ubiquitinated in unstressed cells such as Hsp90, Hsp70, Hsp60, actin, β-tubulin and vimentin, indicating no relationship to oxidative stress **(Publication III)**. Maybe the elevation of ubiquitinated proteins upon H_2O_2 treatment is independent from the proteasomal system, but rather functioning as a signal for autophagy.

4.4 The role of ubiquitin during proteotoxic stress and the maintenance of proteostasis

The used stress stimuli in this work to introduce proteotoxic stress are H_2O_2/UV irradiation to induce oxidative stress and Lactacystin to inhibit the proteasome, leading to an unnatural accumulation of ubiquitinated proteins. As it is suggested and extensively discussed above, ubiquitin plays a minor role in the degradation of oxidized proteins. Nevertheless, there is an augmentation of ubiquitinated proteins in the later phase after treatment with H_2O_2 **(Publication II and III)**. Additionally, there are some new identified ubiquitinated proteins upon H_2O_2 treatment compared to the control **(Publication III)**. Interestingly, among these “H_2O_2-triggered” ubiquitinated proteins are the heterogeneous nuclear ribonucleoprotein U (hnRNP U), nucleolin or nucleophosmin. hnRNP U is a mRNA binding protein with regulating functions during the initial phases of the transcription by interaction with the RNA Pol II [216]. Nucleolin (also called C23) and nucleophosmin (also called B23) are two important multiple function proteins of the nucleus, participating in ribosome biogenesis, cell cycle regulation, chaperones of the nucleus and DNA repair [217,218]. Moreover, nucleophosmin has the ability to interact with both hnRNP U [219] and nucleolin [220]. Ubiquitination of these ribosomal associated proteins, especially of nucleophosmin, is a well known regulation mechanism [217,221]. The role of nucleophosmin ubiquitination and its cellular significance is not fully elucidated until yet. Although, ubiquitinated nucleophosmin is a substrate for proteasomal degradation, it is reported that ubiquitination can also result in increased protein stability of nucleophosmin [222]. Furthermore, it is suggested that K63-linked ubiquitination of ribosomal associated proteins regulates the translation efficiency of ribosomes [223]. Because nucleolin and nucleophosmin are two abundant proteins, the consequences of their ubiquitination are

only speculative and not clear at present. However, it seems obvious that ubiquitination of such proteins upon oxidative stress are a fine tuned, regulative mechanism and no random degradation mechanism of some oxidative proteins. Certainly it is thinkable that ubiquitination after 8h, 16h or 24h after oxidative stress serves to degrade the up-regulated stress response proteins in the cytosol and in the nucleus in a controlled and regulated manner.

Inhibition of the proteasome, as it was used in Publication II-IV, is automatically associated with the accumulation of undegraded, polyubiquitinated proteins. Accumulation of polyubiquitinated proteins leads to proteotoxic stress and acts coincidently as a multivariable signal, initiating a number of response mechanisms that cope with proteasomal inhibition. Increasing masses of polyubiquitinated proteins tend to form larger protein aggregates if proteasomal inhibition remains not responded or last over a longer period of time [224,225]. Since the amount of (mono-) ubiquitin molecules is limited in the cell, there is a shortage of free available ubiquitin molecules after or during proteasomal inhibition. Moreover, the reduction of free available ubiquitin in the cytoplasm results in a shift of ubiquitin between the two major ubiquitin pools. Due to the lack of available ubiquitin in the cytoplasm there is an observed redistribution of ubiquitin from the nucleus to the cytosol, accompanied by a coincident deubiquitination of histones [226,227]. Besides acetylation and methylation, ubiquitination is a major regulator of histone function. In mammalian cells ubiquitination is mainly found on the histones H2A and H2B which is associated with condensed DNA, gene silencing, regulation of RNA polymerase II and transcription regulation [228-230]. Thus, it is suggested that histone deubiquitination and the accompanied activation of gene expression is a calculated response to the proteotoxic stress of proteasome inhibition.

In addition, the accumulated polyubiquitinated proteins function as signal which can be detected by HDAC6, resulting in the activation of several response mechanisms: (i) stabilization and prevention of aggregate formation by the induction of classical Hsps, (ii) elimination of polyubiquitinated proteins/aggregates by HDAC6 mediated aggresome formation and autophagy, (iii) containment of inflammation by the induction of HO-1 and Nrf-2 pathway and (iv) reduction of proteotoxic stress mediated apoptosis by the induction of classical Hsps and HO-1 **(Publication IV)** [231-233] (Fig. 5). The particular effects and mechanisms of HDAC6, Hsps and HO-1 are discussed in detail in the following chapters below.

4.5 The role of heat shock proteins during proteotoxic stress

In unstressed situations protein homeostasis is balanced by the controlled degradation of proteins by the proteasome as well as the folding and stabilization of proteins by the constitutive expressed chaperones of the Hsp family [234]. In stress situations, affecting proteostasis, it is absolutely indispensible that the particular preservers of proteostasis have the ability to adapt by increasing their activity or their total amount. Concerning Hsps, there is a drastic increase of several Hsps, highly regulated and initiated by inducible HSF-1 or in case of HO-1 induction by Nrf-2 [235,236] (**Publication IV**). Proteotoxic stress can be evoked for example by heat shock, oxidative stress or proteasome inhibition, resulting in an accumulating mass of unfolded/misfolded, ubiquitinated, aggregated and unfunctional proteins which jeopardize cell viability. In case of proteasome inhibition, which is the stress stimulus in Publication IV, there is in accordance with previous studies an up-regulation of Hsp27, Hsp70 and HO-1 mRNA and proteins **(Publication IV)** [237-241]. Although it seems evident that there is a responsive induction of chaperones, the kinetic of this induction varies between different cell lines and species. In the chicken erythoblastic cell line HD6 Hsp70 induction reaches its peak already after 2 and 4 hours upon proteasome inhibition with MG132 [242], whereas inhibition with Lactacystin in xenopus laevis A6 cells, induction of Hsp70 and 30 was significant elevated after 12 hours [243]. The latter is in accordance to the results of **Publication IV**, suggesting a dependency to the given proteasome inhibitor and cell line. Elevation of HO-1 seems also to be a slower response, due to its first significant up-regulation after about 12 to 48 hours [244,245] (**Publication IV**).

The general effects of HO-1 activity can be divided into two major parts: its antioxidative properties and the anti-inflammatory/anti-apoptotic properties. Although the increase of oxidative stress upon proteasome inhibition is limited, which is demonstrated by DCF assay in Publication IV (**Publication IV**), and seems not to be the major reason for HO-1 induction, the anti-oxidative effects are also important during proteasome inhibition. HO-1 oxidizes with the help of NADPH cytochrome P450 reductase free heme to biliverdin, free Fe^{2+} and CO [246,247]. Fe2+, which is itself a reactive inducer of ROS by the involvement of the Fenton Reaction [118], induces the expression of antioxidative ferritin. Biliverdin is immediately reduced to bilirubin, functioning verifiable as a scavenger of ROS and RNS[248-250]. The anti-apoptotic effect and anti-inflammatory effect are mainly mediated by the liberation of CO. CO

acts as a second messenger, reducing the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, MIP-1β. On the other side CO mediates the up-regulation of IL-10 prolonging cell survival [251]. Moreover, there are some evidence that HO-1 related augmentation of CO inhibit p53 dependent cell death and the release of cytochrome C [252,253]. Recent findings demonstrated that p53 is a major factor of proteasome inhibition dependent apoptosis [254,255]. Because p53 is also a substrate of the UPS, p53 accumulates in the nucleus upon proteasome inhibition resulting in apoptotic cell death. Moreover, Latonen et al. demonstrated that ubiquitin is necessary to transfer p53 between nuclear and cytosolic compartments, resulting in a nuclear accumulation of p53 in the nucleus when there is a lack of free available ubiquitin during proteasomal inhibition [256]. Therefore, HO-1/CO mediated interaction with p53 is a regulating counterpart to the p53 mediated apoptosis upon proteasome inhibition.

The suggested regulation pathway in Publication IV, where HO-1 is induced by Nrf-2 and p38MAPK activation is in accordance with some other studies (**Publication IV**). Wu et al. [257] also found evidence for the involvement of activated p38MAPK in HO-1 up-regulation upon proteasome inhibition. Using SB203580 as a p38MAPK inhibitor, HO-1 induction reaches after proteasome inhibition only the same levels than the untreated controls [258]. In another study treating cells with proteasome inhibitors, it was shown that cellular Nrf-2 content is augmented and Nrf-2 is needed for the up-regulation of HO-1 upon proteasome inhibition [259]. The latter was confirmed by the co-transfection of transgenic KEAP-1 in combination with proteasome inhibition, resulting in a significant decrease of ARE-dependent genes, including HO-1 [260].

Focusing on oxidative stress, HO-1 is a very important cellular antioxidative defense mechanism, diminishing ROS and the resulting oxidative stress. Hence, cells lacking HO-1 or possess inhibited HO-1 activity, exhibit greater ROS concentrations and tend more to undergo cell death [261,262]. Interestingly, in **Publication III** major representatives of classical Hsps (Hsp60/70/90) have been found in the ubiquitin fraction, but HO-1 was not ubiquitinated. This fact leads to some suggestions: either HO-1 is degraded in an ubiquitin independent way, HO-1 has been degraded much quicker than 16h and does not appear in the ubiquitin fraction after16h, HO-1 is not degraded due to its importance in the antioxidative defense mechanism or HO-1 content in the ubiquitin fraction was too low for its determination. Due to the fact that HO-1 is an ER-membrane located protein, it is normally degraded by the ubiquitin-proteasome dependent ERAD process [263], which disproves the suggestion that HO-1 is not a

substrate for ubiquitination. Further on, in **Publication III** melanoma cells were also treated with LC inhibiting proteasomal degradation, resulting in the accumulation of all ubiquitinated proteins over the period of 16h. Thus, if HO-1 is ubiquitinated during that time, it had to be detected in that fraction, which was obviously not the case **(Publication III)**. Therefore it must be assumed that HO-1 is not ubiquitinated during (oxidative) stress situations to help keeping up cell homeostasis, function and survival. Certainly it is possible that HO-1 was not detected in the ubiquitin fraction because of a too low protein content of HO-1 in this fraction, however, this seems very unlikely due to the high induction of HO-1 during stress conditions **(Publication IV)** [264-266] and the mentioned accumulation of UPS substrates in LC treated cells.

The role of the classical Hsps during proteotoxic stress is a little bit different compared to HO-1. The observed up-regulation of Hsp27 and Hsp70 in Publication IV, initially provoked by the accumulated amount of ubiquitinated proteins, seems to be a compensational attempt of the cell to cope with the emerging amount of unfolded proteins. Increasing amounts of unfolded (polyubiquitinated) proteins, also augment the likelihood of the formation of protein aggregates, which can occur after proteasome inhibition or oxidative stress [267]. Protein aggregates are toxic, disrupt cell functionality and can lead finally to cell death. Thus, formation of protein aggregates correlates with the occurrence of several age-dependent diseases, such as neurodegeneration [268], AMD [269], cataracts [270], skin ageing [271] and atherosclerosis [272] (**Publication I**). Preventing protein aggregation is therefore one major task of Hsps. Especially the observed up-regulated Hsps 27 and 70 are crucial in the disaggregation process. It was shown by the group of Liberek that Hsp70 is the chaperone that participates in the initial step of disaggregation by disentangle polypeptides from aggregates [273,274]. Moreover, there is evidence that small Hsps (sHsps) support the disaggregation reaction of Hsp70 by their incorporation in high molecular protein aggregates. Thus, sHsps loosen the aggregate structure and make the incorporated polypeptides more susceptible to Hsp70 mediated disaggregation [275,276]. The co-localization of small Hsps with several cellular proteins such as actin- and intermediate filaments, ubiquitin ligases, DNA-binding proteins, HO-1 and proteasomal subunits after stress, elucidate the importance of sHsps in their function to keep proteins in a soluble confirmation [277]. It is suggested that about 33% of all cytosolic proteins retain in a soluble state by the help of sHsps in yeast [278].

In many organisms (bacteria, yeast, plants) Hsp70 transfer the disentangled polypeptide to the Hsp104/CIp machinery, which is a high molecular weight Hsp trying to refold the aggregated polypeptides [279]. For a long time there was no evidence for such a disaggregase in mammalian cells. p97/VCP, being also a part of the complex with HDAC6, Hsp90 and HSF-1 (**Publication IV**) [280], has shown to collaborate with Hsp70 and Hsp40 to refold soluble misfolded proteins and prevent protein aggregation [281]. However, there was no convincing demonstration of p97- catalyzed disaggregation using pure components [282]. In a recent published work from Shorter et al. [282] it was indicated that cell lysates from rat liver as well as from HeLa cells have the ability to disaggregate protein aggregates. Moreover, it was demonstrated that this disaggregase activity is mediated by the constitutive form of Hsp110 – Apg2 [282]. In this model Hsp110 interacts with Hsp70 and Hsp40, forming a disaggregase machinery, which is comparable to the yeast model. Although, the Hsp110- dependent disaggregase was able to disaggregate disordered, amorphous aggregates, it failed to catalyze the reduction of amyloids, suggesting that such aggregates have to be removed by autophagy [282].

Like HO-1, Hsp27 and Hsp70 have also anti-apoptotic properties when they are up-regulated. Hsp70 prevents apoptosis by inhibition of the apoptosis inducing factor (AIF), formation of the apoptosome and prevention of caspase 3 activation [283]. Hsp27 prevents the release of cytochrome c, interacts also with caspase 3 and is involved in the degradation of I-κBα, resulting the activation and translocation of NF-κB and thus in increased cell survival [284,285]. Further on, it was shown that Hsp27 possesses an ubiquitin binding domain and is able to enhance the activity of the 26S proteasome by its chaperone domain [286]. This would be of special interest for newly synthesized proteasomes to cope with the accumulated mass of ubiquitinated proteins after proteasome inhibition. In regard to this, Hsp70 has also the ability to support the UPS when it has recovered. Like it was mentioned in the introduction and in Fig. 2, Hsp70 is together with its co-chaperones Bag-1 and CHIP involved in the ubiquitination of unfolded proteins and their transport to the proteasome, enhancing the efficiency of the UPS.

The role of the classical Hsps during and after oxidative stress is indifferent and a broadly discussed topic. As it is shown in **Publication III**, there is a reduction of Hsp70 and Hsp90 immediately after the treatment with H_2O_2. Not until 16h after the treatment with H_2O_2, protein levels of Hsp70 and Hsp90 remain under the protein levels of the

untreated controls. Due to the fact that Hsps are high inducible upon proteotoxic stress this is a surprising result. Maybe it is a matter of the used cells and the source producing ROS. Wallen et al. treated cells with heat, menandione, H_2O_2 and a xanthine/xanthine oxidase system. Only heat stress and the treatment with menandione led to an induction of Hsp70, but not the treatment with H_2O_2 and xanthine/xanthine oxidase [287]. In accordance to this Adachi et al. also failed to induce Hsp70 with H_2O_2. Treatment with 0.25mM H_2O_2 for 1h, 2h and 5h resulted in no induction of Hsp70 [288]. Moreover, treatment with 44°C for 20min together with H_2O_2 led to a much lower Hsp70 protein content than heat treatment alone, indicating a H_2O_2 induced degradation of Hsp70 or a disrupted induction via HSF1 [288]. Therefore it can be suggested that oxidative stress leads to no induction of Hsp70 or Hsp90 (at least not in the first hours after the cell treatment), but rather to a degradation of the two Hsps. Like it was already mentioned most oxidative protein modifications are irreversible and the affected proteins have to be degraded by the proteasomal system. Thus, Hsp70 or Hsp90 are unable to repair/refold these modified proteins during the acute phase during and after oxidative stress. However, in **Publication III** it is observed that Hsp70 and Hsp90 recover 16h after H_2O_2 treatment. In this late phase after oxidative stress, it can be supposed that Hsp70 and Hsp90 are needed to support the ubiquitin dependent degradation of proteins and disaggregate oxidative stress mediated protein aggregates. The reason for the degradation of the two chaperones during the "first phase" after H_2O_2 treatment is unclear. One thinkable reason is an oxidative mediated damage of the chaperones, resulting in their proteasomal degradation.

4.6 The role of HDAC6 in maintenance of protein homeostasis

Since HDAC6 was identified as an unusual histone deacetylase, possessing two catalytic domains, an ubiquitin binding domain and a preferred localization in the cytosolic compartment, it was shifted into the focus of posttranslational protein modification and regulation [289]. Recent findings show evidence that HDAC6 mediates the removal of misfolded proteins and favors their accumulation into cellular aggresomes by interaction with ubiquitin and dynein motors. HDAC6 has the ability to bind coincidently to ubiquitinated proteins via the BUZ domain (also called ZnF-UBP domain [290]) and to the dynein motor component $p150^{glued}$ via a DMB domain, functioning as a linker between ubiquitinated proteins and the tubulin-dynein-motor system [291]. Because HDAC6, lacking its catalytical domain failed to rescue aggresome formation and tubulin is a major target of HDAC6 deacetylation, it is

suggested that HDAC6 also facilitates the transport of the dynein-misfolded-complex to the aggresome by deacetylation of microtubules [292]. Aggresomes are inclusion bodies next to the nucleus at the proximity end of the microtubule organizing center. Aggresomes are the endpoint of the microtubule dependent transport of polyubiquitinated protein aggregates, degrading long lived cytosolic proteins, overload or damaged cytoplasmatic organelles and damaged, abnormal protein aggregates [293]. Further support of the role of HDAC6 in autophagy, was demonstrated by the direct dependency on HDAC6 for the autophagic clearance of CFTR [294] or mutant Huntingtin aggregates [295] and the HDAC6 mediated recruitment of the autophagic machinery to aggresomes [296].

Furthermore, Boyault et al. demonstrated that HDAC6 related polyubiquitin binding acts as a "masking function", hampering the interaction with other ubiquitin binding factors like USPs or proteasomal subunits [297]. As a consequence HDAC6 has the ability to reduce the polyubiquitin turnover, which would lead to an attenuation of ubiquitin- proteasomal degradation and an excessive accumulation of polyubiquitinated protein aggregates in aggresomes. To keep the balance of the fate of polyubiquitinated proteins, HDAC6 interacts with the AAA-ATPase chaperone p97/VCP [298]. p97/VCP plays an important role in the preparation and transport of polyubiquitinated proteins to the proteasome and dissociates the HDAC6-ubiquitin complex, counteracting the HDAC6 promoted accumulation of polyubiquitinated in aggresomes and support their degradation by the proteasome. Thus, the HDAC6-p97/VCP complex is a crucial regulator of the cellular fate of misfolded-polyubiquitinated proteins. Dissociation of HDAC6 from p97/VCP, liberates HDAC6 for the binding of new polyubiquitinated proteins and its interaction with other substrates [299,300].

Besides the involvement in autophagy and aggresome- related degradation of polyubiquitinated proteins, HDAC6 and its connection to p97/VCP is also a crucial factor for the up-regulation of heat shock proteins upon proteasome inhibition. In another study Boyault et al. used different HDAC6 mutant/knockdown cell lines to examine the involvement of HDAC6 in the induction pathway of classical heat shock proteins [301]. In absolute accordance to the results of **Publication IV**, they revealed that HDAC6 presence is absolutely essential for the up-regulation of heat shock proteins upon proteasomal inhibition. Same observations have been made by RNAi mediated suppression of HDAC6 **(Publication IV, Fig. 3)** Moreover, it seems that the ubiquitin-binding domain is also essential for the observed Hsp induction, due to the fact that cell

possessing a mutant HDAC6 with an unfunctional ubiquitin binding domain also have no Hsp induction upon proteasome inhibition [302]. This could also be confirmed by suppression of ubiquitin activating enzymes E1, resulting as well in a clear attenuation of Hsp induction after LC treatment **(Publication IV, Fig. 3)**. Deacetylase activity of HDAC6 seems to play no role in Hsp induction, due to the fact that cell lines with HDAC6 deficient catalytic domains show also a clear induction of Hsps after proteasome inhibition. Further on, they found evidence for a complex formed out of Hsp90, HSF-1, p97/VCP and HDAC6. In case of proteasomal inhibition there is a consequent accumulation of ubiquitinated proteins, which are sensed by the ubiquitin-binding domain of HDAC6. Binding of ubiquitinated proteins induces the dissociation of the HDAC6-p97/VCP complex [303-305]. Following the liberation of HDAC6 and p97/VCP, the latter uses either its ATP dependent segregase activity to dissolve the bond between Hsp90 and HSF-1 directly or p97/VCP stimulates the Hsp90 ATPase activity, which results in the release of HSF-1 and the consequence up-regulation of several heat shock proteins [306]. Thus, ubiquitin is a strong regulator of HDAC6-p97/VCP interaction and the related cell response.

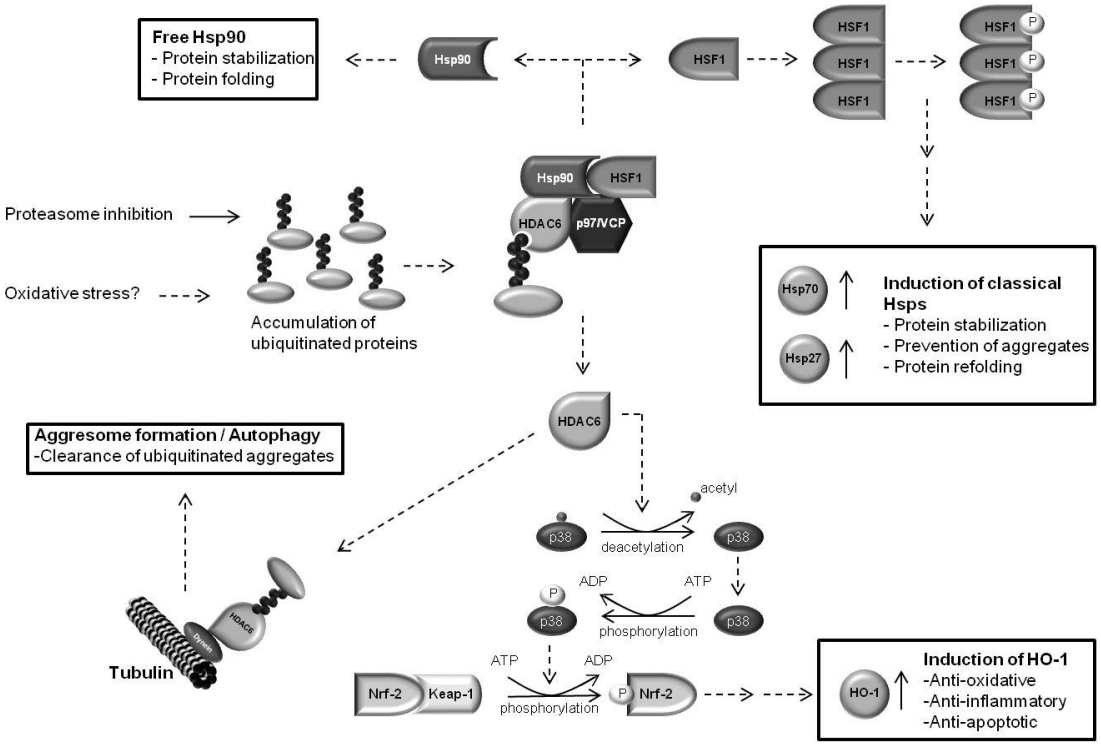


Figure 5: Role of HDAC6 in the maintenance of protein homeostasis.

Proteasome inhibition leads to the accumulation of polyubiquitinated proteins. These were sensed by the ubiquitin binding domain of HDAC6. Binding of HDAC6 leads to the dissociation of a complex, made out of HDAC6, p97/VCP, Hsp90 and HSF-1. Unbound HSF-1 trimerizes and functions as an activating

transcription factor for classical Hsp. HDAC6 initiates the formation of aggresomes and the autophagic clearance of ubiquitinated protein aggregates. HDAC6 is also needed for the p38/Nrf-2 dependent activation of HO-1 by the deacetylation and indirect activation of p38. Hsp90 functions also as molecular chaperone, supporting protein folding and stabilization.

Beyond the up-regulation of classical Hsps upon proteasome inhibition, it is demonstrated in Publication IV, that HDAC6 is also involved in an induction of HO-1 after proteasome inhibition **(Publication IV)**. Initiation of this pathway is also the detection of ubiquitinated proteins and the release of HDAC6. Previous studies could show that the induction of HO-1 upon proteasome inhibition is mediated by a p38MAPK-dependent activation of Nrf-2, which is the most important transcriptional activator of HO-1 gene translation [307]. In **Publication IV** it is demonstrated for the first time that p38 is a substrate for HDAC6 mediated deacetylation, and this deacetylation step is necessary for p38 phosphorylation and thus for the activation of Nrf-2 and HO-1 **(Publication IV)**. There is only limited knowledge about the acetylation/deacetylation of p38. In a paper from Shin et al. application of the unspecific HDAC class I and II inhibitor Trichostatin (TSA), results in a diminished phosphorylation/activation of p38 [308]. Contrarily in a publication stimulating mouse macrophages (RAW 264.7) with the bacterial endotoxin lipopolysaccharide (LPS), HDAC6 seems to attenuate the phosphorylation of p38, while repression of HDAC6 via siRNA results in an augmentation of phosphorylated p38 after LPS stimulation. However, comparing with the control siRNA the results seems to be inconclusive [309].

Summarized HDAC6 is an essential regulator of protein homeostasis, functioning as a sensitive sensor for the status of ubiquitinated proteins in the cellular system. Due to its involvement in the initiation of aggresome formation and autophagic clearance of ubiquitinated aggregates as well as the induction of classical Hsps and HO-1, HDAC6 plays a major role in the degradation of ubiquitinated damaged proteins, stabilization of misfolded/unfolded proteins and prevention of cell death [310-314] (Fig. 5)

4.7 HDAC6 and the proteasome in cancer

Due to the central part of the proteasome in the maintenance of proteostasis, it is also a promising target for cancer therapy. Inhibition of the proteasome by the FDA-accepted proteasome inhibitor bortezomib has successfully been tested for multiple myeloma

(MM) in several clinical studies [315-317]. Inhibition of NF-κB activation, inhibition of myeloma cell adherence and the accumulation of toxic, polyubiquitinated, unfolded/misfolded proteins have been proposed as possible mechanism of action [318]. At the moment bortezomib is also tested for the therapy of other solid tumors, such as lung cancer or prostate cancer [319,320].

However, it is frequently reported that there are many cancer cells exhibiting a resistance against bortezomib treatment with the result of diminished treatment success. Induction of heat shock proteins or makroautophagy are two major reasons for the resistance of cancer cells against proteasome inhibition [321,322]. Chauhan et al. demonstrated in human SUDHL lymphoma cells by inhibition of Hsp27, using Hsp27 antisense RNA, increased apoptosis after treatment with bortezomib [323]. Moreover, in a review from Wu et al. [324] it is precisely explained that there are several pathways inducing compensating makroautophagy upon proteasome inhibition, diminishing the efficiency of bortezomib treatment. Makroautophagy as well as the up regulation of heat shock proteins prevent the unfolded protein response in the endoplasmatic reticulum and subsequent apoptosis by the compensating degradation, folding or stabilization of unfolded, polyubiquitinated proteins and toxic aggregates. A key player in the regulation of these two response mechanisms is HDAC6 [325,326] **(Publication IV)**. Since it is demonstrated in **Publication IV**, that HDAC6 is also involved in the induction of HO-1, which also contributes to the anti- apoptotic response to proteasome inhibition, HDAC6 should be an interesting target for a combined treatment with bortezomib in cancer therapies. This could be of particular interest in tumors overexpressing HDAC6. Because HDAC6 is an estrogen regulated gene [327] the deacetylase is drastically overexpressed in estrogen-dependent breast cancer, but also in squamous cancer cell lines or ovarian carcinomas [328]. Besides the induction of proteotoxic response mechanisms, HDAC6 is also crucial for oncogenic transformation of cancer cells. Results from Lee et al. strongly indicate the requirement of HDAC6 for Ras-induced oncogenic transformation by providing anchorage-independent proliferation of cancer cells, which is a hallmark of malignant transformation [329]. Interestingly, in this study HDAC6 knockdown shows a significant decrease in phosphorylation of AKT and ERK1/2, supporting the thesis that HDAC6 has the ability to interact with kinases of the MAPK pathway.

Thus, specific HDAC6 inhibitors, like Tubacin or its derivative NK84, were developed and successfully evaluated for its antitumor activity in several cancer types, such as

MM, lymphoblastic leukemia, pancreatic cancer, lung cancer or breast cancer [330,331]. Combined treatment of ovarian cancer cells with bortezomib and the specific HDAC6 inhibitor NK84 resulted in a drastic decrease of cell viability compared to single bortezomib treatment. Moreover, combined treatment allows much lower bortezomib concentrations to achieve equal outcomes [332]. Increased amounts of ubiquitinated proteins, decreased cell motility and migration after cell scratching experiments and the increase of apoptotic markers like PARP cleavage indicate the augmentation of proteotoxic stress, leading to increased cell death of cancer cells upon combined treatment with bortezomib and Tubacin/NK84. Similar results have been made for MM and pancreatic cancer cells after combined treatment with bortezomib and Tubacin [333,334]. Due to the fact that high dose bortezomib therapy leads to different stages of neuropathies as a side effect, combined treatment with Tubacin would allow to lower bortezomib concentration, which would demonstrably reduce neuropathy during and after the cancer therapy [335].

5. Conclusion

Maintenance of protein homeostasis is crucial for keeping up cell functionality and cell viability. The ubiquitin proteasomal system as well as HDAC6 are two components of the maintenance system to keep up protein homeostasis. The aim of this work was to elucidate the role of these systems during several stress situations, in particular the responsibility of the ubiquitin proteasome system during oxidative stress and the stress response mechanisms during and after proteasome inhibition.

As a result following points can be summarized:

- Oxidative stress leads to increased amounts of oxidized proteins and ubiquitinated proteins. However, it becomes obvious that most oxidized proteins are not ubiquitinated after oxidative stress
- By isolation of ubiquitinated proteins before and after oxidative stress and following determination of these proteins by MALDI-TOF mass spectroscopy it can be suggested that ubiquitination after oxidative stress is a regulative mechanism and affects only distinct proteins, e.g. Hsps or regulative nuclear proteins
- Inhibition of the proteasome results in a compensative induction of classical Hsps, such as Hsp27 or Hsp70 and the stress response proteins HO-1
- Induction of these stress response proteins is mediated by an accumulating mass of ubiquitinated proteins and its detection by HDAC6
- The HDAC6 dependent induction upon proteasome inhibition of classical Hsps is mediated by Hsf-1
- HDAC6 dependent induction upon proteasome inhibition of HO-1 is mediated by an p38MAPK/Nrf-2 pathway
- Activation of p38 is mediated via direct deacetylation of p38 by HDAC6

In regard to these results it is obvious that both, the UPS as well as HDAC6, are two major players of the cellular stress response. Especially HDAC6 has the ability to induce several different stress response mechanisms to cope with increasing proteotoxic stress levels. Knowing the stress response mechanism upon proteasome inhibition reveals for example new targets to overcome proteasome inhibitor resistance in cancer therapy.

6. References

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7. Abstract/Zusammenfassung

7.1 Abstract

The control and maintenance of protein homeostasis is a crucial factor to maintain cell functionality, cell viability and cell division. To cope with this challenge the cell obtain a large variety of repair- and stabilization proteins, meaning especially the family of heat shock proteins, as well as proteases such as the ubiquitin proteasomal system (UPS), which degrades defect, modified, unused and irreparable proteins. There are different kind of stress stimuli, disturbing protein homeostasis and therefore the entire cell physiology. In this work cells were stressed with hydrogen peroxide (H_2O_2) to generate oxidative stress or Lactacystin to inhibit the proteasome. For both cases the role of the UPS was investigated. Additionally, it was elucidated which compensatory mechanisms are activated when the proteasome is inhibited.

Methodical this comprises the isolation of proteins via different immunoprecipitation techniques, the identification of the proteins by MALDI-TOF mass spectrometry and diverse immunoblotting techniques, gene silencing of distinct gene/proteins by siRNA transfection, different protein activity assays and RT-qPCR to verify the expression at mRNA levels.

As a result it is shown that after treatment with H_2O_2 most oxidized proteins are not ubiquitinated. Although, there is an increase of ubiquitinated proteins 16h after H_2O_2 treatment, it can be suggested due to the mass spectrometry results that ubiquitination upon proteasome inhibition is a regulatory mechanism affecting only distinct proteins and not the bulk of oxidized proteins.

After inhibition of the proteasome there is also a drastic accumulation of ubiquitinated proteins. The accumulating mass of undegraded, ubiquitinated proteins acts as a marker or signal for proteotoxic stress. Interaction of ubiquitinated proteins with the histondeacetylase 6 (HDAC6), results in the compensatory up-regulation of several chaperones/Hsps and heme oxygenase-1 (HO-1). Induction of Hsps is mediated by a HDAC6 dependent release of the heat shock factor 1 (HSF1). Induction of HO-1 is mediated by a p38MAPK/Nrf-2- dependent pathway, whereas p38 is directly deacetylated by HDAC6 upon proteasome inhibition. Deacetylation of p38 prepares the kinase for further phosphorylation and activation. Up-regulation of Hsps and HO-1

upon proteasome inhibition stabilizes ubiquitinated proteins, reduces the risk of aggregation and function as well anti-apoptotic, anti-inflammatory and anti-oxidative.

Due to these results it can be suggested that the UPS and HDAC6 play a central role in the maintenance of protein homeostasis and function as an essential connection to the down-stream located stress response mechanisms.

7.2 Zusammenfassung

Die Kontrolle und Aufrechterhaltung der Protein-Homöostase ist ein wichtiger Beitrag zum Erhalt der Zellfunktion, Zellvitalität und der Zellteilung. Um diese Aufgabe zu erfüllen besitzt die Zelle ein breites Spektrum an Reparatur- und Stabilisationsproteinen, allen voran die stressinduzierbaren Hitzeschock Proteine (Hsps), sowie multi-katalytische Proteasen, wie das Proteasom, die zum Abbau defekter, veränderter, nicht mehr gebrauchter und irreparabler Proteine beitragen. Verschiedene Arten von Stress sind in der Lage die Protein-Homöostase aus dem Gleichgewicht zu bringen und somit auch die allgemeine Zellphysiologie zu stören. In der hier vorliegenden Arbeit wurde der zelluläre Stress zum einen durch Hydrogenperoxid-induzierten Oxidativen Stress zugefügt und zum anderen durch die Inhibition des Proteasoms mittels Lactacystin. In beiden Fällen wurde die Rolle des Proteasoms als auch die der Ubiquitinierung untersucht. Zusätzlich wurde herausgearbeitet welche alternativen und kompensierenden Mechanismen aktiviert werden, wenn das Proteasom gehemmt ist.

Methodisch beinhaltet dies, Proteine und deren Expression auf vielfältigste Weise zu untersuchen: Isolation verschiedener Proteine aus verschiedenen Zelllinien mittels unterschiedlicher Immunoprezipitations-Techniken, die Identifizierung der isolierten Proteine mittels MALDI-TOF-Massenspektroskopie, verschiedene Immunoblott-Techniken, das Blockieren von Genen/Proteinen durch den Einsatz von RNAi-Techniken, verschiedene Aktivitätstest einzelner Proteine sowie die Expressionskontrolle der mRNA ausgesuchter Proteine.

Als Resultat konnte gezeigt werden, dass nach oxidativem Stress ein Großteil der oxidierten Proteine nicht ubiquitiniert wird. Obwohl sich 16h nach oxidativem Stress ebenfalls ein Anstieg von ubiquitinierten Proteinen zeigt, kann aufgrund der massenspektroskopischen Analyse davon ausgegangen werden, dass es sich hierbei um gezielte, regulatorische Ubiquitinierungen handelt.

Durch Inhibition des Proteasoms kommt es ebenfalls zu einer drastischen Akkumulation von polyubiquitinierten Proteinen. Die Akkumulation von ubiquitinierten Proteinen kann als Marker oder Signal für proteotoxischen Stress angesehen werden. In einem Histondeacetylase 6 (HDAC6) abhängigen Schritt führt die Detektion der akkumulierten ubiquitinierten Proteine zu kompensatorischer Induktion von verschiedenen Chaperonen und der Hämoxygease- 1 (HO-1). Die Induktion der Hsps erfolgt über die HDAC6 gesteuerte Freisetzung des Hitzeschock-Faktor 1 (HSF1). Über einen p38MAPK/Nrf-2 abhängigen Signalweg kommt es zur Induktion der HO-1,

wobei dabei p38 direkt durch die HDAC6 deacetyliert und somit aktivierbar wird. Die Zunahme an Hsps und der HO-1 trägt zu Stabilisierung der ubiquitinierten Proteine bei, vermindert deren Aggregation und wirkt anti-apoptotisch, anti-inflammatorisch und anti-oxidativ.

Durch die vorliegenden Ergebnisse lässt sich schlussfolgern, dass das Ubiquitin-Poteasomale-System und die HDAC6 eine zentrale Rolle in der Aufrechterhaltung der Proteinhomöostase einnehmen und als wichtige Knotenpunkte für die weitere kompensatorische Stressantwort fungieren.

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Lebenslauf

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unverheiratet, keine Kinder

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- Erfahrung in der Methodenentwicklung und -validierung
- Erfahrung in der Dokumentation/Präsentation von Versuchsergebnissen
- Sehr gute Fähigkeiten in wissenschaftlichem Schreiben
- Qualifikation:
  -Promotion mit biochemischem/zellbiologischem Schwerpunkt
  -Diplomarbeit mit biochemischem/zellbiologischem Schwerpunkt
  -Industriepraktikum im Fachbereich QA/QC
  -Wissenschaftliche Hilfskraft mit Schwerpunkt auf molekularbiologischen Methoden

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10/2009 – 06/2012

Promotion an der Biologisch-Pharmazeutischen Fakultät
Schwerpunkte: Labortätigkeit (u.a. Immunoblotting, ELISA, Immunopräzipitation, Durchflusszytometrie, Zellseparation, Enzymaktivitätsassays, ICC/IHC, RT-qPCR, Zellkultur), Industrieprojekt zu Haut schützenden Substanzen, Methodenentwicklung, wissenschaftliches Publizieren, anleiten von TAs und Studenten
Titel: Role of the ubiquitin-proteasomal system and HDAC6 in the maintenance of protein homeostasis.
Voraussichtliche Verleihung des Doktor Grades: Juni 2012, vorläufige Note nach Disputation: 1,0 (einstimmig vorgeschlagen für summa cum laude)

Universität Hohenheim

04/2009 – 09/2009

Diplomarbeit am Institut für Biologische Chemie und Ernährungswissenschaften
Schwerpunkte: Verbesserung einer Bestrahlungstherapie für diverse Hauttumore, Labortätigkeit (Immunoblotting, Durchflusszytometrie, ELISA, Enzymaktivitätsassays, Zellkultur)
Titel: Inhibition ausgewählter Zell-Schutzmechanismen und die Auswirkung auf die PDT
Note: 1,0

10/2004 – 09/2009

Studium der Ernährungswissenschaften
Schwerpunkte: organische Chemie, Biochemie, Lebensmitteltechnologie, Ernährungstoxikologie
Abschluss: Diplom (1,0)

Praktika und weitere relevante Tätigkeiten

09/2008 – 03/2009

Praktikum bei Glanbia Nutritionals Deutschland GmbH, Abteilung: QA/QC,

	78359 Orsingen-Nenzingen Schwerpunkte: Probenanalytik (Photometrie, AAS, HPLC), Methodenetablierung, Methodenvalidierung unter ISO-Richtlinien
10/2007 – 07/2008	**Wissenschaftliche Hilfskraft am Institut für Physiologie der Universität Hohenheim** Schwerpunkt: verschiedene Protein- und DNA Analysen

Auslandsaufenthalte

10/2011	**Gastwissenschaftler am Institut für Biochemie und Molekulare Biologie, Universität von Süddänemark; Odense, Dänemark** Schwerpunkt: Identifikation von Proteinen mittels MALDI-MS/MS
06/2010	**FEBS/SFRR-E/IUBMB - Advanced Lecture Course**; Spetses, Griechenland Schwerpunkte: Oxidativer Stress, Aging, Methodenerweiterung Aktive Teilnahme durch Posterpräsentation

Zivildienst

08/2003 – 04/2004	**Zivildienst bei der Gemeinde Volkertshausen** Schwerpunkt**:** Landschaftspflege/-gestaltung

Schulische Ausbildung

09/2000 – 07/2003	**Ernährungswissenschaftliches Gymnasium** Mettnauschule Radolfzell, Abschluss: Abitur (1,3)

Zusatzqualifikationen

EDV-Kenntnisse	**MS Office**, **GraphPad Prism 5, Reference Manager, diverse Gerätesoftware**	
Sprachen	**Englisch**	fließend in Wort und Schrift
	Spanisch	Grundkenntnisse
	Deutsch	Muttersprache
Fortbildungen	**aktive Teilnahme an nationalen und internationalen Kongressen**	

Mitgliedschaften und Freizeitinteressen

Mitgliedschaften	**Musikverein Weiterdingen e.V.** (ehemaliges Vorstandsmitglied) **Narrenverein Weiterdingen e.V.**
Freizeitinteressen	**Triathlon, Fußball, Musik, Kochen**

Hilzingen, 15. Juni 2012

Wissenschaftliche Arbeiten

Poster

2010*, Spetses, Griechenland*

Inhibition of cell protection mechanisms and the effect on Photodynamic Therapy, Kästle, M. et al.

2012, 49. Wissenschaftlicher Kongress der DGE

Die Rolle der Histondeacetylase 6 (HDAC6) für die zelluläre Protein-Homöostase nach Proteasominhibierung, Kästle, M. et al.

Vortrag

Selective melanoma cell treatment by combination of PDT and inhibitor treatment; Kästle, M., 2011, 3rd Postgraduate symposium on Cancer Research; *Dornburg, Deutschland*

Publikationen

Kästle,M., Grimm,S., Nagel, R.; Breusing, N., Grune.T.; Combination of PDT and inhibitor treatment affects melanoms cells and spares keratinocytes; *Free Radic Biol Med*.; 2011, 50: 305-312

Kästle,M., Grune, T.; Protein bearing oxidation-induced protein carbonyl groups are not preferentially ubiquitinated; *Biochimie*;2011;93: 1076-1079

Kästle,M., Grune,T.; Protein oxidative modification in the ageing organism and the role of the ubiquitin proteasomal system; *Cur Pharm Design*; 2011, 17(36):4007-22

Kästle,M., Woschee,E., Grune,T.; Histone deacetylase 6 (HDAC6) plays a crucial role in p38MAPK-dependent induction of the

oxygenase-1 (HO-1) in response to proteasome inhibition; *Free Radic Biol Med.*; 2012, submitted

Kästle M., Reeg S., Rogowska-Wrzesinska A., Grune T.; Chaperones, but not oxidized proteins, are ubiquitinated after oxidative stress; Free Radic. Biol. Med.; 2012, in press

Buchkapitel

Kästle M., Grune T.; Interaction of the proteasomal system with chaperones – protein triage and protein quality control; The Proteasomal system in aging and disease; Elsevier, Academic Press; 2012, in press

Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass die hier vorliegende Arbeit im Rahmen der mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich Schiller Universität Jena angefertigt wurde.

Die vorliegende Arbeit wurde von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt. Des Weiteren versichere ich, dass keinerlei Hilfe eines Promotionsberaters in Anspruch genommen wurde.

Die Arbeit wurde in keiner Form einer anderen Prüfungsbehörde vorgelegt und auch nicht veröffentlicht.

Hilzingen, 15. Juni 2012

Marc Kästle