

"Impact of Mdm2-p53 on the proteasome
assembly and disassembly
Role of the ubiquitination of some 19S subunits"

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ZUSAMMENFASSUNG

Das Ubiquitin Proteasom System „UPS“ ist ein grundlegender Bestandteil für den Abbau von zellulären Proteinen. Defekte dieses Systems sind mit verschiedenen menschlichen Erkrankungen verbunden und Proteasom-Inhibitoren werden bereits zur Behandlung diverser Krebsarten verwendet. Das UPS beinhaltet zwei wichtige ATP-abhängige Schritte, das Markieren des Substrats durch Polyubiquitin-Ketten und seinen Abbau durch das 26S Proteasom. Das 26S Proteasom, die bedeutendste zelluläre Protease, besteht aus einem katalytischen Teil (20S), welcher von einem oder zwei regulatorischen Komplexen bedeckt wird (19S).

In unserem Labor wurde herausgefunden, dass die „RING“ E3 Ubiquitin Ligase Mdm2 mit einigen 19S Untereinheiten (S2, S4, S5a, S6a, S6b, S8, S10b und S12) eine Verbindung eingeht und den Abbau des Tumorsuppressors p53, das am besten bekannte Substrat von Mdm2, fördert, indem ein ternärer Komplex zwischen Mdm2, p53 und den 19S Untereinheiten entsteht. Da Mdm2 mit einigen 19S Untereinheiten nicht nur wegen des Abbaus von p53, sondern auch für die Ubiquitinierung von einigen 19S Untereinheiten (S2, S4, S5a, S6a, S6b and S8) zu interagieren scheint, war das Ziel dieser Arbeit, die Rolle von Mdm2 und seinem Substrat p53 in Bezug auf die 19S proteasomalen Untereinheiten aufzuklären.

Meine Ergebnisse deuten darauf hin, dass die Ubiquitinierung nicht die zum Abbau von Substraten benötigte Ubiquitin-Kette zur Folge hatte und dass sie nicht den proteasomalen Abbau der 19S Untereinheiten fördert. Überraschenderweise steigerte die Anwesenheit von Mdm2 und seines Substrates p53 die Bereitstellung von proteasomalen Untereinheiten und dadurch die Bildung des 26S Proteasoms. Dieses Ereignis erhöhte wahrscheinlich die Effizienz des Abbaus seiner Substrate. Die Auswirkung auf den Zusammenbau des Proteasoms könnte auch auf andere E3 Ligasen, wie Siah-1 und c-Cbl ausgeweitet werden. Allerdings ist die Ubiquitinierung der 19S Untereinheiten durch Mdm2 nicht mit dem Zusammenbau des Proteasoms, sondern eher mit seinem Auseinanderfallen verbunden. Die Deubiquitinierung der S8 und S6b Untereinheiten des 19S Proteasoms durch die Deubiquitinase USP2 fördert deren Interaktion untereinander und den Aufbau des 19S Proteasoms.

Zusammengefasst zeigen meine Ergebnisse einen neuen Aspekt, um die Aktivität des 26S Proteasoms durch eine E3 Ligase und ihres Substrates zu regulieren, da sie erstens eine Auswirkung auf dessen Zusammenbau haben und zweitens einige 19S Proteine ubiquitiniert werden, die mit dem Auseinanderfallen des Proteasoms verbunden sind.

ABSTRACT

The ubiquitin proteasome system “UPS” is a fundamental actor for the proteolysis of cellular proteins. Defects of this system are associated with diverse human diseases, and proteasome inhibitors are already used in cancer therapies. The UPS involves two main ATP-dependent steps, the targeting of the substrate by polyubiquitin chains and its degradation by the 26S proteasome. The 26S proteasome, the major cellular protease in cells, is formed by a catalytic particle (20S proteasome) capped by one or two regulatory complexes (19S proteasome).

Data from the laboratory demonstrated that the “RING” E3 ubiquitin ligase Mdm2 associates with some 19S subunits (S2, S4, S5a, S6a, S6b, S8, S10b and S12) and promotes degradation of the tumor suppressor p53, the most well-known substrate of Mdm2, by forming a ternary complex between Mdm2, p53 and 19S proteins. Since Mdm2 seems to interact with proteasomal subunits not only for the degradation of p53 but also for the ubiquitination of some 19S subunits (S2, S4, S5a, S6a, S6b and S8), the aim of this work was therefore to clarify the role of Mdm2 and its substrate p53 on the 19S proteasomal subunits.

My results indicated that this ubiquitination did not imply the ubiquitin chain generally involved in the degradation of substrates and did not promote the proteasomal degradation of the 19S proteins. Surprisingly, the presence of Mdm2 and its substrate p53 increased the recruitment of proteasomal subunits, and thus, the formation of the 26S proteasome. This phenomenon probably enhanced the efficiency of substrates degradation. The effect on the proteasome assembly could be extended to other E3 ligases such as Siah-1 and c-Cbl. However, the ubiquitination of the 19S subunits by Mdm2 is not associated with the assembly of the proteasome but rather with its disassembly. The deubiquitination of S8 and S6b subunits of the 19S proteasome by the deubiquitinase USP2 favors their interaction and formation of the 19S proteasome. Taken together, my data revealed a novel aspect to regulate the activity of the 26S proteasome via an E3 ligase and its substrate, as first having an impact on the assembly, and second, ubiquitinating some of 19S proteins that are associated with proteasome disassembly.

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ABBREVIATIONS

aa	aminoacid
APS	ammonium persulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumine
°C	degrees Celsius
Cbl	Casitas B-lineage lymphoma
cDNA	complementary DNA
C-terminal	carboxy- terminal
CP	Core Particle
CRULs	Cullin ring ubiquitin ligases
Da	dalton
DMEM	dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	deoxynucleosides triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
eg.	exempli gratia, for example
et al.	<i>Et alii</i> , and others
FBS	fetal bovin serum
Fig.	figure
g	gram
GST	Glutathion-S-transferase
h	hour
HRP	horseradish peroxidase
HECT	homologous to E6-AP C-Terminus
IP	immunoprecipitation
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobases
kDa	kilodalton
l	liter
LB	Luria-Bertani
M	molar
μ	micro
m	milli
Mdm2	mouse double minute 2
min	minute
mRNA	messenger RNA
MW	molecular weight
n	nano
NaCl	sodium chloride
NES	nuclear export sequence

NLS	nuclear localisation signal
NP-40	nonident P-40
N-terminal	amino- terminal
OD	optical density
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	posphate buffer saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PHD	plant homeodomain
PMSF	phenylmethanesulphonylfluoride
PVDF	polyvinylidene difluoride
RC	Regularory Complex
RING	really interesting new gene
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	second
SDS	sodium dodecyl sulfate
Siah1	seven in absentia homolog 1
siRNA	small interfering RNA
TBS	tris buffered saline
TEMED	tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminometane
u	units
Ub	ubiquitin
U-box	UFD2-homology domain
USP2	ubiquitin specific protease 2
UV	ultra violet
V	volt
WB	Western blot

1. INTRODUCTION

The Ubiquitin Proteasome System “UPS” is a fundamental cellular actor due to its central role in apoptosis, its importance for the rapid destruction of key regulatory proteins such as cell cycle regulators, transcription factors but also for the elimination of defective proteins (Coux et al., 1996; Hoffman and Rechsteiner, 1996; Voges et al., 1999; Hershko et al., 2000; Pickart and Cohen, 2004; Devoy et al., 2005). The crucial function of the UPS in cellular biology has been recognized with the Nobel Prize for Chemistry which was awarded to Avram Hershko, Aaron Ciechanover and Irwin Rose in 2004.

Furthermore, certain tumor cells have been shown to be more sensitive to proteasome inhibitors than normal cells (Dick et al., 2010). Therefore, a better understanding of the regulation of the “UPS” (delivery and degradation of substrates, assembly and disassembly of the proteasome...) may help to develop new drugs for cancer therapies. The interaction between polyubiquitinated substrates and the proteasome constitutes an important aspect of the UPS pathway. A failure of this regulation leads to proteasome dysfunction and to substrate accumulation (Finley, 2009).

One example of protein regulation by the proteasome is the p53 tumor suppressor (Jain and Barton, 2010). This protein induces cell cycle arrest or apoptosis after exposure to stress signal such as DNA damage or oncogene activation. In unstressed cells, p53 is maintained at a low level due to tight regulation by the E3 ubiquitin ligase such as Mdm2. The Mdm2 protein associates with some proteasomal subunit proteins and induces the degradation of p53 by promoting a ternary complex between Mdm2, p53 and the proteasomal proteins (Kulikov et al., 2010). However, mechanisms of p53 delivery to the proteasome and regulation of the proteasomal proteins by Mdm2 are still under constant investigation.

1.1 The Ubiquitin Proteasome System “UPS”

The ubiquitin proteasome system allows the specific proteolysis of cellular proteins and is present in both the cytoplasm and the nucleus (Hershko et al., 1998). Many cellular functions are modulated by proteasome-dependent proteolysis including apoptosis, cell cycle progression, DNA transcription and repair, differentiation and development, immune response, neural and muscular degeneration, ribosome biogenesis or viral infection (Rock and Goldberg, 1999; Schubert et al., 2000; Kodadek, 2010). The UPS pathway (*Fig.1*) usually functions in two main ATP-dependent steps with first ubiquitination of target proteins. Ubiquitination consists in protein modifications by ubiquitin, a small regulatory protein expressed in all eukaryotic cells (Kimura and Tanaka K, 2010). This step is followed by the degradation of ubiquitinated substrates through the 26S proteasome (Ferrell et al., 2000).

1.1.1 The ubiquitin conjugation pathway

1.1.1.1 Ubiquitination of substrates

Ubiquitination of proteasome substrate requires a cascade of three to four enzymes, E1, E2, E3 and E4 (*Fig.1*) (6). The reaction begins with the sequential binding of Mg^{2+} -ATP and ubiquitin to the ubiquitin activating enzyme (E1) to form an adenylate ubiquitin intermediate. The ubiquitin is then transferred to the catalytic cysteine of the E1 via the formation of a thioester bond (*step 1, Fig.1*). Afterwards, the activated ubiquitin is transferred to the cysteine in the active site of an ubiquitin conjugating enzyme (E2) (*step 2, Fig.1*). The E2 enzyme catalyses the covalent attachment of ubiquitin directly on substrates (RING finger or RING finger like E3s families), or when it acts in partnership with an E3 ubiquitin ligase, ubiquitin is transferred on the E3 ligase (HECT E3s family) (*step 3 and 4, Fig.1*). An amino-isopeptide bond is formed between the carboxyl group of glycine 76 of ubiquitin and a ϵ -amino group of an internal lysine residue of the substrate protein.

The eukaryote genome encodes only two E1 enzymes (Xu et al., 2010) and around 36 genes encoding for E2 enzymes have been identified in the human genome (Van Wijk et al., 2010). In contrast to E1 or E2 enzymes, E3 ligases are quite numerous with more than 600 enzymes (Deshaies et al., 2009). Hence, one E2 enzyme usually interacts with several E3 ligases. For example, the E2 conjugating enzyme UbcH5A associates with RING E3 ligases

such as c-Cbl (Joazeiro et al., 1999) or Mdm2 (Rajendra et al., 2004). Vice versa an E3 ligase can interact with several E2 enzymes. As an example, UbcH5A, -B, and -C and E2-25K support Mdm2-mediated ubiquitination of p53 (Saville et al., 2004).

For some ubiquitin conjugation reactions, a polyubiquitin chain conjugation factor (E4) takes part in the extension of ubiquitin chains (Koegl et al., 1999) (*step 5, Fig.1*). Concerning p53, the acetylating/ubiquitin E4 factor proteins p300/CBP has been shown to be a chain assembly factor for p53 (Shi et al., 2009). Finally, the substrate is recognized and degraded by the proteasome (*step 6, Fig.1*) and ubiquitin molecules are recycled (*step 7, Fig.1*).

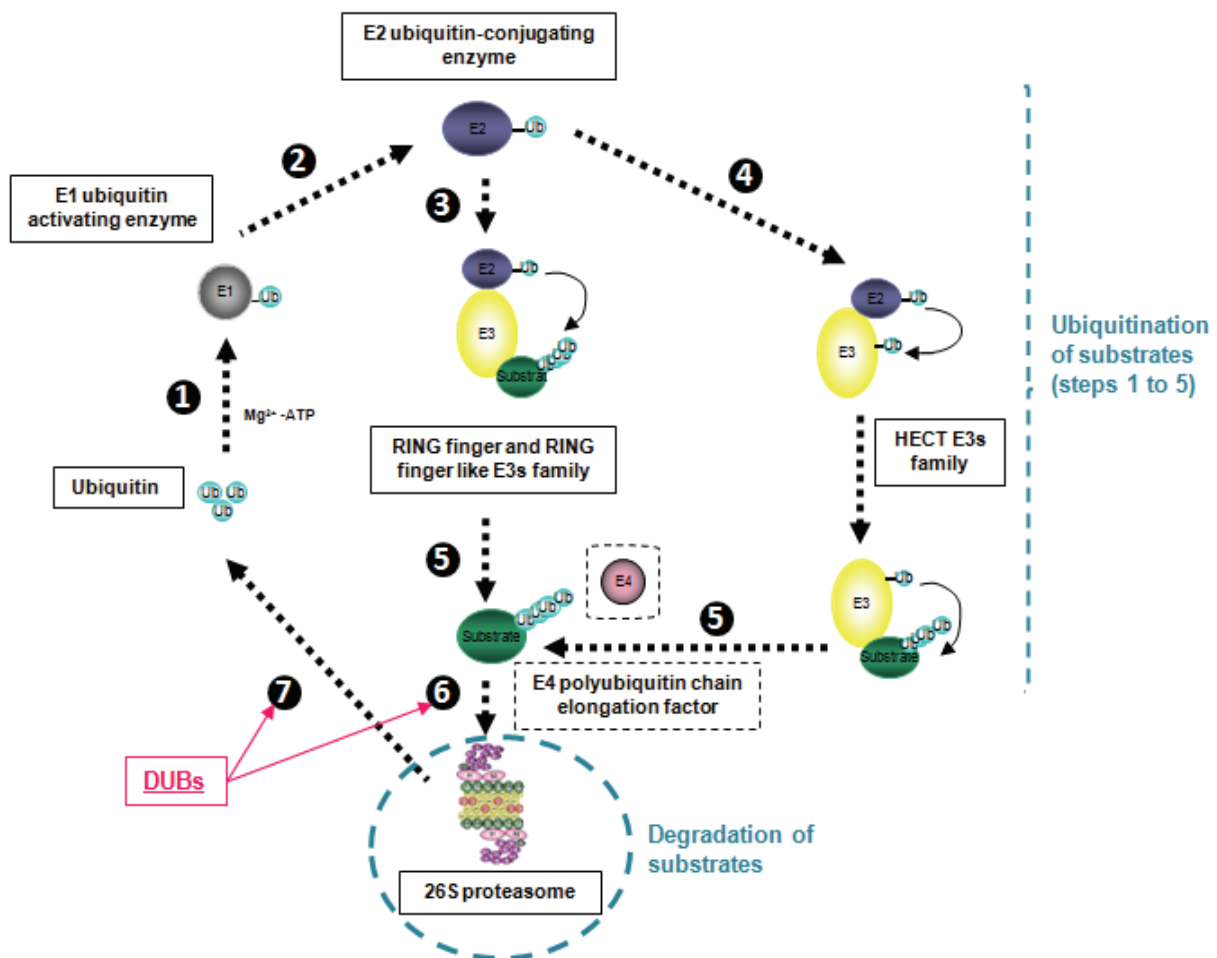


Figure 1: The ubiquitin proteasome pathway. The ubiquitin molecule is first activated by the ubiquitin activating enzyme (E1) in the presence of Mg^{2+} and ATP via formation of a high energy thioester bond (1). The activated ubiquitin molecule is then transferred to the ubiquitin conjugating enzyme (E2) by formation of a second high energy thioester bond with ubiquitin (2). Subsequently, the ubiquitin molecule is either transferred directly to the substrate by the RING-type E3 ligase (3) or associated to the HECT-type E3 ligases by formation of a third high energy thioester bond with the catalytic cysteine of the HECT domain before to be attached to the substrate (4). This phase is followed by the extension of the ubiquitin chain. In some cases, E4 enzyme (dotted) takes part in the elongation of ubiquitin chains (5). The ubiquitinated substrates are then recognized and degraded by the 26S proteasome (6) and the ubiquitin molecules recycled by deubiquitinating enzymes (DUBs) which can also protect substrates from proteasomal degradation (7).

1.1.1.2 E3 ubiquitin ligases

E3 enzymes could be divided into three families (RING-E3s, HECT-E3s and RING-finger related E3s) depending on their mode of action:

- **The HECT E3s** (Homologous to E6-AP C-Terminus) form an ubiquitin thioester intermediate through the catalytic cysteine of the E3 before they transfer ubiquitin to a lysine of the substrate. This family was discovered in studies on the degradation of the tumor suppressor protein p53 by the oncogenic E6 protein of human papillomaviruses (HPVs) (Huibredtse et al., 1995). The HECT domain of E6-AP possesses approximately 350 amino acids and shows homology to many proteins. This domain contains a conserved catalytic cysteine required for the E3 activity. The N-terminus contributes to the recognition of substrates whereas the HECT domain binds to E2 enzymes and catalyses the ubiquitination (*step 4, Fig.1*). (Rotin et al., 2009)

- **The RING (Really Interesting New Gene) E3s**: The RING finger domain acts as adaptor proteins and brings the associated E2 enzymes close to the substrate to allow its ubiquitination (*step 3, Fig.1*). This domain harbors eight conserved histidine and cysteine residues which are coordinated by two zinc ions (Borden et al., 2000).

✓ **Monomeric RING finger E3s**: Both the substrate binding site and the catalytic RING domain are on the same protein. This is why they are called single-subunit RING E3 ligases. One of the most described monomeric RING E3 is the Mdm2 protein, a protein mostly known for its ability to target p53 for proteasomal degradation (Honda et al., 1997). Other single-subunit RING E3s such as Cbl family, Siah-1 or Parkin are identified (Freemont, 2000).

✓ **Multimeric RING finger E3s**: Another RING E3 contains several multisubunit protein complexes including the SCF (Skp1-cullin-F-box), the CBC (cullin-elongin B and C) or the APC (anaphase promoting complex) complexes.

The cullin-RING ubiquitin ligases (CRULs) family represents the current model for multimeric RING ligases (Jackson et al., 2009). In human, seven cullins are known; they are associated with one specific E3 ligase monomeric and are connected with different subunits for the binding of substrates. As an example, the SCF complex consists of several proteins (Rbx1/Skp1/Cullin1/F-box protein). Rbx1 is essential for the E3 activity of the complex recruiting the E2 enzyme. The F-box protein which mediates the substrate specificity is variable. Three classes of F-box are identified in function of the domain that interacts

with the substrate: F-box FBWs (WD40 repeated motifs), F-box FBLs (leucine-rich repeats) and F-box FBXs. (Ho et al., 2008)

The other CRULs are composed by different receptors such as the SOC proteins (suppressor of cytokine signaling) (Tyers et al., 2000).

- **Two RING finger-related E3s:**

✓ The U-box (UFD2-homology domain) is a domain of ~70 amino acids. The structure of the U-box E3s (CHIP, UFD2) is very similar to the structure of the RING domain even if U-box domains do not have conserved cysteine and histidine residues (Ohi et al., 2003). The prototype U-box protein, yeast Ufd2, was identified as a ubiquitin chain assembly factor (E4) that cooperates with E1s, E2s, and an E3s to catalyze the formation of a ubiquitin chain on artificial substrates. (Hatakeyama et al., 2001)

✓ The PHD (plant homeodomain) represents a variant of the RING finger with eight similarly spaced cysteines and histidines (Lu et al., 2002; Coscoy and Ganem, 2003).

1.1.1.4 Ubiquitin and ubiquitin like modifications

1.1.1.4.1 Ubiquitin chains

The ubiquitin protein is highly conserved among eukaryotes but is absent in archaea (Kimura and Tanaka K, 2010). The ubiquitin molecule is found free or conjugated to other proteins in cells, and thus, can modify the biochemical properties of the target protein. The active form of ubiquitin is generated from a high molecular weight precursor by the action of ubiquitin C-terminal hydrolase (UCH), which release the mature 8 kDa protein. After cleavage, ubiquitin exposes glycine 76, which is involved in the isopeptide bond formation with a lysine residue on target substrates. (Catic et al., 2005)

Ubiquitin is a protein of 76 amino acids which contains seven lysine residues (K6, K11, K27, K29, K33, K48, K63) (*Fig. 2A*). When conjugated to preceding ubiquitin moieties, ubiquitin can form chains of ubiquitin (polyubiquitination), using one or several of the seven internal lysine residues (*Fig. 2B*) (Pickart et al., 2000). In addition to the formation of polyubiquitin chains, conjugation of a single ubiquitin on a single lysine residue (monoubiquitination) or on multiple lysine residues (multimonoubiquitination) can occur. Monoubiquitination of target proteins can have a role in trafficking, endocytosis, transcription or histone function (Sigismund et al., 2004).

Considering that an ubiquitin molecule contains seven lysine, seven possible isopeptide linkages can be formed. One of the most frequent ubiquitin linkages is the K48-linked chain which is a recognition signal for protein degradation. At least four molecules of ubiquitin are required to target substrates for degradation by the proteasome (Thrower et al., 2000). The K63-linked chains take usually part in DNA repair, signal transduction, trafficking to multivesicular body, kinase activation or stress responses (Spence et al., 1995; Lauwers et al., 2009). In addition K63-linked chains can support proteasomal degradation (Yasushi et al., 2009). The K11- and K29-linked chains are involved in proteasome-dependent degradation (Jin et al., 2008). The existence of K6-, K27- and K33- linked chains have been reported, but their functions are not clear (Glickman et al., 2002). Some studies highlighted the possibility of “mixed” (containing all possible isopeptide linkages) or “branched” linkages (Kirkpatrick et al., 2007). Proteins modified by this class of “non-conform” chains are under the control of the E2-E3 pairs and seems to be poorly degraded by the proteasome (Kim et al., 2006).

A- Ubiquitin sequence:

MQIFV^{K₆}TLTG^{K₁₁}TITLEVEP^SDTIENV^{K₂₇}^{A₂₉}IQD^{K₃₃}EGIPDQQLRIFAG^{K₄₈}QLEDGTLSDYNIQ^{K₆₃}ESTLHLVLR^LRGG

B-

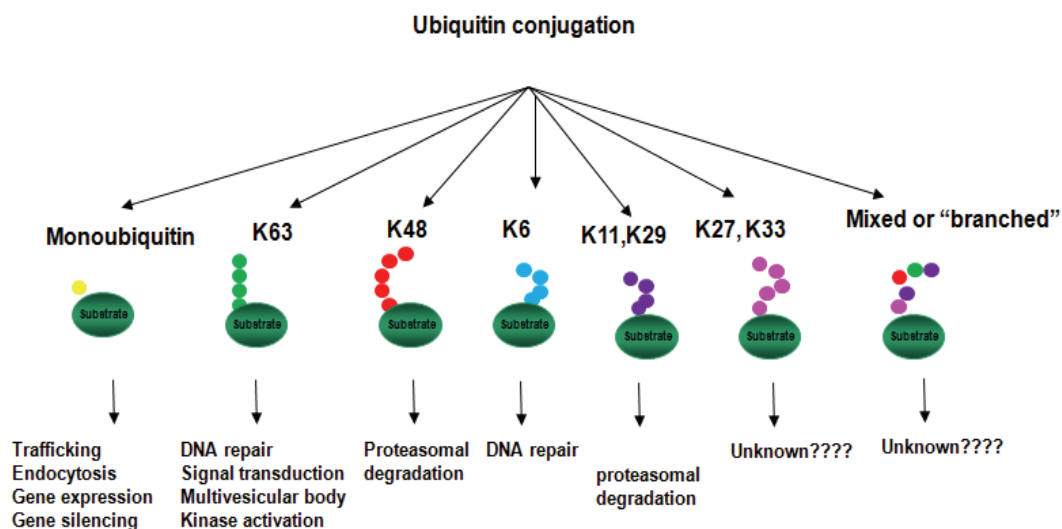


Figure 2: Sequence of ubiquitin and ubiquitin linkages. **A-** Amino acid sequence of ubiquitin. Lysines are highlighted in red. **B-** Polyubiquitin chains are built by formation of an isopeptide bond between the seven potential lysines of ubiquitin (K6, K11, K27, K29, K33, K48 or K63). The K48 linkages are highly abundant and represent the main signal for proteins degradation by the proteasome. The other lysine linkages are less abundant. The question marks indicate that the functions of K27, K33 and “mixed/branched” chains are largely unknown.

1.1.1.4.2 Ubiquitin-like “UBLs” modifications

Other small ubiquitin-like proteins, called UBLs, including SUMO, Nedd8, ISG15 or Atg8 can also modify proteins. They are crucial regulators of many cellular processes such as transcription, DNA repair, signal transduction, autophagy or cell-cycle control (Bae and Park, 2010; Hock and Vousden, 2010; Dou et al., 2011; Boh et al., 2011; Duverger et al., 2011). Modified proteins by UBLs possess a conjugating pathway similar to ubiquitin conjugation but do not target proteins for proteasomal degradation. Furthermore, the UBL conjugation can inhibit the formation of polyubiquitin chains since UBLs and ubiquitin modifications target the same lysine (Kerscher et al., 2006). Three different SUMO proteins exist in humans, SUMO-1 is generally conjugated to proteins as a monomer, while SUMO-2 and SUMO-3 are able to form high molecular weight polymers on proteins. SUMO modifications seemed to be implicated in cell cycle progression, in transcriptional regulation (p53, c-Jun, c-myc), and in modulation of protein-protein interactions (Andreou et al., 2009). Nedd8, another UBL protein takes part in cell cycle progression and cytoskeletal regulation. A few RING finger family of E3s such as c-Cbl (Oved et al., 2006) and Mdm2 (Xirodimas et al., 2004) have been reported to catalyze the attachment of Nedd8 to specific substrates.

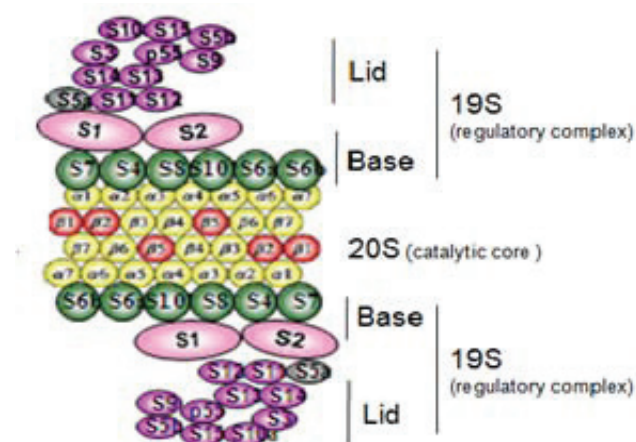
1.1.2 Degradation of targeted substrates by the 26S proteasome

Structure and function of proteasomes are conserved from archaeobacteria to eukaryotes (*Fig. 3*). It is essential for cell and organism viability (Fujiwara et al., 1990). Other evolutionarily conserved complexes have been described, among them the immunoproteasome, composed by 20S proteasome and 11S activator, involved in the antigen processing (Hill et al., 2002) or the Cop9 Signalosome which has a role in regulation of ubiquitination and cell signaling (Kato and Yoneda, 2009). However, the 19S activator which binds to the 20S proteasome to form the 26S proteasome, is the only proteasome activator that is known to stimulate degradation of protein substrates (Ferrell et al., 2000).

1.1.2.1 The 26S proteasome

The 26S proteasome (~2500 kDa) (*Fig. 3*) is a cellular multisubunit ATP-dependent protease that plays a crucial role in cellular regulation and protein homeostasis, degrading proteins modified by polyubiquitin chains (Coux et al., 1996). It is composed of a 20S

proteasome or catalytic particle (CP, ~700 kDa) capped by one or two 19S proteasome or regulatory complexes (RC, ~ 900 kDa) and assembles in an ATP-dependent manner (Hirano et al., 2005).



Tanaka, 6th Proteasomes Workshop 2005.

Figure 3: The 26S proteasome. The proteasome is the major cellular protease in cells. It is composed of a catalytic core (core particle CP, 20S proteasome) and one or two regulatory complexes (RC, 19S proteasome). The 19S proteasome is divided into two subcomplexes: the base and the lid. The 19S proteasome is in charge of substrate recognition, deubiquitination, denaturation, translocation and subsequent degradation in the 20S core. The 20S proteasome is composed of 2 outer rings of α subunits and 2 inner rings of β subunits. The β subunits carry on the proteolytic activity. (Tanaka, 6th Proteasomes Workshop 2005)

19S Proteasome

Nomenclature Dubiel, et al. ²	Gene Human	Sequence length (aminoacid)
19SATPase subunits		
S7	PSMC2	432
S4	PSMC1	440
S6b	PSMC4	418
S10b	PSMC6	389
S6a	PSMC3	439
S8	PSMC5	406
19Snon-ATPase subunits		
S2	PSMD2	908
S1	PSMD1	953
S3	PSMD3	534
p55	PSMD12	455
S9	PSM11	421
S10a	PSMD6	389
S12	PSMD7	324
S11	PSM13	376
S5a	PSMD4	377
S13	PSMD14	310
S14	PSMD8	257
S5b	PSMD5	503
S15	PSMD9	223

20S Proteasome

Nomenclature Baumeister, et al. ¹	Gene Human	Sequence length (aminoacid)
20Sα-type subunits		
α 1	PSMA6	246
α 2	PSMA2	233
α 3	PSMA4	261
α 4	PSMA7	248
α 5	PSMA5	241
α 6	PSMA1	263
α 7	PSMA3	254
20Sβ-type subunits		
β 1	PSMB6	239
β 2	PSMB7	277
β 3	PSMB3	205
β 4	PSMB2	201
β 5	PSMB5	208
β 6	PSMB1	241
β 7	PSMB4	264

Table 1: Proteasome nomenclature. The nomenclature is based on the work of ¹Baumeister et al. (1998) for the 20S subunits and on the work of ²Dubiel et al. (1995) for the 19S subunits. The 20S subunits are divided in α and β subunits whereas the 19S is composed of ATPases and non-ATPases subunits. In this table are reported as well the gene (human) and the sequence length corresponding to each subunits.

1.1.2.1.1 The 20 S proteasome

Structure

The 20S proteasome carries the catalytic activities. The cylindrically shaped structure is formed by four stacked rings of α (1 to 7) and β (1 to 7) subunits (*Table 1*). The subunits β ₂, β ₅ and β ₁ possess the proteolytic activity, harboring respectively trypsin-like, chymotrypsin-like, and caspase-like peptidase activities. Outer α rings of the 20S form a gating channel (Groll et al., 2000), which interacts with the base of the 19S, composed of six distinct AAA-type ATPases, S1 and S2 (Braun et al., 1999). (*Fig. 3*)

Assembly

Proteasomal subunits should be connected into a functional complex, the proteolytic active sites have to mature and be controlled during the assembly process. The assembly of the proteasome is regulated by numerous proteins (chaperones) and is highly conserved from yeast to human. The formation of the 20S proteasome starts with the assemblage of a ring of α -subunits which should be error prone with the help of at least four chaperones (PAC1 to PAC4). Then, the β -subunits are incorporated, a fifth chaperone POMP is required that functions as quality control agent. Finally, two sets of α/β -rings are connected accompanied by the maturation of the β -subunits in order to form functional 20S proteasomes (Bedford et al., 2010).

1.1.2.1.2 The 19S proteasome

Structure

The 19S proteasome can be dissociated into two subcomplexes: the base that binds directly to the 20S core, and an external lid. The base and the lid are stabilized by the S5a subunit. The base is formed by six distinct AAA-type ATPases (S4, S6a, S6b, S7, S8 and S10b) and non-ATPases subunits (S1 and S2) (*Table 1*). The lid is composed by non-ATPases subunits (S3, S9, S10a, S11, S12, S13, S14, S15 and P55). (Gorbea et al., 1999) (*Fig. 3*)

Assembly

Assembly of the 19S proteasome requires four chaperones (Nas2/p27, Nas6/gankyrin/p28, Rpn14/PAAF1, Hsm3/S5b) which can stabilize 19S subcomplexes, facilitate the incorporation of these subcomplexes into 19S proteasome precursors and act like quality control proteins. Several studies, in yeast and human, reported subcomplexes formation such as S10b-S6a-p27,

S4-S7-S2-S5b, S8-PAAF1 and S6b-p28. Thereafter, these subcomplexes might assemble together with S1 into a 19S precursor. The last step consists of the linkage of S5a and the lid to form the 19S proteasome (Fig. 4) (Saeki et al., 2009; Le tallec et al., 2009; Kaneko et al., 2009; Funakoshi et al., 2009).

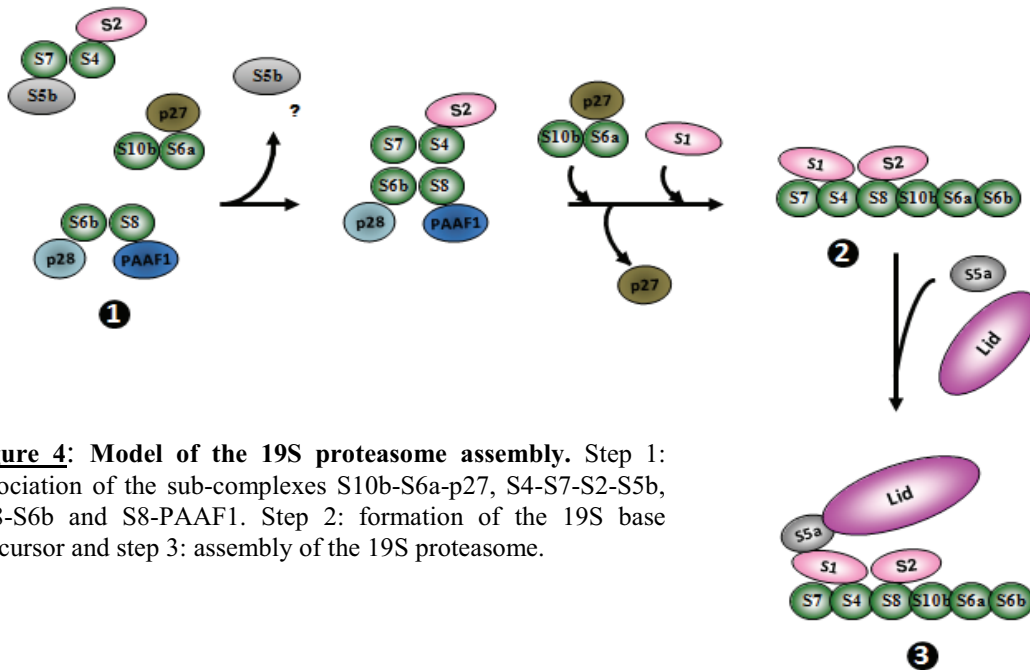


Figure 4: Model of the 19S proteasome assembly. Step 1: association of the sub-complexes S10b-S6a-p27, S4-S7-S2-S5b, p28-S6b and S8-PAAF1. Step 2: formation of the 19S base precursor and step 3: assembly of the 19S proteasome.

1.1.2.1.3 Assembly of the 19S and 20S to form the 26S proteasome

In vitro experiments demonstrated that the 26S proteasome can dissociate into the 19S and 20S proteasome and reassociate in an ATP-dependent manner (Liu et al., 2006; Isono et al., 2007). It appeared with *in vivo* experiments that the 26S proteasome can be assembled both *de novo* and, by analogy with the *in vitro* experiments, from the 20S proteasome and the already assembled 19S proteasome (Kiss et al., 2005; Babbitt et al., 2005). In fact, a dynamic equilibrium occurs between 20S, 19S and 26S proteasomes that can be modified depending on conditions in the cell (Bajorek et al., 2003).

1.1.2.2 Signaling of protein degradation

The 19S proteasome drives ATP-dependent substrate recognition, deubiquitination, processing and delivery to the 20S core where the proteolytic activity of the proteasome is located (Ferrell et al., 2000).

1.1.2.2.1 Recognition of substrates

For degradation of substrates, polyubiquitinated protein must be recognized by specific multiubiquitin receptors or by adaptor proteins that subsequently bind the proteasome (Hartmann-Petersen et al., 2004). Many Ubiquitin Binding Domains (UBDs) were characterized: UIM (ubiquitin-interacting motif), DUIM (double UIM), UBA (ubiquitin-associated) or PRU (pleckstrin-like receptor for ubiquitin) among the most important ones.

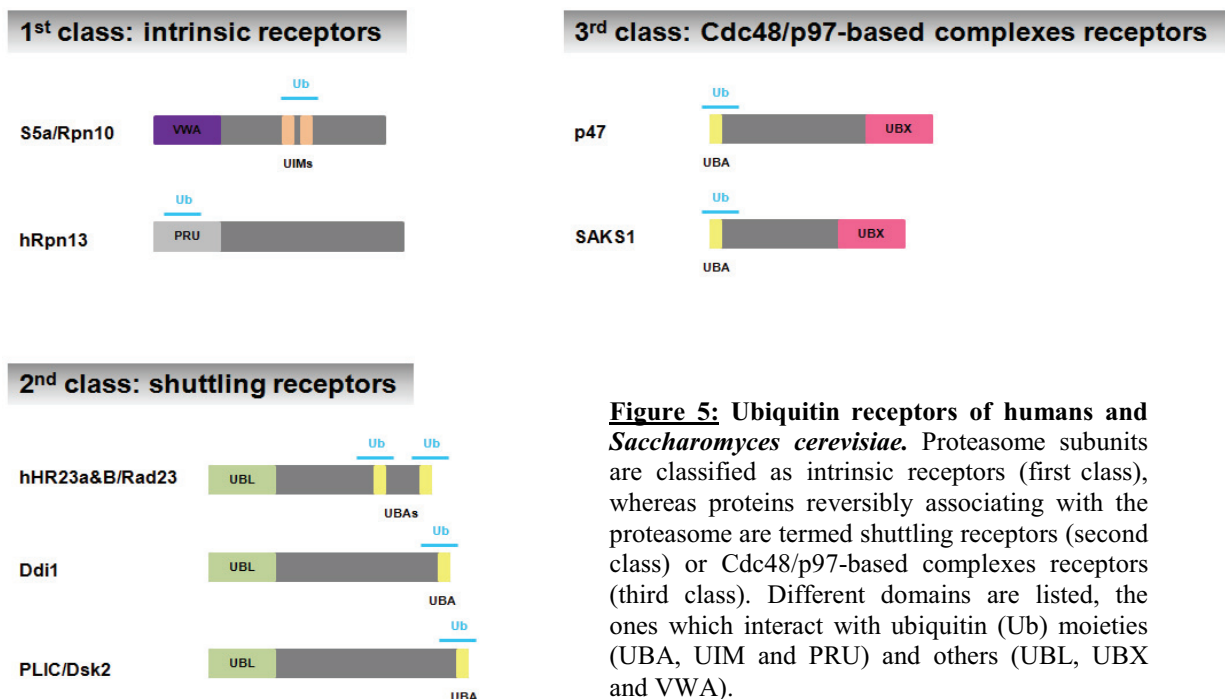


Figure 5: Ubiquitin receptors of humans and *Saccharomyces cerevisiae*. Proteasome subunits are classified as intrinsic receptors (first class), whereas proteins reversibly associating with the proteasome are termed shuttling receptors (second class) or Cdc48/p97-based complexes receptors (third class). Different domains are listed, the ones which interact with ubiquitin (Ub) moieties (UBA, UIM and PRU) and others (UBL, UBX and VWA).

One of the first class of multiubiquitin receptors discovered contains the S5a protein. In most organisms this protein exists as a free subunit as well as a subunit of the 26S proteasome. S5a binds polyubiquitin chains (Deveraux et al., 1994), and thus, directly recruits substrates to the proteasome (Verma et al., 2004; Van Nocker et al., 1996). The property of S5a to bind ubiquitin-conjugates is linked to two independent ubiquitin interacting motifs (UIMs), while a VWA (vonWillebrand A) domain in the N-terminus thought to mediate interaction with the proteasome (Kang et al., 2007). Thereafter, a second subunit of the 19S proteasome was identified as a multiubiquitin receptor *in vitro*, the S6a AAA-ATPase (Lam et al., 2002). HRpn13 constitutes also another intrinsic ubiquitin receptor of the proteasome and it binds ubiquitin via a PRU domain (pleckstrin-like receptor for ubiquitin). (*first panel, Fig. 5*)

A second class of multiubiquitin receptors involves ubiquitin-like domain (UBL)-containing shuttle factors, including Rad23/hHR23 A and B, Dsk2/PLIC and Ddi1 (*second*

panel, Fig. 5). These UBL factors are known as shuttle factors because they target ubiquitinated substrates to the 26S proteasome via the C-terminal ubiquitin associated (UBA) domain, which then interact with S5a or/and hRnp13 in the 19S proteasome via the N-terminal ubiquitin like (UBL) domain (Saeki, et al. 2002). Furthermore, some studies have shown that S2 base subunits function as plausible receptors of shuttle factors ubiquitin-like domain (Leggett et al, 2002).

A third class of multiubiquitin receptors involves Cdc48/p97-based complexes (Elsasser et al., 2005) (*third panel, Fig. 5*). Cdc48/p97 is an ATP-dependent chaperone consisting in six identical subunits and can bind ubiquitinated substrates via adapter molecules containing UBX (Ubiquitin regulatory X)-UBA domains (Ufd1–Npl4). These complexes could be involved in degradation of cytosolic substrates such as I κ B α and HIF1 α (Alexandru et al. 2008).

1.1.2.2.2 Deubiquitination, unfolding, translocation into the 20S and degradation of substrates

Once the substrate is delivered to the proteasome, polyubiquitin chain is removed. Therefore, three deubiquiting enzymes (paragraph 1.1.3, Deubiquitinases “DUBs”) are associated with the 19S proteasome. Among them, is a metalloprotease, the S13 subunit of the proteasome (Verma et al., 2002). Uch37 which belongs to the UCH family (Yao et al., 2006) and USP14, ubiquitin specific proteases and they are associated with the proteasome (Borodovsky et al., 2001). Uch37 and USP14 are activated by their binding to the S1 subunit of the proteasome (Crosas et al., 2006). S13 cleaves at the base of the ubiquitin chain, where it is linked to the substrate. While Uch37 and S14 remove ubiquitin moieties from the distal end of the chain. These two proteins can inhibit the degradation of low ubiquitinated proteins and function as an editing activity for the proteasome (Amerik et al., 2004).

After deubiquitination, one or more of the six AAA-ATPases located in the base of the 19S proteasome catalyze the unfolding of the substrate, the opening of the gate and the translocation of substrates into the 20S catalytic chamber. The 19S proteasome unfolds ubiquitinated proteins most likely by binding to an unstructured segment of the substrate, known as an initiation site, and using energy from ATP hydrolysis, by targeting the substrate into a channel that leads to the 20S proteasome (Orlowski et al., 2010). The denatured polypeptide is then translocated into the degradation chamber of the proteasome, where it is cleaved into small peptides (Kenniston et al., 2004).

Although target proteins for proteasomal degradation are usually polyubiquitinated, some substrates can also be degraded without ubiquitin modification, as examples the ornithine decarboxylase protein (ODC) or p21^{CIP} (Murakami et al., 2000; Sheaff et al., 2000).

1.1.3 Deubiquitinases “DUBs”

Deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin or ubiquitin-like moieties from target proteins. The human genome encodes around 95 putative DUBs divided into five families (Nijman et al., 2005; Komander et al., 2009). Four families contain cysteine proteases (ubiquitin specific proteases (USP), ubiquitin C-terminal hydrolases (UCH), Machado Joseph disease proteases (MJD) and otubain proteases (OTU)) while the JAMM belong to a family of Zn-dependent metalloproteases (JAB1/MPN/Mov34 metalloenzyme, one working on the ubiquitin-like protein Nedd8) (Komander et al., 2010). The cysteine proteases perform the reaction via a catalytic triad formed by cysteine, histidine and aspartic acid residues. The DUBs play several roles in the ubiquitin pathway such as after substrate degradation occurs or when ubiquitin-mediated function ends, ubiquitin molecules are recycled and ubiquitin moieties are removed from modified proteins to maintain a free ubiquitin pool, step fundamental for an efficient proteolysis (Johnston et al., 2006).

1.1.4 Other roles of proteasomal subunits in cells

Proteins of the 19S proteasome can have additional roles in cells distinct from proteasomal functions. The 19S ATPase proteins independent of 20S, called APIS complex, in particular S8, S6a and S6b, have been reported to be recruited to certain promoters and to actively participate in transcription, in yeast (Sun et al., 2002) and in human (Truax et al., 2010). In addition, S8, S6a and S6b proteins have been described as critical regulator for histones (Koues et al., 2009).

1.2 Mdm2 and its role in p53 regulation

The diverse activities and the central role of the UPS in apoptosis have made the proteasome an important target for drug development (Navon and Ciechanover, 2009). One example of protein regulation by the 26S proteasome is the p53 tumor suppressor that has been shown to be regulated by the E3 ubiquitin ligase Mdm2 via its association with some 19S proteasomal subunits (Kulikov et al., 2010).

1.2.1 The “RING” E3 ubiquitin ligase Mdm2

1.2.1.1 Generalities

The *mdm2* (murine double minute 2 or *hdm2* for human) gene was initially identified as one of the genes responsible for the spontaneous transformation of the BALB/c mouse cell line NIH 3T3-DM (Cahilly-snyder et al., 1987). The three genes *mdm1*, *mdm2*, and *mdm3* were located on a short acentromeric extra-chromosomal body, called double minute chromosome. The second (*mdm2*) has transforming abilities (Fakharzadeh et al, 1991). Mdm2 can be classified as an oncogene and over-expression have been observed in a wide variety of human tumors such as sarcoma, glioblastoma, leukemia or non-Hodgkin's lymphoma (Oliner et al., 1992; Bueso-Ramos et al., 1993).

Mdm2 is the main E3 ligase controlling the stability of p53 that plays a role in cancer development (Honda et al., 1997). It has become clear since several years that if Mdm2 is present at high level, other cellular pathways, distinct from p53, can be activated that lead to tumorigenesis (at least 5-10% of all human tumors have abnormal Mdm2 over-expression) (Honda et al., 1997). Mdm2 can interact and controls others proteins than p53 (*Fig. 5*), and some data describe Mdm2 as a regulatory protein involved in cell cycle arrest. For instance, it has been shown that Mdm2 inhibits the activation of cell-cycle checkpoints due to interaction with p21, Rb and E2F-1 (Zhang et al., 2004; Sdek et al., 2005; Mundle et al., 2003). Mdm2 also has a role in DNA repair owing its binding to Nbs1 (Bouska et al., 2008) or Tip60 (Sapountzi et al., 2006). In addition Mdm2 has an impact on apoptosis via the regulation of FOXO3a stability (Yang et al., 2008), through the regulation of insulin-like growth factor 1 receptor (IGF-1R) and the AKT signaling pathway (Tao al., 2007).

1.2.1.2 Structure of Mdm2

The Mdm2 protein (90kDa) consists of several domains (*Fig. 6*) that are conserved between species from human to zebrafish. The first domain, in the N-terminal of Mdm2, is the p53 interaction domain which is sufficient to bind p53 and inhibits its capacity to interact with the transcriptional machinery. Downstream of the p53 binding domain, is a nuclear localization signal (NLS) and a nuclear export signal (NES). These two domains mediate the ability of Mdm2 to shuttle between the nucleus and the cytoplasm (Roth et al., 1998). The central domain of Mdm2 contains an acidic region, responsible for the interaction with a range of regulatory proteins such as CBP/p300 (Argentini et al., 2001), ribosomal proteins L5, L11, L23 (Marechal et al., 1994, Zhang et al., 2009), ARF (Tao et al., 1999) or YY1 (Sui et al., 2004) and supports the second binding site for p53 (Freedman et al., 1999). Finally, at the C-terminus, a zinc finger domain, a RING finger domain, a Walker A motif and a nucleolar signal sequence are present. The RING domain of Mdm2 can bind to RNA and nucleotides (Elenbaas et al., 1996) or functions as an E3 ligase that catalyzes the transfer of ubiquitin or Nedd8 to target substrates including p53 (Honda et al., 1997; Xirodimas et al., 2004). (*Fig. 6*)

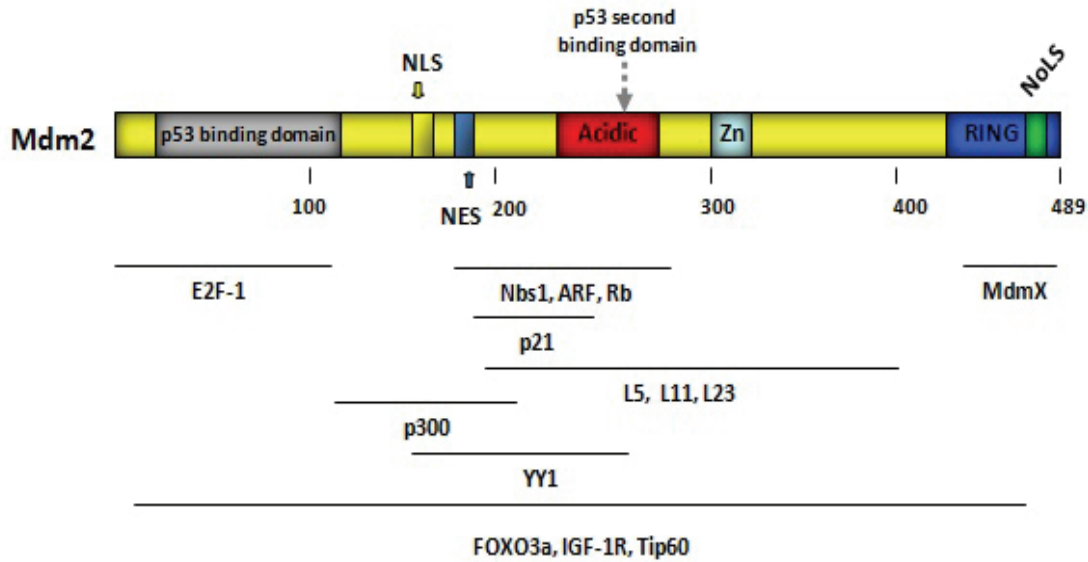


Figure 6: Mdm2 structure and interacting partners. From N to C terminus of Mdm2, the boxes represent respectively the p53 binding (p53), acidic, zinc finger (Zn) and E3 ubiquitin ligase ring finger (RING) domains. The nuclear localization signal (NLS), the nuclear export signal (NES) and the nucleolar export signal (NoLS) are as well indicated. The regions where interacting partners of Mdm2 bind are underlined with black lines.

1.2.1.3 Post-translational modifications of Mdm2

Mdm2 is highly regulated through many types of post-translational modifications such as ubiquitination, phosphorylation or sumoylation that have a positive or a negative impact on its ability to influence p53 activity (Maya et al., 2001; Ogawara et al., 2002; Meek et al., 2003). Furthermore, Mdm2 is a RING E3 ligase for itself, promoting its auto-ubiquitination. After stimuli such as DNA damage, Mdm2 promotes its auto-ubiquitination, targeting itself for proteasomal degradation which leads to the activation of p53 (Fang et al., 2000). Sumoylation of Mdm2 modulates its E3 ligase activity and decreases Mdm2 self-ubiquitination (Buschmann et al., 2001).

1.2.2 P53 "guardian of the genome"

Despite its early discovery in 1979 (Levine et al., 1983), p53 is still one of the most studied tumor suppressor proteins (55639 PubMed-listed publications). Through its anti-proliferative activity, p53 "the guardian of the genome" (Lane, 1992) is an important target for cancer therapy, particularly for those possessing the wild type protein.

Mutations in *p53* increase susceptibility to cancer and may be somatic or inherited. Germ line mutations in *p53* contribute to Li-Fraumeni syndrome, which causes predisposition to a diversity of tumors (Hollstein et al., 1994; Cho et al., 1994; Goh et al., 2011).

P53 is a short-lived nuclear phosphoprotein (half-life of less than 20 min) (Olson et al., 1993). The low level of p53 is maintained by several RING finger E3 ligases among them: Mdm2 (Honda et al., 1997), PirH2 (Leng et al., 2003), Mule (Chen et al., 2005), Cop1 (Dornan et al., 2004), E6-AP (Beer-Romero et al., 1997) and Topors (Rajendra et al., 2004). Subsequent studies revealed that activated p53 (high level of p53) is a sequence specific DNA binding transcription factor and regulates a huge number of target genes (Hoh et al., 2002). Moreover, under certain conditions p53 is also subjected to a plenty of post-translational modifications (acetylation or phosphorylation) different from ubiquitination that can both activate or stabilize p53 (Siliciano et al., 1997; Hupp, 1999; Liu et al., 1999; Rodriguez et al., 2000; Vogelstein et al., 2000; Barlev et al., 2001; Li et al., 2002; Brooks et al., 2003; Chuikov et al., 2004).

1.2.3 The Mdm2-p53 pathway

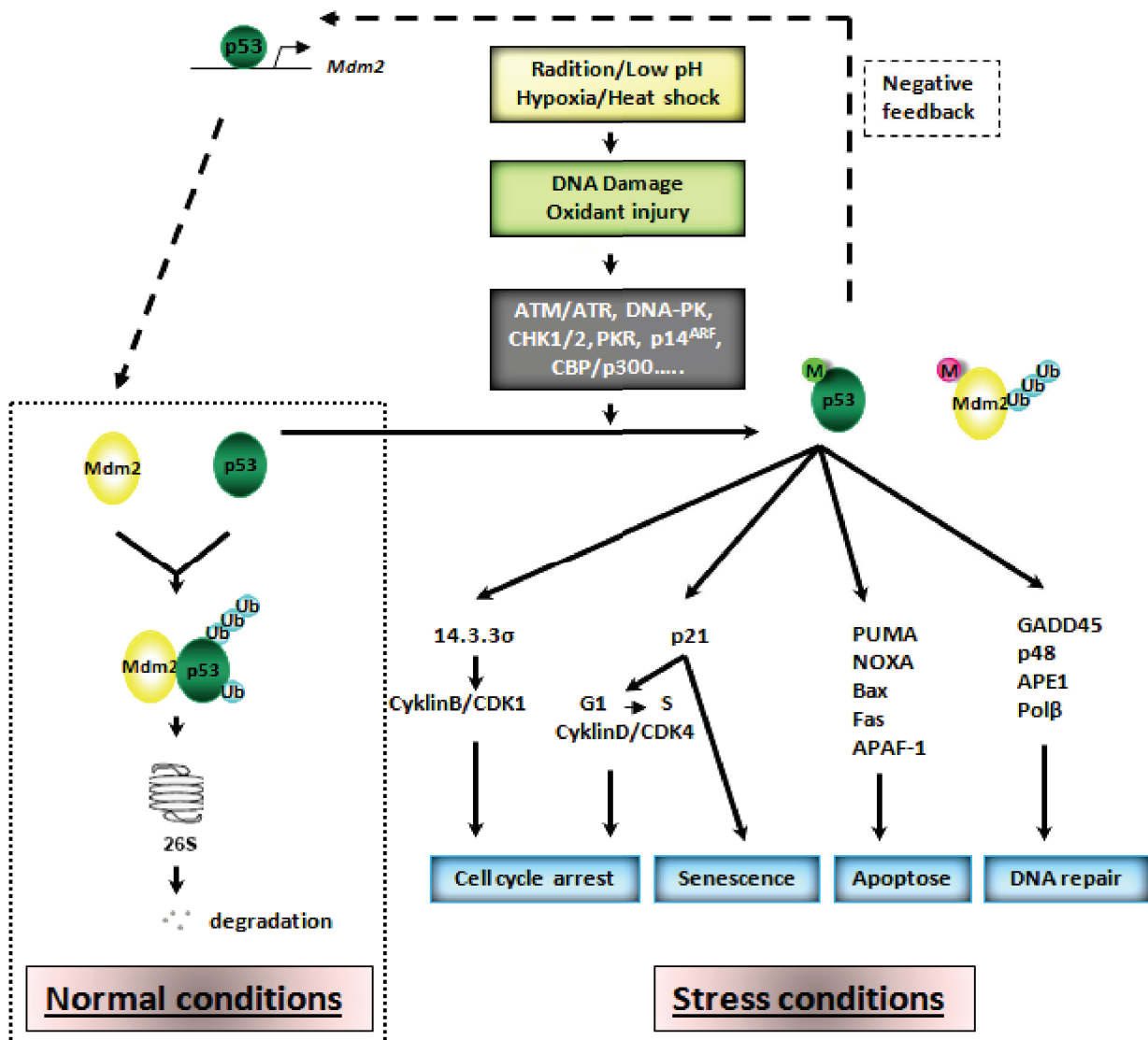


Figure 7: General scheme of p53 regulation. Under normal conditions, p53 is maintained at a low level in cell via its degradation by the proteasome and Mdm2 is the most important E3 ligases for p53. Upon stress conditions, p53 becomes stabilized and activated by post-translational modifications (M: phosphorylation or acetylation or methylation or neddylation or sumoylation, Ub: ubiquitination) and other signaling pathways, which activate transcription of genes implicated in cell cycle arrest, senescence, apoptosis or DNA repair.

1.2.3.1 In normal conditions, p53 is one of the major substrate of Mdm2

As mentioned above, several E3 ubiquitin ligases take part in p53 degradation but the main regulator is Mdm2 which controls the transcriptional activity of p53. Under normal conditions (Fig. 7, normal conditions), Mdm2 interacts with the transactivation domain of p53 and thus

inhibiting the binding of transcriptional co-activators (for example, p300 and CBP) (Wadgaonkar et al., 1999; Chen et al., 1993), and inducing its ubiquitination followed by its degradation by the 26S proteasome. Mdm2 provides a second binding site within the central acidic domain (Kulikov et al., 2006).

Concerning p53, initial studies on the p53-Mdm2 interaction identified the N-terminal region as a binding site for Mdm2 (Sakaguchi et al., 1997), but others have described a second binding site within the DNA-binding domain (Shimizu et al., 2006). P53 harbors as well a tetramerization domain that enhances its interaction with Mdm2 and thus, its degradation (Kubbutat et al., 1998). Thereafter, several researches have shown that binding of Mdm2 to p53 without E3 ligase activity was not enough to inhibit p53 activity (Itahana et al., 2007), implying a central role for the “RING” E3 ligase function of Mdm2 for the negative regulation of p53. Thus, Mdm2 inhibits p53 activity through two main mechanisms: blocking the transcriptional activation of p53 and promoting its degradation (Momand et al., 1992).

1.2.3.2 The p53-Mdm2 pathway upon stress condition

The tumor suppressor p53 maintains genome integrity and prevents inappropriate cell proliferation due to genotoxic stress (Vogelstein et al., 2000). After a cellular exposure to a variety of stimuli, such as DNA damage (including IR ionizing radiation, UV radiation, cytotoxic drugs or chemotherapeutic agents, or infectious virus), heat shock, hypoxia, or oncogene over-expression, p53 is activated and accumulates in the nucleus where it binds specific sites in the regulatory region of p53 responsive genes (*Fig. 7, stress conditions*). Thus, activated p53 constitutes a pivotal regulatory protein which activates several biological responses (Levine, 1997). The activation of p53 involves an increase of the p53 protein level as well as numerous changes in the protein through plethora posttranslational modifications, resulting in induction of p53 targeted genes (Fritsche et al., 1993). In response to DNA damage or oxidant injury which causes double strand breaks in DNA (DSBs), the protein kinase ATM (ataxia telangiectasia mutated) is activated and can in turn activate the Chk2 kinase (Matsuoka et al., 1998). This event leads to the phosphorylation of p53 at distinct sites promoting cell cycle arrest or apoptosis dependent of p53 (Morgan et al., 1997). Furthermore, DNA damage involves also replication blockage, inducing the kinase ATR (ATM and Rad3-related). Subsequently, the kinase Chk1 is activated and can phosphorylate and activate p53 (Shieh et al., 2000). Many genes are activated by p53, nearly more than hundred genes contain p53 responsive elements, and these genes promote diverse signaling pathways. It

includes genes implicated in cell cycle arrest (14.3.3 or p21), DNA repair (GADD45, p48, APE1, pol β), senescence (p21) or apoptosis-related genes (PUMA, Bax, Fas, NOXA, APAF-1) (Vogelstein et al., 2000) (*Fig. 7, stress conditions*). However, an elevated level of p53 can also induce repression of gene expression like for bcl-2, bcl-X, cyclin B1 and survivin, some of them are negative regulators of apoptosis (Mack et al., 1993).

1.2.3.3 Monoubiquitination versus polyubiquitination of p53

It is becoming clearly evident that the ubiquitination of p53 by Mdm2 is not so easy to understand than initially thought. Otherwise, according to the levels of Mdm2 proteins and the kind of ubiquitination (poly versus mono), the ubiquitination of p53 regulates not only the degradation of p53, but also affects its localization and activity (Li et al., 2003). The monoubiquitination and the nuclear export of p53 are thus carried by low level of Mdm2 which constitute a way of regulation of p53 in unstressed cells (Boyd et al., 2000,). In contrary, high levels of Mdm2 induced polyubiquitination of p53 followed by its nuclear degradation by the proteasome and take part in the inhibition of the p53 functions during the later stage of cellular stress response or when Mdm2 is overexpressed in tumors (Shirangi et al., 2002). The mechanism is not well elucidated whether the monoubiquitination of different lysines in the C-terminal region and/or in the DNA binding domain can liberate the NES (Nuclear export signal), leading to the nuclear export (Nie et al., 2007) or if export machinery such as Crm1 is required.

1.2.3.4 The feedback loop

Importantly, the rates of Mdm2 are modulated by p53 which stimulates the expression of the *mdm2* gene by a negative feedback loop (*Fig. 7, upper part*) (Wu et al., 1993). Therefore, when Mdm2 protein is expressed in a cell where p53 is active, it inhibits further p53 function, resulting in less Mdm2 produced. Factors that alter this loop and disturb the ability of p53 protein to stimulate Mdm2 or inhibit Mdm2 activity, lead to growth arrest. While factors that enhance Mdm2 levels by amplification of this gene or increased Mdm2 activity will induce cell proliferation.

1.2.4 The ternary complex “p53-Mdm2-19S subunits”

Besides targeting ubiquitin chain formation, Mdm2 has an additional role for p53 because mutations in the central domain of Mdm2 still induce the ubiquitination of p53 even if it is not enough for its degradation (Argentini et al., 2001; Blattner et al., 2002). Recent results from our laboratory showed that subunits of the 19S proteasome can associate with E3 ubiquitin ligases such as Mdm2. This interaction seems to be necessary for the transport of p53 and its degradation by the 26S proteasome (*Fig. 8*).

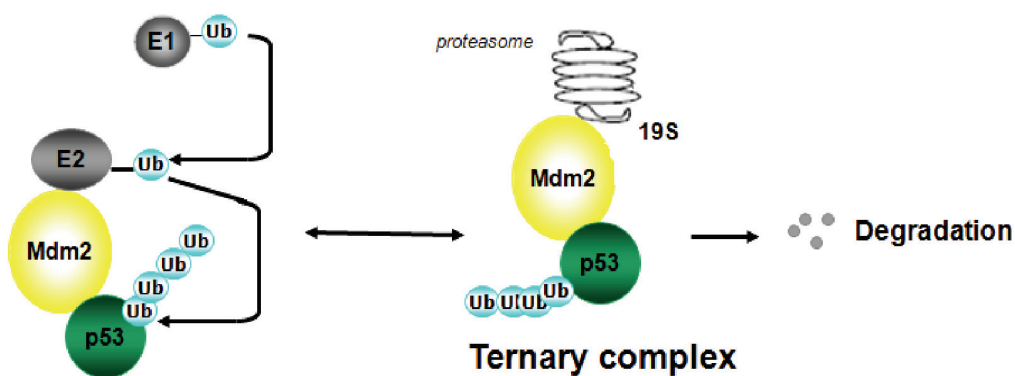


Figure 8: Ternary complex. This complex is formed between Mdm2, p53 and some 19S subunits (S2, S4, S5a, S6a, S6b, S8, S10b, and S12) to deliver p53 into the 26S proteasome.

P53, Mdm2 and the proteasome can therefore form a ternary complex. The binding of Mdm2 to the proteasome is regulated by the central domain of Mdm2 containing an “EDY” motif (E: glutamic acid, D: aspartic acid and Y: tyrosine) that interacts with the C-terminal part of Mdm2. The phosphorylation of this motif or binding of p53 releases the C-terminus of Mdm2 that can associate with the proteasome and induce the degradation of p53. Mdm2 can interact strongly with the 19S subunits S2, S4, S5a, S6a and S6b and weakly with S8, S10b, and S12. All these subunits contain an “EDY” motif (or “EDF”, F: phenylalanine) similar to the one found in the central domain of Mdm2. However, the physiological role of the interactions between Mdm2 and the 19S subunits is still unclear. (Kulikov et al., 2010)

1.3 Aim of the study

Several authors have described the potential interactions existing between the 19S proteins-E3ligases-substrates (Berezutskaya et al., 1997; Sdek et al., 2005 and Corn et al., 2003). However, mechanisms of protein delivery to the proteasome are still poorly understood. Results from our laboratory demonstrated that Mdm2 could interact with 19S subunits (S2, S4, S5a, S6a, S6b, S8 and S10b) to form a ternary complex between p53, Mdm2 and 19S proteins in order to deliver p53 efficiently to the proteasome. This process can be expanded to other E3 ligases like Siah-1 and c-Cbl, which interact likewise with some 19S subunits (S8 and S10b). (Kulikov et al., 2010)

The aim of my PhD was to elucidate the physiological role of the interactions of E3 ubiquitin ligases with the 19S proteasome and to figure out the impact of substrates.

Since Mdm2 is one of the E3 ligases interacting with some 19S proteins, the goal was to determine the impact of Mdm2 and p53 on the modifications of the 19S proteasomal subunits as well as its effect on the assembly/disassembly of the proteasome. The experimental section of this thesis consists of three parts: 1) Confirm and characterize the post-translational modifications (ubiquitination) of some 19S subunits induced by Mdm2, 2) Investigate the effect of the association between Mdm2, p53 and 19S subunits on the 26S proteasome and to test if this principle could be extended to other E3 ligases (Siah-1 and c-Cbl), and 3) Elucidate the functions of the ubiquitinated 19S subunits whether this process is linked to the assembly or the disassembly of the 26S proteasome.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and consumables

Name	Source
Agar	<i>Otto Nordwald, Hamburg</i>
Agarose	<i>Peqlab, Erlangen</i>
Ampicillin/Streptomycin	Gibco-BRL, Karlsruhe
Bacto-agar	<i>Otto-Nordwald GmbH, Hambourg</i>
Bacto-petri dishes	<i>Greiner Labortechnik, Nürtingen</i>
BSA	<i>PAA, Pasching, Österreich</i>
Bradford reagent	<i>Biorad, Munich</i>
DMSO	<i>Fulka, Buchs, Germany</i>
DTT (Dithiotreitol)	<i>Roche, Mannheim</i>
ECL Hyperfilm	<i>Amersham, GE Healthcare, Freiburg</i>
Eppendorf (1,5/2 ml),	<i>Eppendorf, Hambourg.</i>
Falcon (15/50 ml)	<i>Greiner Bio One, Frickenhausen</i>
FBS (Foetales Bovine Serum)	<i>PAA, Pasching, Österreich</i>
Filter paper	<i>Wattman, Optikon (Schweiz)</i>
IPTG	<i>Peqlab, Erlangen</i>
Luminol	<i>Fluka, Buchs, Schweiz</i>
MG132	Proteasome inhibitor, <i>Calbiochem, Darmstadt</i>
Milk powder	Saliter, Obergünzburg
Nonidet P-40 (NP40)	<i>Boehringer, Mannheim</i>

Petri dishes and flasks	<i>Greiner Bio One, Frickenhausen</i>
PBS (Phosphate Buffered Saline 1X and 10X)	<i>Gibco Invitrogen, Karlsruhe</i>
Phosphatase-Inhibitor Cocktail	<i>Roche, Mannheim</i>
PVDF membrane (Immobilon-P [®])	<i>Millipore, Zug (Schweiz)</i>
Triton-X-100	<i>Biorad, Munich</i>

All the other chemicals and consumables were purchased from *Roth* (Karlsruhe), *Sigma-Aldrich* (Taufkirchen), *GibcoTMInvitrogen* (Karlsruhe) and *Merck* (Darmstadt).

2.1.2 Kits

Name	Source
Qiagen [®] Plasmid Mini Purification Kit	<i>Qiagen, Hilden/France</i>
Qiagen [®] Plasmid Maxi Purification Kit	<i>Qiagen, Hilden/France</i>
Lipofectamin TM 2000	<i>Invitrogen, Karlsruhe</i>

2.1.3 Standards

Name	Source
Gene Ruler TM 1 kb DNA Marker	<i>Fermentas, France</i>
Page Ruler TM Protein Marker	<i>Fermentas, St. Leon-Rot</i>
Gel Filtration Calibration Kit HMW	<i>GE-Healthcare, Freiburg</i>

2.1.4 Binding matrices

Name	Source
Protein A Sepharose	<i>Pierce, Thermo Scientific, La Jolla (USA)</i>

Glutathion-Sepharose 4B	<i>GE-Healthcare</i> , Freiburg
NHS Sepharose 4FF	<i>Pharmacia Biotech</i> , France
Ni ²⁺ -NTA Agarose	<i>Qiagen</i> , Hilden

2.1.5 Oligonucleotides

The small interfering RNAs (siRNAs) were purchased from *Eurofins MWG* (Ebersberg).

Name	Sequence
Control	5'-AACCCCUUUUAAAAGGGGCC-3'
Mdm2	5'-ACCAACAUGUCUGUACCUACUTT-3'
S8	5'-ACCAACAUGUCUGUACCUACUTT-3'

2.1.6 Enzymes

Name	Source
Factor Xa	<i>England Biolabs</i> , Beverly (USA)

2.1.7 Cells lines

Name	Source and description
U2OS cells	Human, epithelial, osteosarcoma cell line, WT p53 (ATCC-No. HTB-96™)
H1299 cells	Human, epithelial, lung carcinoma cell line, deficient in p53 (ATCC-No. CRL-5803™)
C2C12 cells	Mouse myoblast cell line (ATCC-No CRL-1772™)

2.1.8 Bacteria

Name	Description
<i>E. coli BL21</i> (for the expression of the Hist- or GST-fused proteins)	Genotyp: F ⁻ , ompT, hsdS (r _B ⁻ , m _B ⁻), gal, dcm (Amersham) <i>GE Healthcare</i> , Freiburg
<i>E. coli DH5α</i> (for routine cloning)	Genotype: F ⁻ , φ80lacZΔM15, Δ(lacZYA-argF) U169, deoR, recA1, endA1, hsdR17(rk ⁻ , mk ⁺) phoA, supE44, thi-1, gyrA96, relA1

2.1.9 Plasmids

Name	Description
pcDNA3.1	for transient transfections into mammalian cells and contained a CMV promoter: <ul style="list-style-type: none"> ✓ Empty vector (<i>Invitrogen</i>, Karlsruhe, Germany) ✓ pcDNA3.1 S1, S2, S4, S5a, S6a, S6b, S8, S9, S10b tagged V5 in C-terminus ✓ pcDNA3.1 S6b tagged Flag in C-terminus ✓ pcDNA3.1 Mdm2, Mdm2 C464, Siah1, c-Cbl tagged Myc ✓ pcDNA3.1 p53 ✓ pcDNA3.1 Ubiquitin tagged Histidine, mutants K6R, K11R, K27R, K29R, K33R, K48R and K63R and the K0 mutant where all lysines are replaced with an alanine. They are all tagged 6x-Histidine
pGEX-5X1	for production of GST-fused proteins: <ul style="list-style-type: none"> ✓ pGEX-5X1-USP2-core (Ventadour et al., 2007)
pET-26d	for production and purification of His-tagged proteins: <ul style="list-style-type: none"> ✓ pET-26d-His₆-MLG (Ventadour et al., 2007)

2.1.10 Antibodies

Primary antibodies:

Name	Description	Experimental conditions
Anti- α_7 , 20S proteasome	mouse, monoclonal, <i>Enzo Life Science</i> , USA	WB 1:5000
Anti-20S proteasome, α/β subunits (MCP321)	mouse, monoclonal, <i>Biomol</i> , USA	WB 1:1000
Anti-polyubiquitin (Fk1)	mouse, monoclonal, <i>Enzo Life Science</i> , USA	WB 1:2000
Anti-polyubiquitin and monoubiquitin (Fk2)	mouse, monoclonal, <i>Enzo Life Science</i> , USA	WB 1:2000
Anti-Flag (M2)	mouse, monoclonal, <i>Sigma-Aldrich</i> , Deisenhofen	WB 1:2000
Anti-Mdm2 (2A10)	mouse, monoclonal, <i>Calbiochem/Merck</i> , Darmstadt	WB 1 :1000
Anti-Mdm2 (4B2)	mouse, monoclonal (Che net al., 1993)	WB
Anti-Myc (9E10)	mouse, monoclonal, <i>Oncogene</i> , Bad Soden	WB 1:1500
Anti-p53 (DO-1)	mouse, monoclonal, <i>Santa Cruz</i> , USA	WB 1:1500
Anti-PCNA (PC10)	mouse, monoclonal, <i>Santa Cruz</i> , USA	WB 1:10000
Anti-S1	mouse, monoclonal, <i>Biomol</i> , USA	WB 1:1000

Anti -S4, anti-S5a, anti-S6a and anti-S8 (p45-110), 19S proteasome subunits	mouse, monoclonal, <i>Biomol</i> , USA	WB 1:2000
Anti-S2, 19S proteasome subunit	rabbit, polyclonal, <i>Calbiochem/Merck</i> , Darmstadt	WB 1:5000
Anti-S6b (TPB7-27), anti-S10b and anti-S9, 19S proteasome subunits	rabbit, polyclonal, <i>Enzo Life Science</i> , USA	WB 1:2000
Anti-Ubiquitin (U5379)	rabbit, polyclonal, <i>Sigma-Aldrich</i> , Taufkirchen	WB 1:15000
Anti-V5	mouse, monoclonal, <i>Serotec</i> , Martinsried	WB 1:5000

Secondary antibodies:

All secondary antibodies were HRP-conjugated and were purchased from *DAKO Diagnostic GmbH* (Hamburg, Germany).

The clean-blot IP detection reagent (HRP) (*Thermo Scientific*), a specific secondary antibody was used for co-immunoprecipitation experiments.

2.2 Methods

A number of protocols and recipes for common buffers used in this project were taken from the laboratory manual of molecular cloning (Maniatis et al., 1989) unless otherwise stated. Aqueous solutions were prepared with water purified by the Milli-Q plus water purification system (*Millipore*, Molesheim).

2.2.1 DNA methods

2.2.1.1 Agarose gel electrophoresis

Agarose gel electrophoresis was performed with 1% agarose gels in 1X TAE buffer (0.04 M Tris pH 7.2, 0.02 sodium acetate, 1mM EDTA) submerged in a horizontal electrophoresis tank containing 1X TAE buffer with Ethidium bromide to a final concentration of 0,3 µg/ml. The samples were mixed with DNA sample buffer (5 mM EDTA, 50% glycerol, 0,01g bromophenolblue) and loaded onto the gel. The GeneRuler DNA ladder mix (*Fermentas*) was used as standard ladder. The electrophoresis was carried at 100 V and the gel was analyzed using a UV light source.

2.2.1.2 Transformation of DNA into bacteria

Usually, 100 µl of chemically competent bacteria DH5α or BL21 were incubated with 1 µl of the purified plasmid DNA for 30 min on ice. The bacteria were heat-shocked at 42°C for 50 sec, incubated 2 min on ice and diluted to 1 ml LB medium. Then, 1 ml of LB medium without ampicillin was added for 30 min at 37°C on a shaker. Finally, the mix or an aliquot was spread onto selective agar plates, supplemented with ampicillin or kanamycin and grown overnight at 37° C.

2.2.1.3 Small-scale purification of DNA

Plasmids were purified using the Qiagen Plasmid Mini Kit®. Transformed bacteria were cultured in 2 ml of LB 1X with the adequate antibiotics overnight at 37°C under constant

shaking. Bacteria were collected by centrifugation at 5000 rpm for 5 min. Thereafter, the supernatant was removed and the pellet was resuspended in 250 µl Buffer P1. Bacteria were lysed by adding 250 µl of solution P2 and incubating for 5 min at RT. Then, 350 µl Buffer N3 (neutralization buffer) was added and mixed immediately, but gently by inverting the tube. The mixture was centrifuged at 10000 rpm for 15 min at 4°C (Eppendorf centrifuge 5415R) and the supernatant was applied to a QIAprep spin column, centrifuged for 60 sec 13000 rpm (Eppendorf centrifuge 5415R). The QIAprep spin column was washed by adding 0,5 ml PB Buffer and then by the PE Buffer. The flow-through was discarded, and centrifuged for an additional minute to remove residual wash buffer. The DNA was eluted from the QIAprep column with 50 µl EB Buffer. Subsequently, the concentration of DNA was determined using a spectrophotometer to measure absorbance at a wavelength of 260 nm.

2.2.1.4 High-scale purification of DNA

For high-scale purification of DNA, the Qiagen Plasmid Maxi kit was used. Bacteria were cultured as described above with 200 ml of LB 1X with antibiotics at 37°C using a shaker. Thereafter, bacteria were collected by centrifugation at 10000 rpm for 15 min at 4°C in a fixed angle rotor (J2-HS centrifuge) and the pellet was resuspended in 10 ml of P1 buffer containing RNase A for 10 min at RT. Alkaline lysis was performed by adding 10 ml of P2 buffer for 5 min on ice and neutralized by 10 ml of P3 buffer, 20 min on ice. The suspension was centrifuged at 4000 rpm, 20 min at 4°C (Beckman Avanti J-20) and the supernatant was applied into a Qiagen Tip 500 column pre-equilibrated with QBT buffer. Next, the column was washed twice with QC buffer. The DNA was eluted from the resin by QF buffer and then precipitated using isopropanol and centrifuged at 10000 rpm for 20 min at 4°C. The pellet was washed with 70% Ethanol and centrifuged at 12000 rpm for 10 min at 4°C. Finally, the DNA pellet was dried and resuspended in TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 8) at a final concentration of 1 mg/ml.

2.2.1.5 Quantification of plasmid DNA

To quantify the amount of DNA the optical density (OD) at 260 and 280 nm was measured with the NanoDrop®. An $OD_{260}=1$ corresponds to 50 µg/ml of double-stranded DNA. A ratio OD_{260}/OD_{280} between 1.8 and 2 corresponds to a standard purity of the nucleic acid.

2.2.2 Cell culture and transfection methods

2.2.2.1 Maintenance of mammalian cell lines

All mammalian cells were cultured in standard conditions at 37°C under 95% humidity and 5% CO₂ in an incubator (*Forma Science*). U2OS, C2C12 and H1299 cells were cultured in DMEM supplemented with 10% FCS and adequate antibiotics (5% penicillin and streptomycin).

Adherent cells were grown to a confluence of 80-90%, then the culture medium was removed, the cells were washed twice with PBS 1X and incubated with trypsin solution at 37°C until they detached from the culture dish. Fresh medium was added to the dish to inhibit the trypsin solution and the cell suspension was centrifuged 2 min at 1000 rpm (*Heraeus Megafuge 10*). Cells were collected and seeded in a new dish at a lower concentration.

Freezing cells: 10 cm dishes at 90% of confluency were trypsinized and collected by centrifugation. The cells were thereafter resuspended in freezing medium (DMEM, 20% FCS, 10% DMSO). Cryovials were first stored at -80°C for 24 h and then transferred in liquid nitrogen.

Thawing cells: cells were thawed rapidly at 37°C and transferred in fresh medium containing adequate FBS and antibiotics. The following day, the medium was replaced with fresh culture medium.

Treatment of cells: MG132 was used at a final concentration of 10 µM for the indicated times.

2.2.2.2 Transfection with Calcium Phosphate reagent (Chen and Okayama, 1987)

The day before transfection, $2 \cdot 10^6$ cells were plated onto 10 cm culture dishes. 10 µg of DNA were diluted in sterile water with CaCl₂ (250 mM) and 2X HBS buffer (280 mM NaCl, 1,5 mM Na₂HPO₄, 50 mM HEPES, pH=7.05) which was mixed dropwise to the cells. The calcium phosphate-DNA precipitate was added to cell culture dishes and mixed by gentle swirling. The day after transfection, the culture medium was removed and cells were

incubated 5 min with 15% glycerol in PBS 1X, washed once in PBS 1X and resuspended in fresh culture medium for further 24 h.

2.2.2.3 Transfection using Lipofectamine 2000

Cells were seeded onto 6 cm plates one day before transfection to ensure 90% confluency on the day of transfection. For transfections into U2OS cells, the ratio of Lipofectamine 2000 to DNA was maintained at 1:1 ($\mu\text{l}/\mu\text{g}$). Before applying the DNA/lipofectamine mixture onto the cell, the growth medium was removed and replaced by DMEM medium without serum and antibiotics. Then, 4 to 6 h after transfection, the medium was changed and normal growth medium with serum and antibiotics was added.

2.2.3 Protein methods

2.2.3.1 Preparation of protein lysates from cells

Typically, cells were washed in ice-cold PBS 1X and scraped in 1 ml of PBS 1X for a 10 cm dish. Cells were collected into an eppendorf tube and centrifuged at 4000 rpm 5 min at 4°C (Eppendorf centrifuge 5415R). The supernatant was removed and cells were lysed in 200 μl of NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% NP40, 1 mM PMSF) and incubated 20 min on ice. The protein extract was cleared by centrifugation at 10000 rpm, 15 min at 4°C.

Protein concentration was quantified using a Bradford assay (paragraph 2.2.5.3). Finally, 2X Sample Buffer (80 mM Tris pH 6.8, 2% SDS, 2% β -mercaptoethanol, 10% glycerol, 0.01% bromophenol blue) was added to 40 to 60 μg of soluble proteins and boiled 10 min at 95°C for denaturation of proteins, prior loading onto a SDS-PAGE.

2.2.3.2 Quantification of protein extracts (Bradford Assay)

Proteins concentrations were determined according to Bradford assay, 2 μl of protein extract were diluted in 1 ml of 1X Bradford solution (*Biorad*, Munich). 200 μl of the solution were added into a 96 well plate and the absorbance was measured at λ ⁵⁹⁵ using an ELX 808 UI Ultra Microplate Reader (software KC4 v 3.01). The signal background was determined using

2 μ l of lysis buffer diluted into 1 ml of Bradford solution 1X. The final protein concentration is calculated through a standard curve of defined amount of BSA (0, 2, 4, 6 μ l of 1 mg/ml BSA).

2.2.3.3 Separation of proteins by SDS-PAGE (polyacrylamide gel electrophoresis)

SDS-PAGE analyses were performed according to the protocol of Laemmli (1970). Separating gels of 8 to 15% acrylamide were used to analyze proteins based on their size under denaturing conditions. The Mini-Protean[®]3 system (*Biorad*, München, Germany) was used to cast the polyacrylamide gel. In the most of the cases, 10% polyacrylamide gel was used (for 30 ml of final volume, 11.9 ml of double distilled water, 10 ml of 30% Acrylamide mix, 7.5 ml of Tris 1.5 M pH 8.8, 300 μ l of 10% SDS, 300 μ l of APS, 12 μ l of TEMED). The running gel was poured and overlaid with ethanol 70%. After polymerization, the ethanol was discarded with distilled water and the stacking gel was added (for 10 ml of solution 6.8 ml of double distilled water, 1.7 ml of 30% Acrylamide mix, 1.25 ml of Tris 1 M pH 6.8, 100 μ l of 10% SDS, 100 μ l of APS, 10 μ l of TEMED). A comb was inserted into the stacking gel allowing the formation of wells. Afterwards, the combs were removed, the gel was fixed in an electrophoresis chamber and 1X running buffer was poured over (10X solution: 30.26 g of Tris base, 144.13 g of Glycin, 50 ml of SDS 20% in 1 L of double distilled water). The desired amount of cell lysate diluted in 2X sample buffer was then loaded onto the SDS gel. The gels were electrophoresed at 100 mV during 2 h.

2.2.3.4 Western blotting and immunodetection

Gels were removed from the electrophoresis chambers and incubated in transfer buffer (transfer buffer for 1L: 800 ml of double distilled water, 100 ml methanol and 100 ml of 10X solution, 10X solution: 30.26 g of Tris base, 144.13 g of Glycin in 1 L of double distilled water). Immobilon TM polyvinylidene fluoride (PVDF) membranes were activated with methanol for 1 or 2 min and then incubated in transfer buffer. Western blotting was performed using a liquid transfer apparatus (*bio-rad*) overnight at 30V. After transfer, the membranes were blocked with 5% milk solution in PBST (PBS 1X, 0.2% tween) for 30 min at room temperature. The membranes were then washed, incubated in PBST with primary antibodies

for 2 h, subsequently washed in PBST (3 times, 10 min each) a second time and incubated with secondary antibodies conjugated with horse radish peroxidase during at least 1h30. The membranes were washed once again in PBST (3 times, 10 min each) with a final wash in PBS 1X (10 min) and were developed using ECL (Enhanced Chemiluminescence). Therefore, an equal volume of ECL I (100 mM Tris-HCL, pH=8.5, 2.5 mM Luminol, 400 μ M coumaric acid) and ECL II (100 mM Tris-HCL, pH=8.5, 0.02% H₂O₂) were mixed and applied to the membranes and incubated for 2 min. The signal was developed using ECL Hyperfilm (*GE Healthcare*) and a developer machine from *Fuji*.

Procedure for Stripping an Immunoblot:

The blot was washed to remove chemiluminescent substrate, placed in Western blot stripping buffer (*PIERCE, Thermo scientific*) and incubated for 5-15 minutes at RT. This step was followed by 3 washes in PBS 1X. Then, the membrane was blocked a second time and tested for the complete removal of the immunodetection reagent (HRP label and primary antibody). After determining that the membrane is properly stripped, the second immunoprobng experiment may be performed.

2.2.3.5 Production of the enzyme USP2-core

BL21 bacteria were transformed with the plasmid encoding the GST-USP2-core and pre-cultured overnight in 100 ml LB 1X with ampicillin. The following day, the bacteria culture was expanded to 200 ml of LB 1X with ampicillin and grown until OD₆₀₀=0.5-0.8. The OD was monitored with a spectrophotometer (*Eppendorf, Biophotometer*).

To induce the production of the recombinant protein, isopropyl- β -thiogalactoside (IPTG) was added (final concentration of 1 mM) and incubated for 2 h at 30°C on a shaker. Bacteria were collected by centrifugation at 6000 rpm for 5 min at 4°C using a fixed angle rotor (J2-HS centrifuge) and the pellet was frozen at -80°C. The next day, bacterial pellets were resuspended in a buffer containing: 2 mM EDTA, 2 mM PMSF in PBS 1X, sonicated at Amp 60, 10 pulses, several times. Then, 1% Triton-X-100 was added to have a complete protein extraction. The suspension was incubated at RT for 1 h on a rotating wheel. The solution was centrifuged at 6000 rpm for 15 min at 4°C (*Eppendorf centrifuge 5415R*). Then, 1 ml of glutathione-sepharose 4B slurry (*GE-Healthcare*) was added to the supernatant and incubated at least for 2 h at 4°C on a shaker in order to purify the GST-fusion proteins. Thereafter, beads

coupled to USP2-core were washed three times with PBS 1X to remove non-specific binding proteins and centrifuged at 2000 rpm, 2 min, RT (*Eppendorf* centrifuge 5415R).

USP2-core was eluted two times with factor Xa in PBS to remove the GST moiety. The amount of proteins was determined using a Bradford assay and the efficiency of the purification was checked by SDS-PAGE followed by staining with Coomassie blue. The USP2-core produced migrates at the predicted size of 39.9 kDa. The activity of the enzyme USP2-core deubiquitinase was tested on cell lysates. USP2-core was used at a final concentration of 1 µg/ml in Tris buffer (0.5 M Tris pH 7.4, 2 mM EDTA and 10 mM DTT) for 3 h minimum.

2.2.3.6 Binding assay

The first step of the experiment was carried out followed the same protocol as the production of the enzyme USP2-core but the GST-USP2-core was not eluted from the glutathione-sepharose 4B slurry beads.

To test the interaction between USP2-core and subunits of the proteasome, cells were transfected with plasmids of the 19S proteasome and lysed in NP-40 buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% NP40, 1 mM PMSF).

Then, 500 µg of protein lysate was diluted in 500 µl of lysis buffer and incubated overnight at 4°C, rotating, with 50 µg of GST-USP2-core bound to glutathione-sepharose beads. The sepharose beads were centrifuged at 2000 rpm for 1 min and samples were washed 3 times in PBS 1X. The proteins interacting with USP2-core were eluted with 2X SDS-PAGE sample buffer and analyzed by Western blotting.

2.2.5.7 Production of the MLG affinity matrix and purification of ubiquitinated conjugates

BL21 bacteria were transformed with pET-26b-His₆-MLG. The MLG peptide is a fragment of the S5a protein, composed of the two ubiquitin interacting motifs UIMs. S5a functions as a multiubiquitin receptor by binding to and recognizing polyubiquitinated proteins destined for 26S proteasome degradation via its two UIM motifs.

The expression of His₆-MLG was induced with IPTG and performed as described for USP2-core (*cf.* §2.2.5.6 Production of the enzyme USP2-core). Bacteria were collected and

lysed by sonication at Amp 60, 10 pulses, several times, in a buffer containing: 50 mM NaH₂PO₄ pH: 8.0, 300 mM NaCl, 10 mM Imidazole and 2 mM PMSF. Then, the solution was centrifuged at 6000 rpm for 15 min at 4°C (Eppendorf centrifuge 5415R).

The His₆-MLG was purified using Ni-NTA Agarose beads (*Qiagen*, France) at 4°C overnight. The resin was washed twice with buffer containing 50 mM NaH₂PO₄, 300 mM NaCl, 30 mM Imidazole pH 8.0. The protein His₆-MLG was removed from the beads with 10 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 30 mM Imidazole pH 7.0) and the eluate was dialyzed in PBS 1X. The size of the His₆-MLG (25 kDa) was checked by SDS-PAGE followed by Coomassie staining.

Thereafter, the protein His₆-MLG was covalently bound to NHS-activated Sepharose 4 Fast Flow resin (*Amersham Bioscience*, France) which constitutes the NHS-His₆-MLG affinity matrix used for isolation of ubiquitin conjugates.

To purify the polyubiquitin conjugates with the NHS-MLG affinity matrix, H1299 cells were washed twice with ice-cold PBS and lysed in NP-40 buffer (150 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 1% Nonidet NP-40, 1 mM PMSF, 10 mM NEM). The protein extract was cleared by centrifugation at 10000 rpm at 4°C for 15 min, and the protein concentration was determined by the method of Bradford. Soluble proteins were then incubated with the MLG affinity matrix for 2 h at RT under gentle agitation (1 mg of protein per 1.6 ml of 50% slurry). The beads were washed 3 times with lysis buffer. Elution was performed by 2X SDS-PAGE sample buffer for Western blot analysis.

2.2.3.8 Staining with Coomassie® blue

Gels were washed with distilled water at room temperature to remove salts and then incubated 1 h at RT on a shaker with Coomassie blue reagent (0.15 g Coomassie brilliant blue R250, 40% methanol, 50% H₂O, 10% acetic acid). Finally the gel was washed several times with destaining buffer (40% methanol, 50% H₂O, 10% acetic acid) to remove excess dye.

2.2.3.9 Ubiquitination Assay

H1299 cells at 60% confluency in 10 cm plates were transfected with plasmids encoding different 19S subunits in presence or not of plasmid encoding Mdm2 with a plasmid encoding His-tagged ubiquitin using calcium phosphate reagent (paragraph 2.2.2.2).

After 48 h transfection, H1299 cells were harvested, collected and washed twice in PBS 1X. The cells were then resuspended in 7 ml of PBS 1X and a volume of 1 ml was used for the input control, centrifuged and lysed in 50 μ l of NP-40 lysis buffer. The remaining of cells were centrifuged and lysed in Gd-HCl buffer (6 M guanidinium-HCL, 0.1 M Na₂HPO₄/NaH₂PO₄ pH 8, 0.01 M Tris pH 8, 10 mM β -mercaptoethanol and 5 mM imidazole). Then, 50 μ l of Ni²⁺-NTA agarose beads were added to the lysate. The samples were incubated at RT on a rotating wheel for 2 h and centrifuged at 2000 rpm (*beckman* centrifuge) for 1 min. Thereafter, the beads were successively washed with the following buffers: Gd-HCl buffer, A buffer (8M Urea, 0.1 M Na₂HPO₄/NaH₂PO₄ pH 6.3, 0.01 M Tris pH 6.3, 10 mM β -mercaptoethanol), A buffer with 0.2% Triton and A buffer with 0.01% Triton. Protein elution was carried out with a buffer containing 200mM imidazole in 5% SDS, 0.15 M Tris pH 6.7, 30% glycerol and 0.72 M β -mercaptoethanol for at least 30 min at room temperature on a shaking machine. The eluate was diluted in 2X SDS-PAGE sample buffer and boiled 10 min at 95°C prior Western blot analysis.

2.2.3.10 Sucrose gradient

A total of 6 plates of 10 cm dishes containing H1299 cells at 90% confluency were used per gradient. The cells were transfected with different plasmids (plasmids encoding V5-tagged 19S subunits, Mdm2, Siah-1 or C-cbl) as indicated in the transfection method part (paragraph 2.2.2.2).

Cells were washed, scraped in PBS 1X, collected by centrifugation (Eppendorf centrifuge 5415R) and lysed in 1.5 ml of proteasome lysis buffer (50 mM Tris, pH 7.4, 20 mM NaCl, 10 mM MgCl₂, 0.5% NP40, 5 mM ATP, 10 mM NEM and 1 mM PMSF). To correctly break cells without disturbing cellular complexes (e.g. the 20S or 19S proteasomes), cell lysates were syringed (needle 26G). This step was necessary in order to remove the cell aggregates to obtain a good resolution of the cellular complexes. Protein extracts were cleared by centrifugation at 10000 rpm at 4 °C for 15 min.

Protein concentration was determined by Bradford assay. An equal amount of cell lysate (between 3 to 5 mg of proteins) were loaded on top of a 10–40% sucrose gradient (12 ml, 25 mM Tris, pH 7.4, 50 mM NaCl, 0.05% NP-40, 1mM PMSF) or subjected to prior deubiquitination with the USP2-core enzyme before loading. Then, gradients were centrifuged at 28000 rpm for 18 h at 4 °C using a ultracentrifuge (*Beckman*, rotor: *Kontron TST 41.14* rotor). Fractions of approximately 400 µl were collected from the bottom using a micropipette and a peristaltic pump (*Minipump, Control company, USA*) linked to a fraction collector (*Teledyne ISCO, Foxi®Jr.*).

Equal amounts of every second fraction were loaded onto SDS-PAGE to have the full elution pattern of a protein on the same gel and analyzed by Western blotting. Protein standards (*GE Healthcare*) such as the tyroglobuline (MW: 669 kDa) and the aldolase (MW: 158 kDa) were systematically used to calibrate sucrose gradients.

2.2.3.11 Immunoprecipitation

Concerning the IP against Flag-tagged S6b, 1 µg of an anti-FLAG antibody or IgG control were coupled to protein A sepharose at RT for 1-2 h. IPs were performed with cell lysate fractionated on sucrose gradients. During this incubation, the fractions 4 to 14 from a sucrose gradient (corresponding to the 19S, 20S and 26S) were pooled and diluted to 1 ml in proteasome lysis buffer (50 mM Tris, pH 7.4, 20 mM NaCl, 10 mM MgCl₂, 0.5% NP40, 5 mM ATP, 10 mM NEM and 1mM PMSF). Then, cell pooled fractions from sucrose gradients, treated or not with USP2 (1 µg/ml) or NEM (10 mM), were added to the anti-Flag antibody coupled to protein A sepharose. The mixture was incubated on a rotating wheel ON at 4 °C.

For the IP, the beads were washed three times with NP-40 lysis buffer and the proteins were eluted with 1X SDS-PAGE sample buffer. The samples were heated at 95°C for 10 min before loading onto a 10% SDS-PAGE gel and analyzed by Western blotting.

3. RESULTS

3.1 The E3 ligase Mdm2 ubiquitinates some 19S subunits

3.1.1 Mdm2 co-fractionates with proteasomal subunits

The ubiquitination of p53 by Mdm2 is not the only step for its degradation by the 26S proteasome. A direct interaction between Mdm2 and some 19S subunits is required to deliver p53 efficiently to the proteasome (Kulikov et al., 2010).

Since Mdm2 has been identified as connecting p53 directly with some subunits of the 19S proteasome, the functional relevance of the association between Mdm2 and 19S or 26S proteasome complexes had to be validated in cells. Therefore, an important point was to assess whether Mdm2 and 19S subunits were present in complex including full-assembled 26S (19S + 20S) proteasome, 19S proteasome or single 19S subunits. Sucrose gradient experiments were used to examine the nature of these putative interactions. To facilitate further reading of the result part of my thesis, the principles of the sucrose gradient experiment are described below (*Fig. 9*).

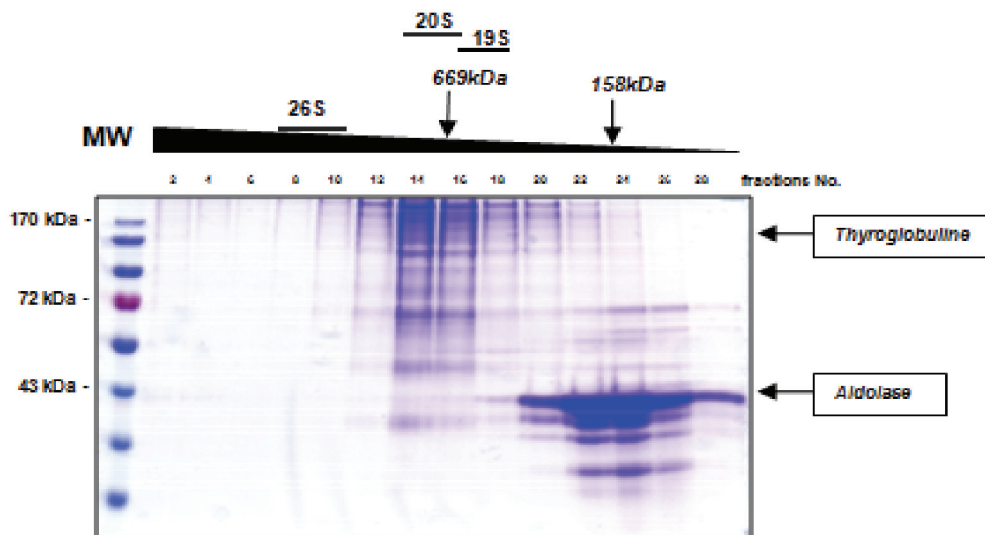


Figure 9: Sizing standards. The molecular weight standards (thyroglobuline 669kDa and aldolase 158 kDa) were used for 10-40% sucrose gradient normalization and ultracentrifuged at 28 000 rpm for 18 h at 4°C. A total of 28 fractions were collected from the bottom of sucrose gradient and 40 µl of every second fractions were resolved by SDS-PAGE. The gel was stained with Coomassie blue. The 20S peaks at 669 kDa corresponding in size to the thyroglobuline marker, the 19S overlaps the 20S and the 26S is located in fractions 8/10. (MW: molecular weight).

Sucrose gradients favours the separation of native complexes that sediment along the gradient and indicate whether a given protein is present alone or in complex with other cellular proteins. Cell lysates (3 to 5 mg of proteins) were loaded on the top of sucrose gradient 10-40% and ultracentrifuged at 28 000 rpm for 18 hat 4°C. The sucrose gradient fractionating discriminates between bigger complexes that sediment in the bottom of the tube and small complexes or single proteins that are located on the top. Fractions of 400 µl were collected from the bottom of gradients. The fraction number two corresponds to the high molecular weight complexes, while the number twenty-eight to the lower complexes. Only 40 µl of every second fraction were loaded onto SDS-PAGE to have the full elution pattern of a protein on the same gel. Molecular weight standards (thyroglobuline 669 kDa and aldolase 158 kDa) were used to calibrate each series of sucrose gradients (*Fig. 9*). Thyroglobuline and Aldolase proteins calibrated both the native step corresponding to the sucrose gradient analysis and the denaturing step related to the Western blot analysis. Furthermore, Ventadour and co-workers demonstrated by silver staining with a mixture of 20S and 19S proteasomes that the 20S was located in higher molecular weight fractions than the 19S proteasome, although the 19S (~ 900 kDa) was bigger than the 20S (~ 700 kDa) (Ventadour et al., 2007). Under these conditions, the 20S proteasome peaked in fractions 14/16 corresponding in size to the thyroglobuline marker, the 19S proteasome in fractions 16/18 and the 26S proteasome in fractions 8/10 (*Fig. 9*). It is important to notice that each of gradient series was calibrated with standards to correct eventual shifts due to variations in the gradient preparation.

To determine the association of Mdm2 with the proteasome, H1299 cells (Human, p53-negative lung carcinoma cell line) were co-transfected with cDNAs encoding Mdm2 or with V5-tagged S8 (19S subunit) and Mdm2 proteins. Cells were lysed in conditions maintaining the integrity of native complexes in buffer containing ATP-Mg²⁺ to favour the formation of native 26S proteasome. Lysates were loaded onto sucrose gradients (10-40%), separated by ultracentrifugation and aliquoted in fractions. Every second fractions were afterwards loaded onto SDS-PAGE. The cell proteins were transferred onto a PVDF-membrane and subsequently, the membrane was incubated with antibodies against endogenous α_7 , Mdm2, V5-tagged proteins (S8), endogenous S8, endogenous S6a, endogenous S2 or endogenous S1 (*Fig. 10*).

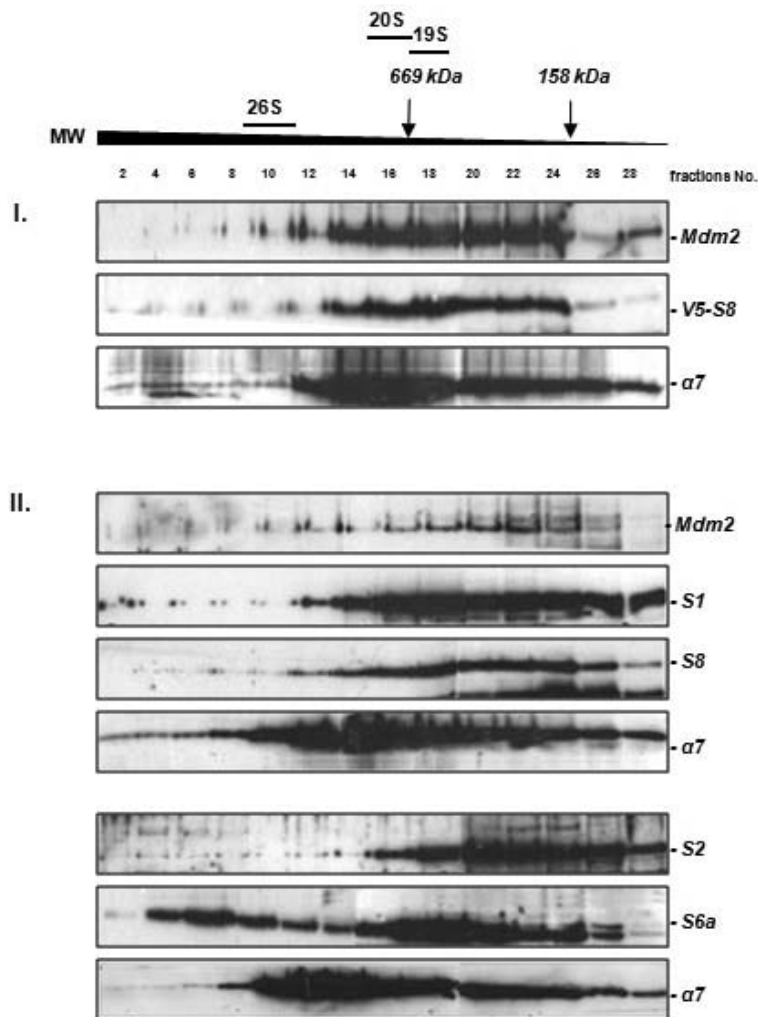


Figure 10: Mdm2 co-elutes with proteasomal proteins. H1299 cells were transfected with 7 μg of a plasmid encoding Mdm2 together with 3 μg of empty vector (panel II) or 7 μg of a plasmid encoding Mdm2 and 3 μg of a plasmid encoding V5-tagged S8 (panel I). 48 h after transfection, cells were lysed in buffer containing ATP-Mg²⁺. Equal amounts of soluble proteins (5 mg) were layered on top of sucrose gradients (10-40%). Gradients were centrifuged at 28 000 rpm for 18 h at 4°C. A total of 28 fractions were collected and 40 μl of every second fractions were resolved by SDS-PAGE. The proteins were transferred onto a PVDF-membrane. The membrane was cutted into 3 pieces. The Western blot membranes of the 1st panel were hybridized with antibodies directed against Mdm2, V5-tagged S8 and $\alpha 7$ (20S) proteins. Concerning the second panel, the upper part of membrane was first incubated with an antibody against S1 or S2 proteins, then the blot was stripped with Western blot stripping buffer (*Thermo scientific Pierce*) and re probed for Mdm2. The middle of membrane was incubated with the anti-S6a or anti-S8 antibodies. The bottom of membrane was incubated with the antibody for $\alpha 7$ (20S) protein. An HRP-linked anti-mouse or anti-rabbit antibody was used. Western blots were developed by ECL. (MW: molecular weight)

As shown in *Figure 10*, the distribution pattern of exogenously expressed V5-S8 was essentially similar to that of endogenous subunit (*panel I and II, compare blots: V5-S8 and S8; Fig. 10*), ranging from fraction 10 to fraction 26. Consequently, the V5 epitope did not disturb the distribution of the 19S subunits and V5-tagged 19S subunits were still incorporated into proteasome complexes. In addition to S8, the elution pattern of other 19S proteins has been

tested. S1, S2 and S6a endogenous proteins shared an analogous distribution from fraction 14 to fraction 24 (*panel II, compare blots: S1, S2 and S6a; Fig. 10*), although these proteins co-immunoprecipitated with Mdm2 (S2, S6a, S8) or not (S1) (Kulikov et al., 2010).

Concerning S6a (*blot S6a; Fig. 10*), another high molecular weight elution peak was observed and might correspond to other functions of S6a, as recent evidences in yeast and mammalian cells supported a novel non-degradative function for components of the 26S proteasome (Truax et al., 2009). As control to validate the sucrose gradient analysis, the elution pattern of 20S proteasomal proteins (α_7) was checked, and α_7 was found in all the fractions with a peak in fractions of the 20S proteasome (*panels I and II, compare α_7 blots; Fig. 10*). The size distribution of Mdm2 was as well characterized and a huge amount of Mdm2 proteins was detected between 158 and 669 kDa in fractions 14-28 (*panels I and II, Mdm2 blots; Fig. 10*), but some Mdm2 proteins eluted with larger complexes in fractions 2-12.

These observations indicated that Mdm2 proteins co-fractionated with endogenous (S8, S2, S6a and S1) or transfected proteasome proteins (V5-S8) which supported their presence in the same complex. In addition, a part of Mdm2 distribution overlapped with the α_7 subunit of the 20S proteasome, suggesting that Mdm2 could be also associated with complexes that contained 20S proteins (*panels I and II, compare blots: Mdm2 and α_7 ; Fig. 10*).

Only few proteasomal proteins eluted in fractions of full-assembled 26S proteasome (fractions 8-12), despite the presence of ATP-Mg²⁺ in the lysis buffer. The majority of proteasomal proteins probably accumulated in subcomplexes in cells and 26S full-proteasome might only assemble when its activity is required. This experiment attests that Mdm2 shares a similar distribution than 26S proteasomal proteins and could be found in fractions corresponding to the full-assembled 19S proteasome complexes.

3.1.2 Characterization of the 19S ubiquitinated subunits

3.1.2.1 Several 19S subunits are ubiquitinated by E3 ligases such as Mdm2, Siah-1 or c-Cbl

Several data obtained in previous study (Kulikov et al., 2010) combined with results from the first paragraph; clearly indicate an interaction between Mdm2 and proteins of the 19S

proteasome. However, it is supposed that these interactions are not only necessary to deliver p53 to the proteasome but also they can have another role in cells such as a direct impact on the 19S proteasome subunits.

A study based on antibody affinity already suggested that some proteasomal subunits (19S and 20S proteins) could be ubiquitinated (Matsumoto et al., 2005). These results were in accordance with that obtained by Ventadour and co-workers that revealed a polyubiquitination of 20S subunits (Ventadour et al., 2006). In the laboratory, some work further indicated that 19S proteins might be ubiquitinated by several E3 ligases such as Mdm2, Siah-1 or c-Cbl, since high molecular weight bands could be detected when 19S subunits are transfected together with these ligases (*Blattner C., unpublished data*).

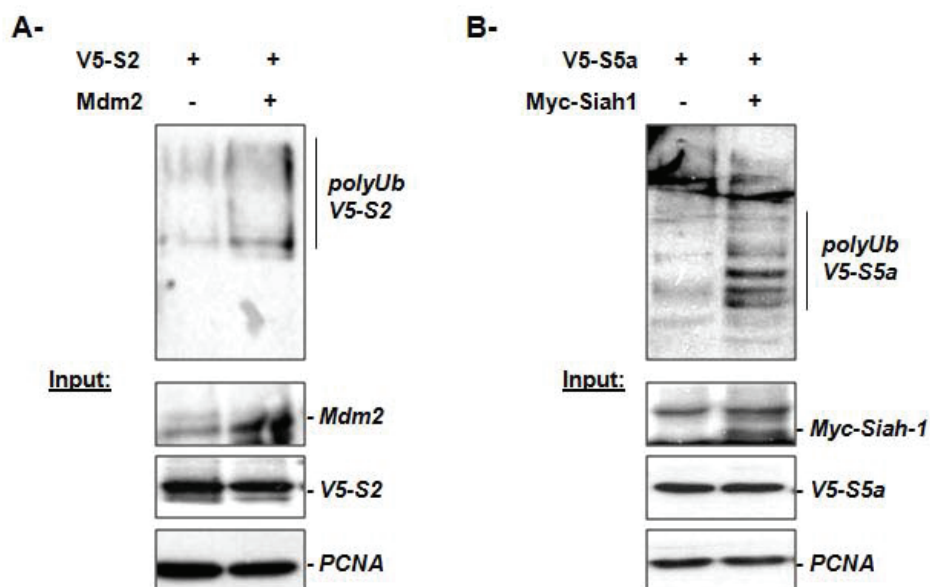


Figure 11: The E3 ligases Mdm2 and Siah-1 ubiquitinate respectively S2 and S5a subunits of the 19S proteasome. H1299 cells were transfected with 3 μg of cDNAs encoding V5-tagged proteins of the 19S proteasome together with 1 μg of cDNA encoding His-tagged ubiquitin protein (His₆-Ub) in the presence or absence of 7 μg of a cDNA encoding Mdm2 (**A**) or Myc-Siah1 proteins (**B**). 48 h after transfection, aliquots of cells (Input) were lysed in NP-40 buffer and analyzed for the presence of Mdm2 (4B2), Myc-Siah-1 (9E10), V5-S5a or V5-S2 by Western blotting. Concerning the input A, the upper part of the membrane was probed with an anti-V5 antibody (V5-S2), the blot was then stripped and reprobed for Mdm2. Equal loading is confirmed by hybridizing the membranes with an antibody directed against proliferating cell nuclear antigen (PCNA). The remaining cells were lysed in guanidinium buffer under denaturing conditions. Ubiquitinated proteins were purified by adsorption to Ni²⁺-NTA-agarose beads, separated by SDS-PAGE and analyzed by Western blotting using an anti-V5 antibody. (Ub: ubiquitin)

Mdm2 as well as Siah-1 and c-Cbl are possibly implicated in the ubiquitination of 19S proteasomal subunit. Then, to validate this hypothesis, an ubiquitination assay was performed. H1299 cells were co-transfected with plasmids encoding the V5-tagged 19S subunits in

presence of Mdm2 or Myc-tagged Siah-1 or with the empty vector as control together with plasmids encoding His-tagged ubiquitin (His₆-Ub). Cells were lysed in 6 M guanidinium buffer which inhibits protein degradation. Ubiquitinated proteins were purified by adsorption on Ni²⁺-NTA-agarose beads via the His₆-tag linked to proteins. Eluted proteins were separated by SDS-PAGE and analyzed by Immunoblotting (*Fig. 11*). For control (Input), aliquots of cellular lysates were loaded on SDS-PAGE and the protein levels of Mdm2, V5-S2, V5-S5a and PCNA was analyzed by Western blotting.

As shown in *Figure 11 A*, Mdm2 induced the ubiquitination the S2 protein of the 19S proteasome with the detection of a pattern of higher-molecular-weight bands in presence of Mdm2, and similar results were found for S5a, S8 and S4. Results obtained in the laboratory (*Blattner C., unpublished data*) demonstrated that S6a and S6b subunits are as well ubiquitinated in presence of Mdm2 and S12 is ubiquitinated in presence or absence of Mdm2, while no ubiquitination of S1, S7, S9, S10b, S11, S15 or p55 has been observed.

Nevertheless, this effect is not specific of Mdm2, as other monomeric RING E3 ligases like Siah-1 are able to ubiquitinate some 19S subunits. Siah-1 plays an important role in regulation of cellular apoptosis (Wen et al., 2010). Similarly to Mdm2, overexpression of Siah-1 resulted in increased polyubiquitination of S5a proteins (*Fig. 12 B*). This effect could be extended to another monomeric RING E3 ligase c-Cbl, described to ubiquitinate the EGF receptor (Wakasabi et al., 2010). Actually, both c-Cbl and Siah-1 induced also the ubiquitination of S6a, S6b and S8 subunits (*Blattner C., unpublished data*).

These results indicate that ubiquitination of 19S subunits might be a common mechanism to several RING E3 ligases, although the 19S subunits involved differ with the E3 transfected. Importantly, the majority of these ubiquitinated proteins are subunits of the 19S base except for S12 which is constitutively ubiquitinated.

3.1.2.2 The ubiquitination of the 19S proteins by Mdm2 is not a recognition signal for their degradation

Polyubiquitination is often a degradation signal for subsequent proteolysis by the proteasome and, considering that Mdm2 ubiquitinated some 19S subunits (S2, S4, S5a, S6a,

S6b and S8); these observations lead to the hypothesis that 19S proteins could be targeted for proteasomal degradation.

To evaluate whether ubiquitinated proteasomal proteins are target for degradation or not, U2OS cells (human, epithelial, osteosarcoma cell line, WT p53) were treated with an inhibitor of the proteasome (MG132) for up to 8 hours and protein levels were analyzed by Western blotting (*Fig. 12*).

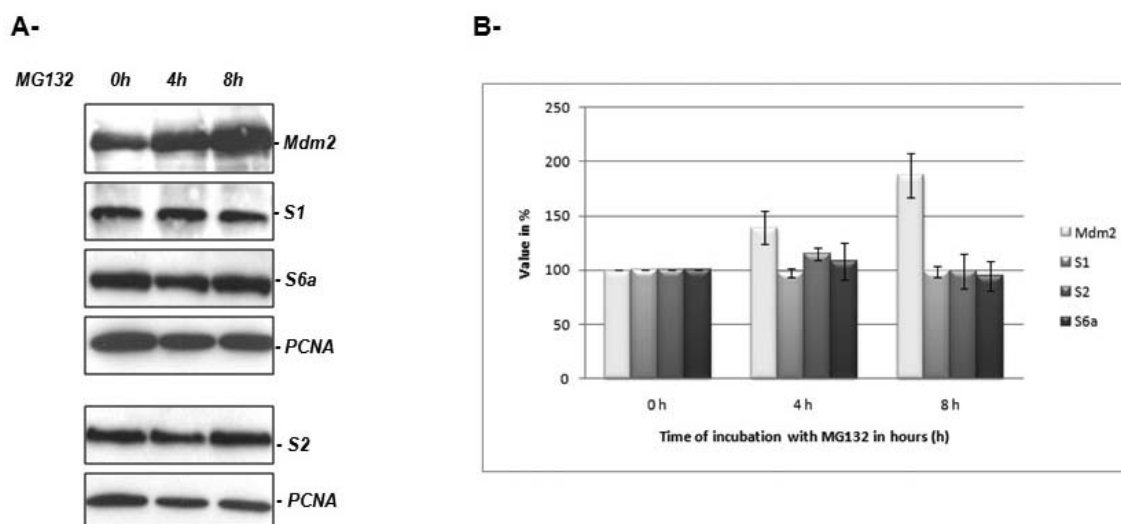


Figure 12: S1, S2 and S6a proteasomal subunits are not substrate of the proteasome. U2OS cells were treated with 10 μ M of the proteasome inhibitor MG132. After the indicated times (0, 4 or 8 h), cells were lysed in NP-40 lysis buffer. **A-** 50 μ g of lysates were separated by SDS-PAGE. After transfer onto a PVDF membrane, the membranes were divided into two or three part. The upper part was incubated with antibodies against Mdm2, endogenous S1 or S2 proteins. For S1 and Mdm2 blots, the membrane was first incubated with an anti-S1 antibody followed by stripping and reprobing with an anti-Mdm2 antibody. The middle part of the membrane was incubated with an antibody directed against endogenous S6a proteins and the lower parts were incubated with an antibody for PCNA and subsequently with HRP-linked anti-mouse or anti-rabbit antibodies. The level of PCNA was used as an indication of equal loading. **B-** Signals for protein expression were quantified by densitometry using the ImageJ software, normalized to an internal control (PCNA) and blotted. The relative value for each protein was expressed in percentage (%).

Upon cell treatment with proteasome inhibitor (*Fig. 12 A*), the abundance of Mdm2, a well characterized substrate of the proteasome (Cho et al., 2001) increased by 46% (quantification by ImageJ software). As expected, the degradation of Mdm2 was triggered through the proteasome pathway. Thus, Mdm2 is used as a control to test the efficiency of the treatment by MG132. In contrast, the level of proteasomal proteins such as S1, S2, S6a remained stable for 0, 4 or 8 h of MG132 treatment. These subunits seemed therefore not to be degraded by the proteasome system.

To have confidence that this experiment was reproducible and that the ubiquitination of the 19S subunits was not a signal for degradation, three independent experiments for each assay have been evaluated by densitometry (ImageJ software, *Fig. 12 B*). The level of proteasomal proteins (S1, S2 and S6a) was constant whereas a clear increase of the amount of Mdm2 could be observed which validated previous observations.

A surprising data contributed to confirm that the ubiquitinated 19S subunits were not targeted for proteasomal degradation. The MLG affinity matrix was tested to isolate the ubiquitinated 19S proteins in order to validate the ubiquitination assay experiment, but this matrix seemed not to recognize them.

The MLG affinity matrix exploits the properties of the S5a subunit of the 19S proteasome, an ubiquitin receptor that allows the purification of ubiquitin conjugates. The MLG peptide is a fragment of the S5a protein, restricted to the two UIM motifs, and that retained the whole binding affinity for polyubiquitin chains. This approach was developed by Dr. Daniel Taillandier (Ventadour et al., 2007).

The His-MLG protein was produced in bacteria, purified and linked to sepharose beads (*upper panel, Coomassie blue staining; Fig. 13 A*). The efficiency of the MLG affinity matrix to purify ubiquitinated proteins was validated for the whole pool of polyubiquitin-conjugates in C2C12 cells (mouse myoblast cell line used as control) with the anti-polyubiquitin antibody (*lower panel; Fig. 13 A*).

To test the ubiquitination of the 19S subunits by Mdm2 with the MLG affinity matrix, U2OS cells were transfected with control siRNA or siRNA targeting Mdm2 or S8. The matrix efficiency was controlled by Western blotting with an antibody against polyubiquitin conjugates (Fk1) to validate the experiment in U2OS cells (*panel: total polyUb conjugates; Fig. 13 B*). Mdm2 and S8 protein levels were significantly reduced when cells were co-transfected with Mdm2 and S8 siRNAs (*Input; Fig. 13 B*). A decrease of 83% and 57% of the expression of these two proteins was respectively detected (quantification by ImageJ software). The depletion of Mdm2 by siRNA, the main E3 ligase of p53, induced a strong decrease in p53 ubiquitination (*eluates from MLG matrix, central panel; Fig. 13 B*). Surprisingly, no ubiquitinated S8 proteins have been detected, in contrary to p53 which was used as control to validate that the experiment was working (*eluates from MLG matrix, upper panel; Fig. 13 B*).

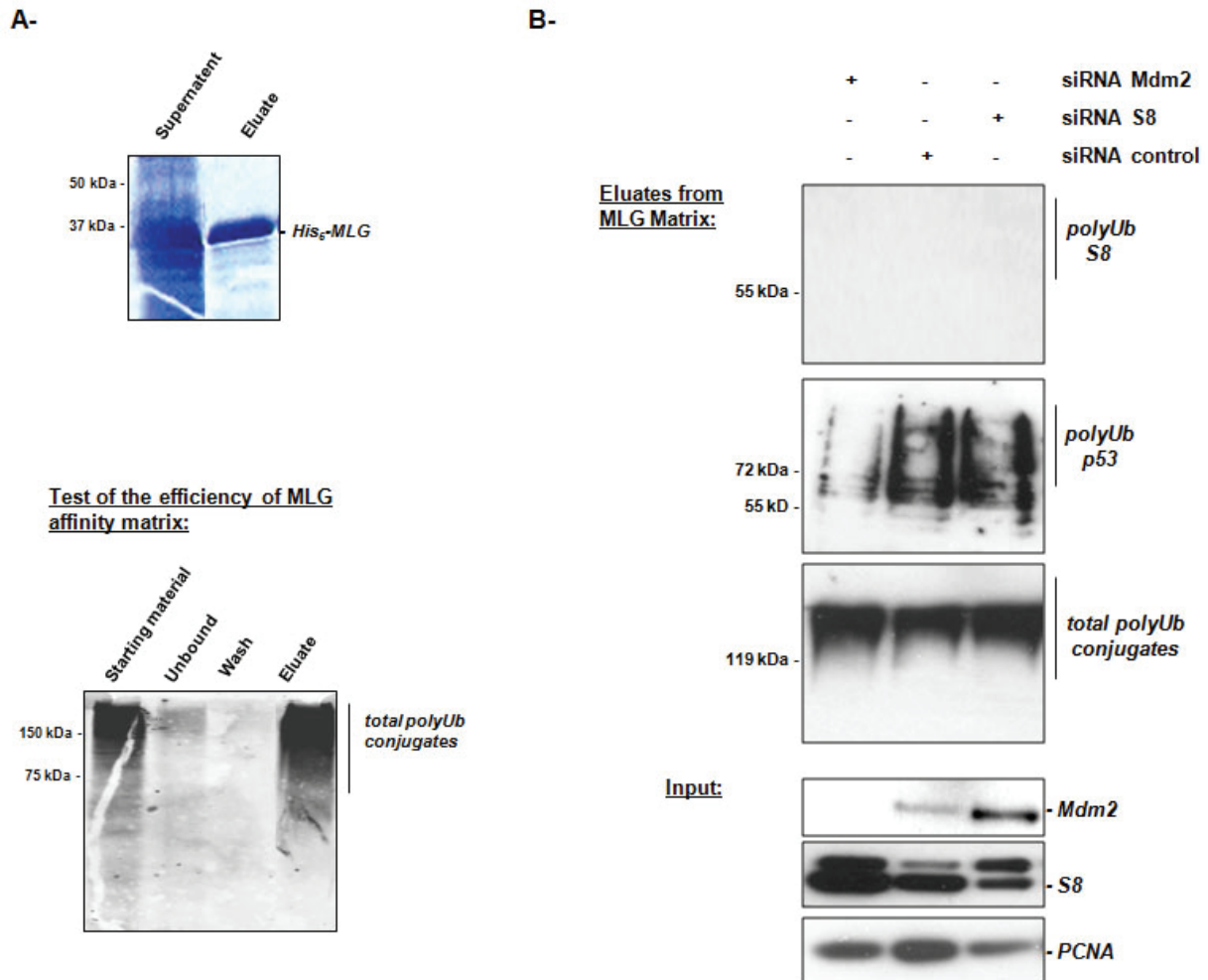


Figure 13: The MLG affinity matrix did not recognize the ubiquitinated S8 subunit. **A- Purification of the MLG affinity matrix.** BL21 bacteria were transformed with pET-26b-His6-MLG. The expression of His6-MLG was induced with IPTG. Bacteria were collected and lysed by sonication. The His6-MLG was purified using Ni-NTA agarose at 4°C overnight. Supernatant and eluate were separated by SDS-PAGE and stained with Coomassie blue. For the MLG-affinity matrix, the protein His₆-MLG was covalently bound to NHS-activated Sepharose 4 Fast Flow. This matrix can be used for the isolation of ubiquitin conjugates. To test the efficiency of the MLG affinity matrix, C2C12 cell lysate (50 µg) was incubated with the MLG affinity matrix (50 µl of 50% slurry) for 2 h at RT. Samples were washed, eluted by adding SDS-PAGE sample buffer, loaded onto a SDS-PAGE gel and, analyzed by Western blotting (Ub: ubiquitin). **B- The ubiquitin chain linked to S8 is not retained by the MLG affinity matrix.** U2OS cells were transfected with siRNA directed against Mdm2 or S8 or with a non-specific control siRNA. Cells were lysed in NP-40 buffer and 50 µg of soluble proteins were incubated with the MLG matrix for 3 h at RT. Proteins were eluted by adding SDS-PAGE sample buffer and analysed by Western blotting using antibodies targeted against S8, p53 (DO-1 antibody) and against ubiquitin conjugates (Fk1 antibody). An aliquot of the cell lysate was probed for the presence of Mdm2 and S8. PCNA was used for loading control (Input).

Unexpectedly, ubiquitinated S8 proteins were not retained by the MLG affinity matrix. In this way, one hypothesis could probably be that S5a not recognizes or captures the kind of ubiquitin chain linked to the S8 subunit. These ubiquitin chains bound to the 19S subunits seemed not constitute a target signal for proteasomal degradation.

3.1.2.3 A “mixed” chain of ubiquitin is linked to S2 and S5a subunits of the 19S proteasome

Previous work reported that Mdm2 induces polyubiquitination of proteins via the lysine K48 to target protein for proteasomal degradation (Ikeda et al., 2008), but no data were available about the ubiquitination of the 19S subunits by Mdm2. Furthermore, my results suggest that proteasomal subunits are not degraded via the proteasome pathway and similarly, that “non-conventional” ubiquitin chain linked to the 19S subunits could explain the lack of binding of polyubiquitinated-S8 to the MLG matrix (previous paragraph).

To elucidate the type of ubiquitin chains linked to the proteasome subunits, an ubiquitination assay was carried out with different mutants of ubiquitin lacking alternatively the different lysine. The subunits S2 and S5a which interact with Mdm2 and are ubiquitinated, were selected for this experiment.

H1299 cells were co-transfected with plasmids encoding for V5-tagged S2 or S5a, with or without Mdm2 and with either His-tagged wild type ubiquitin (His₆-Ub) or ubiquitin mutants. The polyubiquitinated proteins were purified by adsorption to Ni²⁺-NTA-agarose beads and the amount of ubiquitinated S2 and S5a proteins was determined by Western blotting.

Concerning the mutants, the seven lysine were separately replaced with arginine (K6R, K11R, K27R, K29R, K33R, K48R, K63R) or for the K0 mutant all the lysines were exchanged with alanine. The lysine to arginine mutants render ubiquitin unable to form multiubiquitin chains via that specific lysine with other ubiquitin molecules. These proteins can still be linked to the lysine residues on target proteins formed through the remaining ubiquitin lysine residues that have not been mutated. In contrast, the K0 mutant contains no lysine residues and is unable to form ubiquitin chains.

An efficient polyubiquitination of S2 was detectable in the presence of wild type ubiquitin (*Fig.14 A*). In contrary, the K0 mutant was incompetent for S2 ubiquitination; no ubiquitinated forms of S2 were noticed in the higher molecular weight part of the gel demonstrating that S2 is indeed polyubiquitinated and not eventually multimonoubiquitinated (monoubiquitinated at several individual lysine residues).

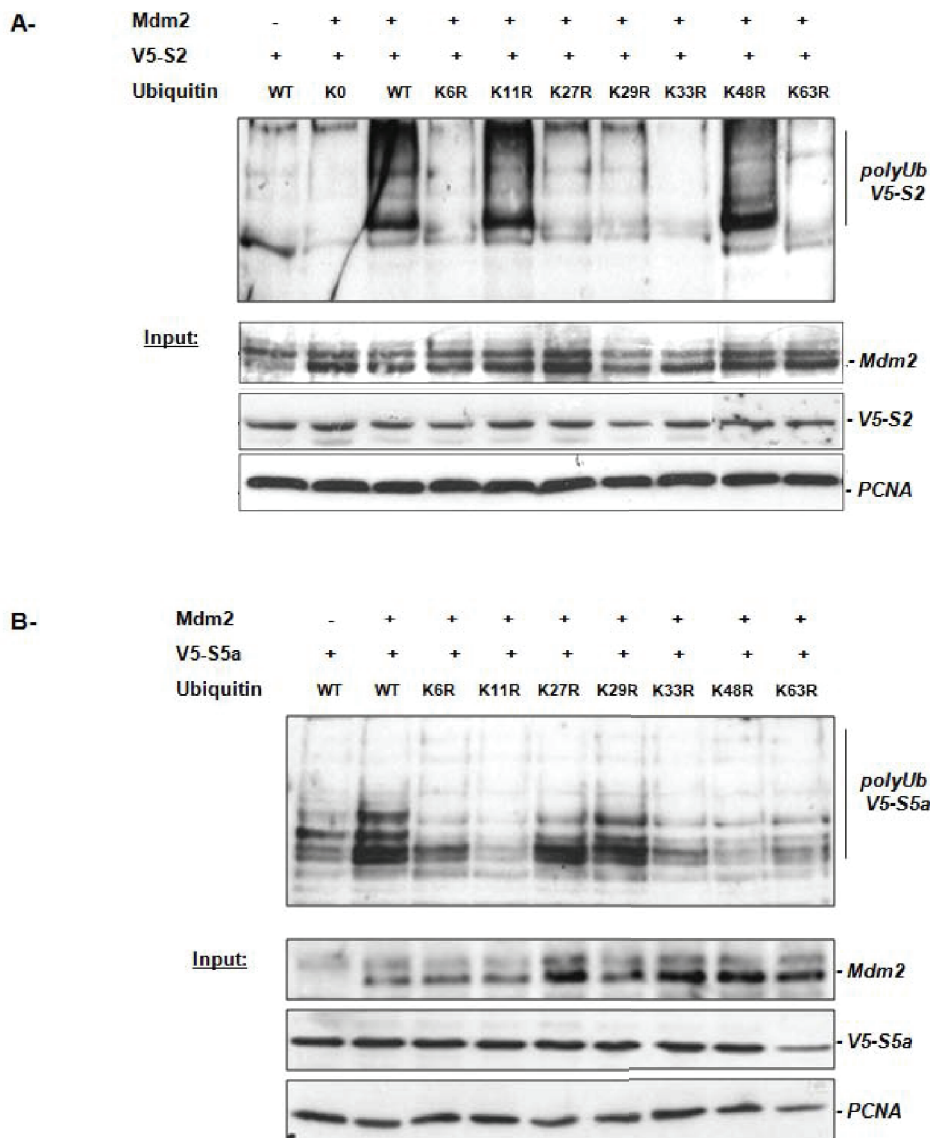


Figure 14: Ubiquitination pattern of S2 and S5a subunits. H1299 cells were transfected with 3 μg of plasmids encoding V5-tagged S2 (**A**) or S5a (**B**), with or without 7 μg of a plasmid encoding Mdm2 and with 1 μg of a plasmid encoding WT or mutant His-tagged Ub. Mutants harboured the indicated mutations (K6R, K11R, K27R, K29R, K33R, K48R and K63R). In the K0 mutant, all lysines have been replaced with an alanine. At 48 h after transfection, cells were harvested and proceeded as described in the legend to *Figure 11*. Inputs (lower panel) were separated by SDS-PAGE and analyzed for the presence of Mdm2 (4B2), V5-S8 or V5-S2 by Western blotting. For the input A, the upper part of the membrane was first hybridised with an antibody directed against V5, stripped and reprobed with an antibody targeted against Mdm2 (4B2). PCNA was used as loading control. Ubiquitinated proteins were also separated by SDS-PAGE and analyzed by Immunoblotting with an anti-V5 antibody (upper panels). (WT: wild type, Ub: ubiquitin)

For the others mutants, clearly the lysine 11 and 48 did not affect the ubiquitination of S2, whereas the lysine mutants 6, 27, 29, 33 or 63 attenuated significantly the rate of ubiquitination of S2. These observations suggest that all these lysines of ubiquitin may contribute to polyubiquitination. In addition the lysine 48 which is the main important linkage for proteasomal degradation is not required for Mdm2-mediated S2 polyubiquitination. This

argument supports results obtained in the previous paragraph, ubiquitinated S2 subunit is not a substrate of the 26S proteasome. Similarly to S2 subunit, the ubiquitination pattern of S5a involved a chain of several lysines of the ubiquitin molecule, but the lysine residues implied are quite different (*Fig. 14 B*). The chain of ubiquitin linked to the S5a subunit is composed of the lysine 6, 11, 33, 48 and 63 without the lysine 27 and 29.

A residual signal of polyubiquitination could be indeed observed for some of the mutants which might be due to the presence of several lysine involved in the formation of the ubiquitin chain. Both subunits S2 and S5a require several lysine residues for polyubiquitination and thus, are linked to an unconventional ubiquitin chain (“mixed” chain).

3.2 Impact of E3 ligases or E3 ligase/substrate on the assembly of the proteasome

3.2.1 Role of Mdm2 and p53 on the formation of the proteasome

3.2.1.1 Mdm2 or Mdm2-p53 complexes increase 19S subunits in higher order complexes

In parallel to work on the ubiquitination of the 19S subunits, the role of Mdm2 and Mdm2-p53 complex on the proteasome was investigated. It was supposed that these proteins could have a direct impact on the distribution of proteasomal subunits and possibly, on the formation of the proteasome. This part of my thesis is focused on one component of the 19S proteasome base, the ubiquitinated S2 subunit, which interacts with Mdm2. The purpose was to compare the elution pattern of S2 alone and S2 co-transfected with Mdm2 and/or p53, using sucrose gradient experiments; in order to demonstrate that E3 ligase and/or substrate could have a role on the assembly of the 26S proteasome. Hence, the requirement of the H1299 cell line that is deficient in p53 to fully monitor the impact of the substrate.

H1299 cells were transiently transfected with V5-tagged S2 or with Mdm2 and/or p53 coding plasmids. As already detailed in the first paragraph, the series of gradients were

normalized using molecular weight standards and the same quantity of proteins was loaded. The protein expression of Mdm2, S2, and p53 was monitored by Western blotting (*input, Fig. 15 A*). Short time exposures of films have been selected to visualize the shift of 19S subunits in the presence of Mdm2 and Mdm2-p53 complexes. To compare each V5-S2 blots to one another, an input control has been used to obtain the same intensity signal. The analysis and time exposure of the films were the same for all the gradients.

An important detail to notice is that NEM (N-Ethylmaleimide, *Sigma*), an inhibitor of deubiquitinase, was used in the lysis buffer to detect the ubiquitin-conjugates. However, no polyubiquitin-conjugates of the 19S subunits have been distinguished using NEM, in presence of Mdm2 and/or p53, even with acrylamide gradient gels 6-15% (*data not shown*). This is probably due to the low abundance of polyubiquitin-conjugates of the 19S subunits and their distributions over high molecular weight (*panels I, II and III, compare blots: V5-S2; Fig. 15 B*).

The elution pattern of Mdm2 remained sensibly identical in presence or not of p53 with a concentration of proteins in fractions 12-22 (*panels II and III, compare blots: Mdm2; Fig. 15 B*). P53 was rather located from fraction 12 to 22 (*panel III; Fig. 15 B*). A huge amount of S2 proteins was found from fractions 14 to 24, but not in higher fractions (*panel I; Fig. 15 B*) suggesting that the majority of S2 proteins eluted with 26S and 19S proteasome complexes. In fact the incorporation of S2 is clearly increased into larger complexes when Mdm2 is overexpressed (fractions 8 to 14) and this process is even more enhanced in presence of the substrate p53 (fractions 2 to 14) (*panels I, II and III, compare blots: V5-S2; Fig. 15 B*). These S2 fractions (12 to 14) matched with the localization of the full-assembled 26S proteasome, implying probably a role of Mdm2 and Mdm2-p53 in its formation. The presence of S2 proteins in fractions 2 to 10, when Mdm2 and p53 are co-transfected, could be explained by the binding of interacting partners of these two proteins with the 26S proteasome (*panel III, blot: V5-S2; Fig. 15 B*). Thus, Mdm2 and Mdm2-p53 complexes shifted the S2 subunit toward higher molecular weight complexes. As control for this experiment, the distribution of endogenous α_7 , subunit of the 20S proteasome was also tested. For the three conditions (I, II and III), the main concentration of α_7 proteins was located from fraction 12 to 20 which corresponds to the 26S and the 20S proteasome (*panels I, II and III, compare blots: α_7 ; Fig. 15 B*). The increase of the S2 subunit in higher order complexes of the sucrose gradient was quantified with the ImageJ software (*Fig. 15 C*) and, therefore attests an improvement of the proteasome assembly by Mdm2 and Mdm2-p53 complex.

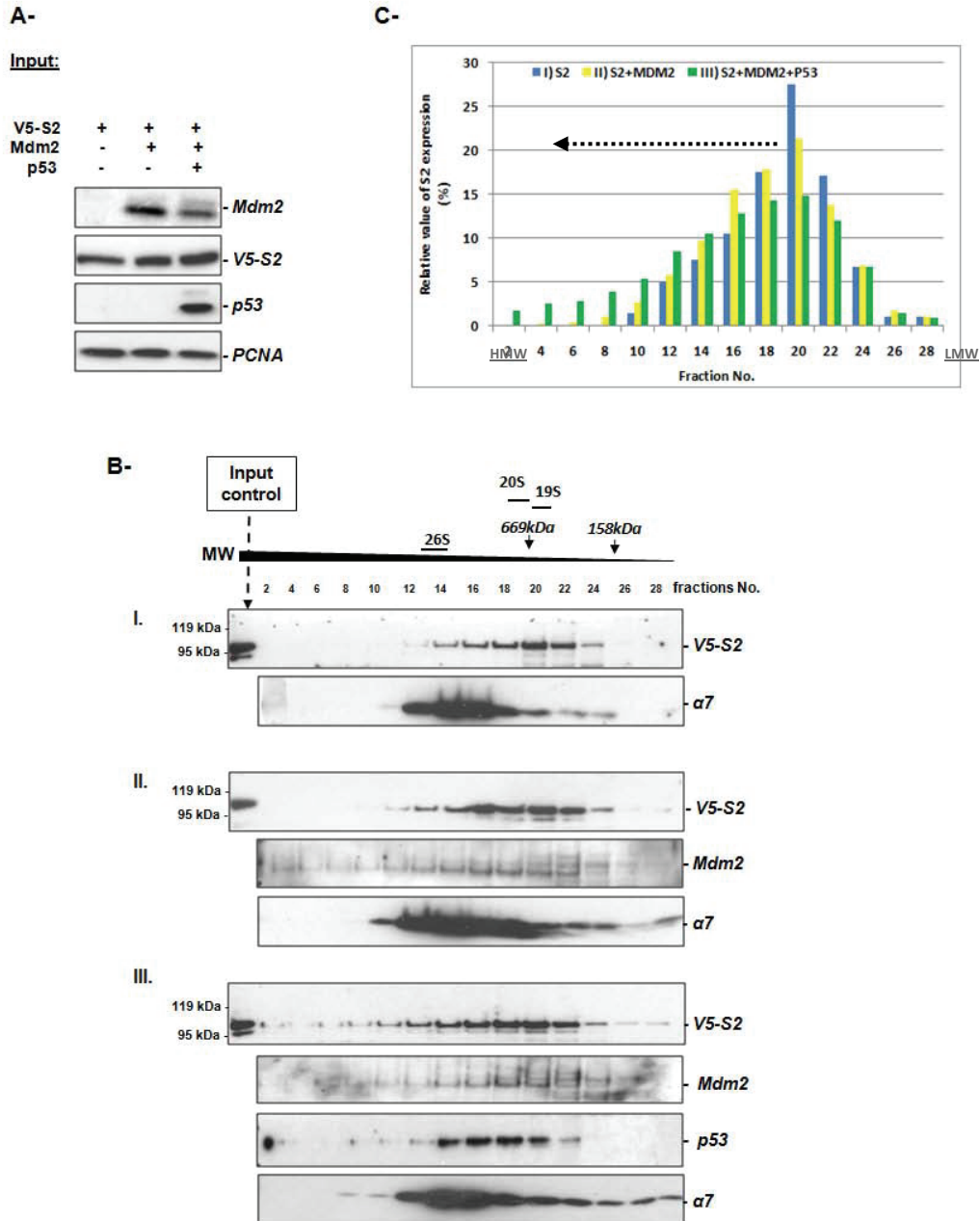


Figure 15: The presence of Mdm2 or Mdm2-p53 shift the distribution of S2 proteins toward higher order complexes. H1299 cells were transfected with 3 μg of a plasmid encoding V5-tagged S2 (panel I) or 3 μg of a plasmid encoding V5-S2 and 7 μg of a plasmid encoding Mdm2 (panel II) or 3 μg of a plasmid encoding V5-tagged S2, 7 μg of a plasmid encoding Mdm2 and 2 μg of a plasmid encoding p53 (panel III). **A-** Cells were lysed in NP-40 buffer containing ATP-Mg²⁺ and 40 μg of soluble proteins were tested for expression of V5-tagged S2, Mdm2, p53 and PCNA which was used as loading control (Input). **B-** Cell lysates (4 mg of proteins) were loaded onto 10-40% sucrose gradient and proceeded as described in the legend to *Figure 10*. Membranes were hybridized with 4B2 (Mdm2), V5 (S2), endogenous $\alpha 7$ and DO-1 (p53) antibodies. The upper part of membranes (blots II and III) were first tested for S2 expression, then the blots were stripped and reprobed for Mdm2. Western blots were developed by ECL. The input control is used to normalize the signal of proteins between all the gradients and is composed of 40 μg of cell lysate corresponding to each gradient. **C-** Signals for S2 protein expression of sucrose gradients were quantified by the ImageJ software, normalized according to the input control and blotted. The relative value for each protein is expressed in percentage (%). The black arrow underlines the shift of S2 toward higher order fractions in presence of Mdm2 and/or p53. (MW: molecular weight, LMW: low molecular weight complexes, HMW: high molecular weight complexes)

Interestingly, equivalent results were found for S8 and S4 subunits of the 19S proteasome, associated with Mdm2 and ubiquitinated, but also for the S9 subunit which did not interact with Mdm2 (*data not shown*). As note, the shift for the subunits S4 and S9 was observed with the Myc-Mdm2 and the experiment was carried out without ATP-Mg²⁺ in the lysis buffer. Thus, the increased amounts of the S4 and S9 into larger complexes corresponded to the 19S proteasome and not to the 26S proteasome. These data underline an impact of Mdm2 and p53 in the recruitment of 19S proteasomal proteins.

All these observations further validate that 19S subunits (S2, S4, S8 and S9) are more incorporated into larger complexes when Mdm2 is overexpressed and this phenomenon is enhanced in presence of the substrate p53. These high molecular complexes corresponded in size to the native 19S and 26S proteasomes.

Obviously, this experiment has been reproduced several times (at least 10 times), each time the shift was detected in the larger complexes of the sucrose gradient and never in the other way. These reproducible data demonstrate the relative importance of the role of E3 ligase and its substrate (Mdm2/Mdm2-p53) on the formation of the proteasome.

3.2.1.2 Mdm2 or p53 do not alter the steady-state levels of 19S proteasomal subunits

One hypothesis has to be rule out, the fact that p53 as well as Mdm2 could act on the protein level of the 19S subunits which might explain the increase of 19S subunits toward higher molecular weight fractions. Furthermore, p53 is a well-known transcription factor which under stress stimuli can activate a plethora of genes (Brooks and Gu; 2003).

To examine whether Mdm2 or p53 affects the steady-state protein levels of the 19S proteasomal subunits, H1299 cells were transiently transfected with V5-tagged S2, S4, S6a or S8 and with increasing amount of Mdm2 (*Fig. 16 A*) or with Mdm2 in presence or not of p53 (*Fig. 16 B*). The protein levels of S4, S6a and S8 remained sensibly identical in presence of increasing amount of Mdm2 (*Fig. 16 A*). Similarly, results obtained with p53 co-transfected in presence of Mdm2 demonstrated that huge amount of p53 did not affect the level of proteasomal proteins such as S2, S8 and S4 (*Fig. 16 B*).

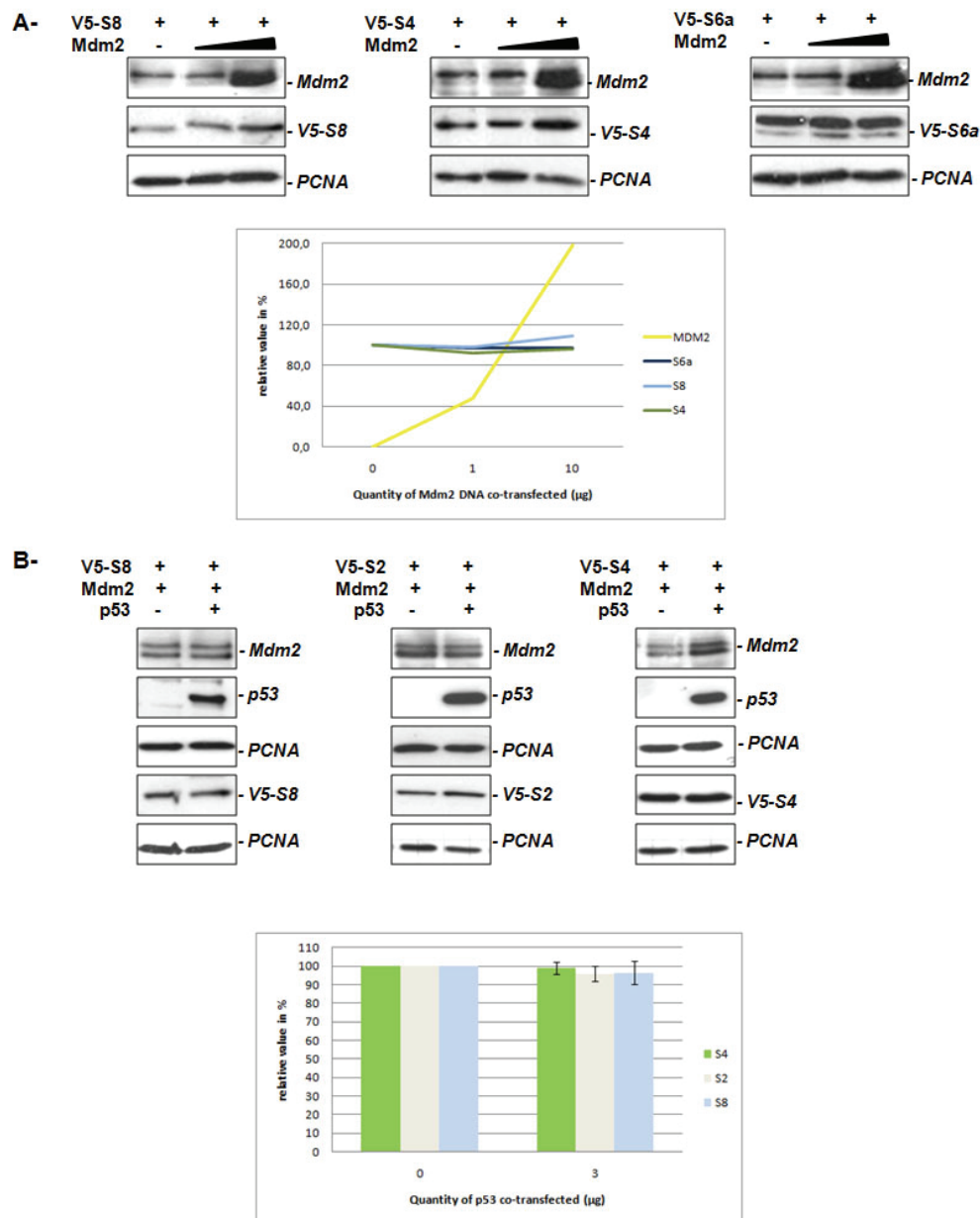


Figure 16: The presence of p53 or Mdm2 does not affect the level of proteasomal proteins. H1299 cells were transfected with cDNAs expressing V5-tagged S2, S4, S6a or S8 (5 µg) in the absence or presence of increasing amounts of Mdm2 (0, 1 or 10 µg) (A) or with Mdm2 (7 µg) in the presence or not of p53 (0 or 3 µg) (B). Cells were harvested 48 h after transfection, and analyzed for the expression of proteasomal proteins, Mdm2 and p53. Concerning the part B for the membrane V5-S2, the upper part was first incubated with an anti-V5 antibody, then the blots were stripped and reprobated for Mdm2. The hybridization with an antibody targeted against PCNA was used for loading control. Western blots were developed by ECL. Signals for protein expression were quantified by densitometry using the ImageJ software, normalized to an internal control (PCNA) and blotted. The relative value for each protein level was expressed in percentage (%).

The quantification experiments by the ImageJ software further validated that Mdm2 and p53 did not modify the protein levels of the 19S subunits S2 and S8 (Graphs, Fig. 16 A and B). These observations lead to the conclusion that Mdm2 and p53 do not enhance proteasomal assembly by increasing steady-state levels of the 19S proteasomal subunits (S2 and S8).

3.2.2 Inhibition of the proteasome does not disturb the assembly of the proteasome

A question remained therefore the role of the substrate degradation on the assembly of the proteasome. An interesting way to test this point was to add onto cells an inhibitor of the proteasome (MG132) which inhibits the degradation of substrates and to monitor the impact of this drug on proteasomal distribution using sucrose gradient analysis.

H1299 cells transfected with plasmids encoding V5-tagged S2, Mdm2 and p53 were treated with MG132 or DMSO as vehicle during six hours prior analysis. The protein expression of Mdm2, S2, and p53 was measured by Western blotting (*Fig. 17 A*). Conditions for sedimentation and analysis of the gradients were the same as previously described in *Figure 10*.

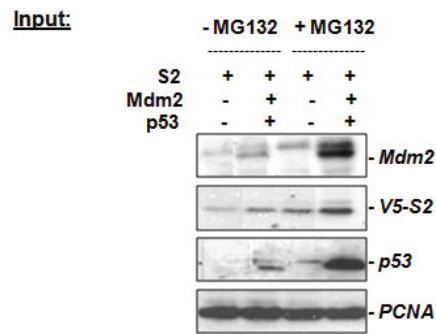
The major amount of p53 and Mdm2 proteins was concentrated from fraction 16 to 28 in presence or not of MG132 (*compare blots: 4, 5, 10 and 11; Fig. 17 B*). These two proteins are well-known substrates of the proteasome (Fang et al., 2000); the MG132 induced therefore an accumulation of p53 and Mdm2 proteins (*compare blots: 5 and 11 for p53, 4 and 10 for Mdm2; Fig. 17 B*). This increase could be observed as well with the protein expression by Western blotting (*Fig. 17 A*).

In contrast, the S2 protein level remained sensibly the same upon MG132 treatment (*Fig. 17 A and B*) and supported the result of the first part indicating that the ubiquitinated 19S proteins are not proteasomal substrates. A shift of S2 proteins was still detected in higher weight complexes from fraction 2 to 14 when Mdm2 and p53 were co-transfected with or without MG132 (*compare blots: 1 and 3, 7 and 9; Fig. 17 B*), which confirmed the role already observed of Mdm2 and p53 on the assembly of the proteasome.

As control for this experiment, the distribution of endogenous α_7 , subunit of the 20S proteasome was also tested for the fourth conditions. After treatment with MG132, the main concentration of α_7 proteins remained still located from fraction 12 to 20 which corresponds to the 26S and the 20S proteasome (*compare blots: 2, 6, 8 and 12; Fig. 17 B*).

These results lead to the supposition that blocking proteasome functions did not affect the recruitment of proteasomal subunits by Mdm2.

A-



B-

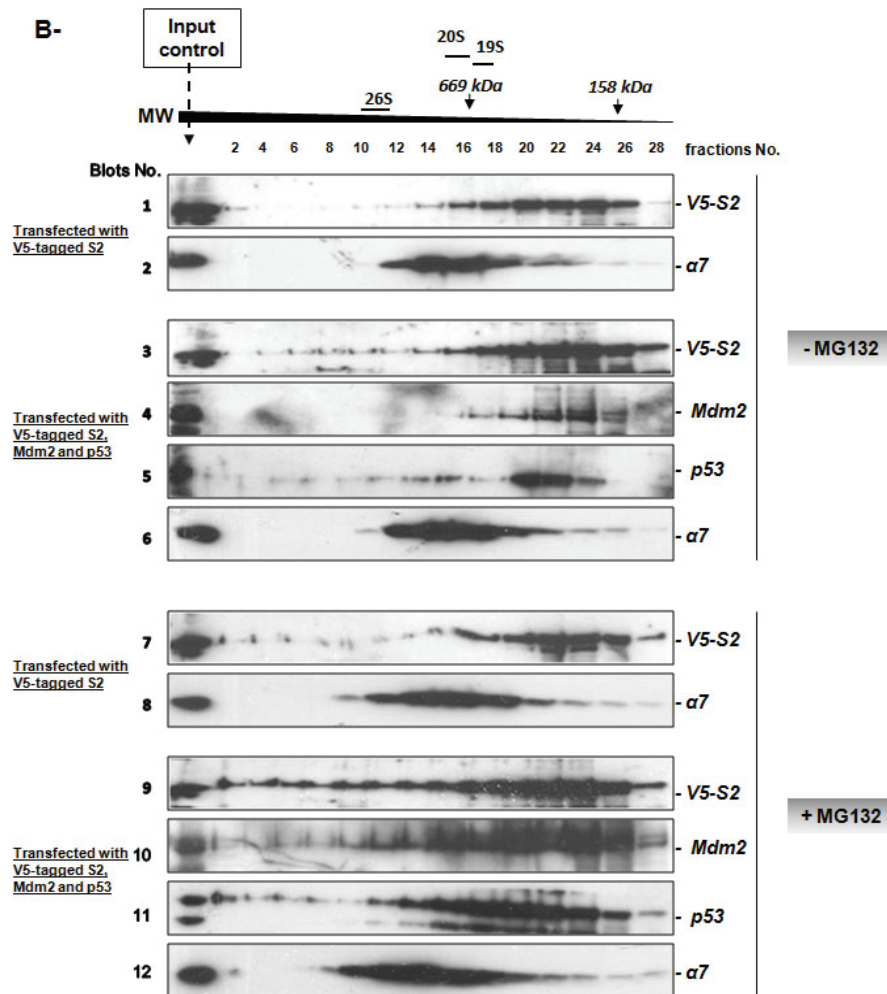


Figure 17: The MG132 inhibitor of the proteasome has no effect on S2 protein distribution. H1299 cells were transfected with 3 μg of a plasmid encoding V5-tagged S2 alone or together with 7 μg of a plasmid encoding Mdm2 and 2 μg of a plasmid encoding p53 for 48 h. 6 hours prior to harvested cells, the proteasome inhibitor MG132 was added to a final concentration of 10 μM (blots 7 to 12) or cells were treated with the same amount of the vehicle DMSO (blots 1 to 6) for control. **A-** Cells were lysed in NP-40 buffer containing ATP-Mg²⁺. For the input, 40 μg of lysates were tested for the expression of V5-tagged S2, Mdm2, p53. PCNA was used for loading control. **B-** The remaining cell lysate (4 mg of proteins) was loaded onto sucrose gradients and analysed as described in the legend to *Figure 10*. The upper part of the membranes (blots 3, 4, 9 and 10) were first tested for S2 expression, then the blots were stripped and reprobred for Mdm2. An HRP-linked anti-mouse or anti-rabbit antibody was used for secondary antibody. Western blots were developed by ECL. (MW: Molecular Weight)

3.2.3 The impact of Mdm2 on the assembly of the 26S proteasome can be extended to other E3s such as c-Cbl or Siah1

To further proof that the influence of Mdm2 on the proteasome formation to target substrates for degradation might be a common mechanism for several E3 ligases, two other monomeric RING E3 ligases Siah-1 and c-Cbl were tested. For this purpose, the S6b subunit of the base of the 19S proteasome has been selected because this subunit is ubiquitinated by the two E3 ligases. The effect of E3 ligases on the assembly of the 26S proteasome was studied by the comparison of the elution pattern of S6b, in presence of Siah-1 or c-Cbl.

The sucrose gradient experiment was performed as previously described in *Figure 10*. H1299 cells were co-transfected with plasmids encoding S6b or S6b and Myc-Siah-1 or S6b and Myc-c-Cbl. The input monitored the level of transfected-proteins (*Fig. 18 A*). The same amount of cell lysates (5.5 mg of proteins) was loaded onto sucrose gradients. Blots were compared via the loading of an input control in order to obtain the same intensity signal (*Fig. 18 B*). The majority of transfected S6b proteins were concentrated from fraction 20 to 26; with few S6b subunits in fractions 16-18 of the 19S proteasome (*blot 1; Fig. 18 B*). The incorporation of the S6b subunit was sensibly increased into larger complexes, for cells overexpressing Siah-1 from fraction 8 to 20 (*compare blots: 1 and 3; Fig. 18 B*) and c-Cbl from fraction 12 to 20 (*compare blots: 1 and 6; Fig. 18 B*). In presence of Siah-1 and c-Cbl, the S6b distribution shifted in higher molecular weight complexes, implying an impact of these two E3 ligases on the proteasome formation. Additionally to S6b, the elution pattern of endogenous α_7 , subunit of the 20S proteasome was checked as control for this assay. The major amount of α_7 proteins was found fractions 8 to 18 (*blots: 2, 5 and 8; Fig. 18 B*) which corresponded to 26S and 20S proteasome fractions. Although a slight shift of two fractions for α_7 proteins was detected in higher molecular weight fractions (fractions 6 and 8, *Fig. 18 B*), the shift of S6b in presence of c-Cbl or Siah-1 remains therefore significant because it consists of an increase of 4 to 6 fractions respectively (*Fig. 18 B*). In contrast to Mdm2 which was encountered in 26S, 20S and 19S fractions (*Fig. 10 and Fig. 15*), the elution pattern of these two E3 ligases (Siah-1 and c-Cbl) was quite different. Despite long time exposure films (*blots: 4 and 7; Fig. 18 B*), the major amount of these proteins was detected in smaller weight fractions (fractions 18 to 28) and only a slight amount co-eluted with 19S proteasome fractions (around fraction 18).

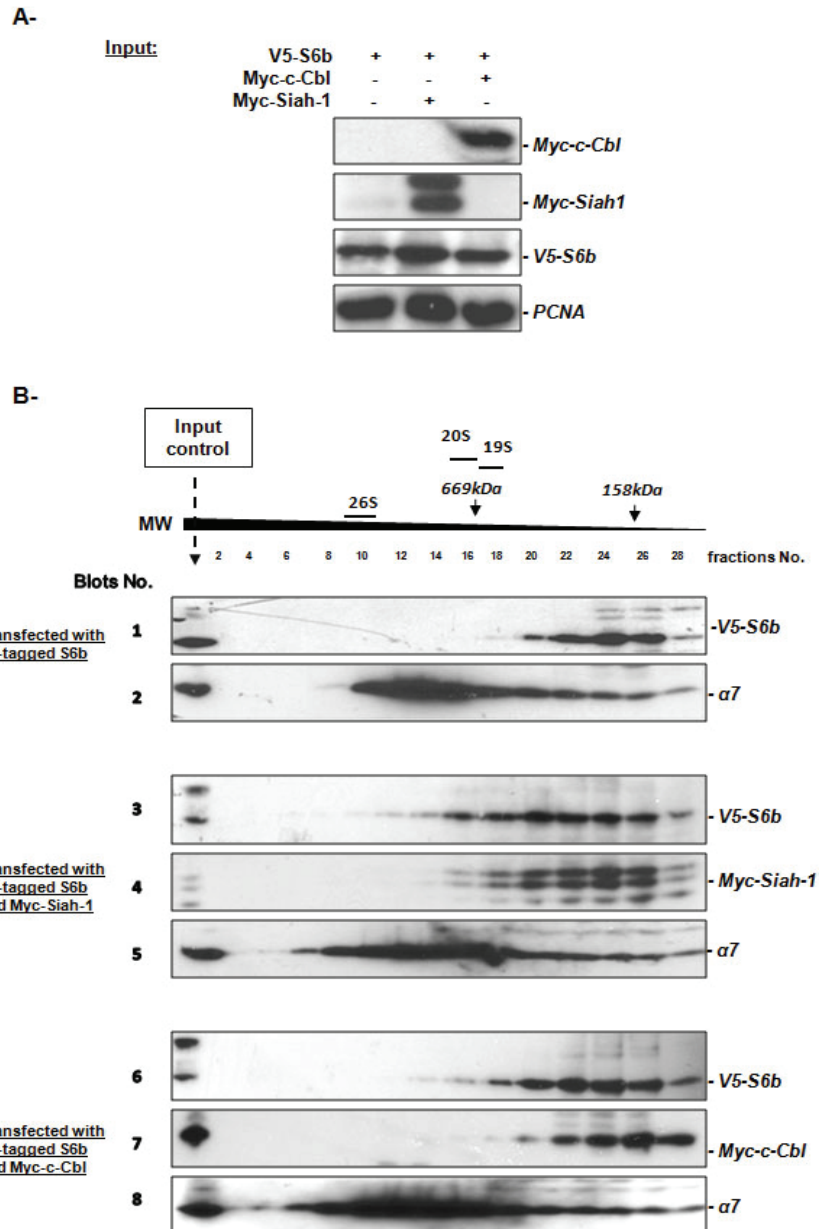


Figure 18: Siah-1 and c-Cbl shift the distribution of S6b protein towards higher order complexes. H1299 cells were transfected with 3 μg of a plasmid encoding V5-tagged S6b or with V5-tagged S6b together with 7 μg of a plasmid encoding Myc-tagged Siah-1 or Myc-tagged c-Cbl. **A-** Cells were lysed in NP-40 buffer containing ATP-Mg²⁺ and 40 μg of soluble proteins (input) were tested for expression of V5-tagged S6b, Myc-tagged Siah1 or Myc-tagged c-Cbl. PCNA was used for loading control. **B-** Conditions for analysis and fractionation of the sucrose gradient are given in the legend to *Figure 10*. Fractions were separated by SDS-PAGE. Proteins were blotted onto a PVDF-membrane and membranes were first tested with the anti-Myc antibody. Then, the blots were stripped and reprobed for V5-S6b and α_7 (blots 3 to 8). An HRP-linked anti-mouse or anti-rabbit antibody was used. Western blots were developed by ECL. The input control normalized the signal of the proteins between all the gradients and was composed of 40 μg of cell lysate corresponding to each gradient. (MW: molecular weight)

Though, the possibility of small amounts of these ligases (undetectable) present around the 26S proteasome fractions should not completely be ruled out. However, these data seemed to corroborate with the fact that Siah-1 and c-Cbl interacted only with S8 and S10b subunits

(Kulikov et al., 2010) although S5a, S6a, S6b and S8 subunits have been ubiquitinated (*paragraph 3.1.2.1*).

3.3 Functions of the 19S subunit ubiquitination by Mdm2

3.3.1 The ubiquitination of the 19S subunits are clearly not implied in the assembly of the 26S proteasome

Considering that polyubiquitination of proteasomal proteins is apparently not associated with their degradation and did not correlate with the assembly of the 26S proteasome, the function of these post-translational modifications is still under investigation. The results obtained with the MG132 treatment already seemed to demonstrate that the ubiquitination of the subunits was not involved in the proteasome assembly because the elution pattern of S2 seemed not to be altered by the MG132.

In order to confirm the role of the ubiquitination of the 19S subunits, the effect of a mutant of Mdm2 (C464), deleted in its E3 ubiquitin ligase functions was tested on the distribution of the proteasomal protein S2. The replacement of the cysteine 464 in the C-terminus of Mdm2 by an alanine (C464A mutant) in its RING domain is known to prevent p53 ubiquitination (Honda et al., 2000). For this experiment, H1299 cells were transfected with V5-tagged S2, Mdm2 wild-type (WT) or mutant (C464A) in presence or absence of p53. Protein levels of V5-tagged S2, Mdm2 WT or mutant, p53 and PCNA were determined by Western blotting (*Fig. 19 A*). As for the other sucrose gradients, the same quantity of proteins was loaded. Conditions for sedimentation and analysis of the gradients are given to *Figure 10*.

The elution patterns of WT and C464A mutant of Mdm2 were equivalent in presence of p53, with the majority of both forms of Mdm2 found from fraction 16 to 24; although a minority of Mdm2 protein eluted with larger protein complexes (*compare blots: 4 and 8; Fig. 19 B*). Concerning the distribution of endogenous α_7 proteins, the major amount of this subunit correctly eluted with 26S and 20S complexes (fractions 12 to 20) (*compare blots: 2, 6 and 10; Fig. 19 B*). The elution pattern of S2 protein remained comparable in presence of the WT or the C464A mutant of Mdm2 (*compare blots: 1, 3 and 7; Fig. 19 B*). The increase of S2 in fractions corresponding to the 26S proteasome is still detected with WT or C464A mutant of Mdm2 in presence of the substrate p53 (*compare blots 1 and 3, 1 and 7; Fig. 19 B*).

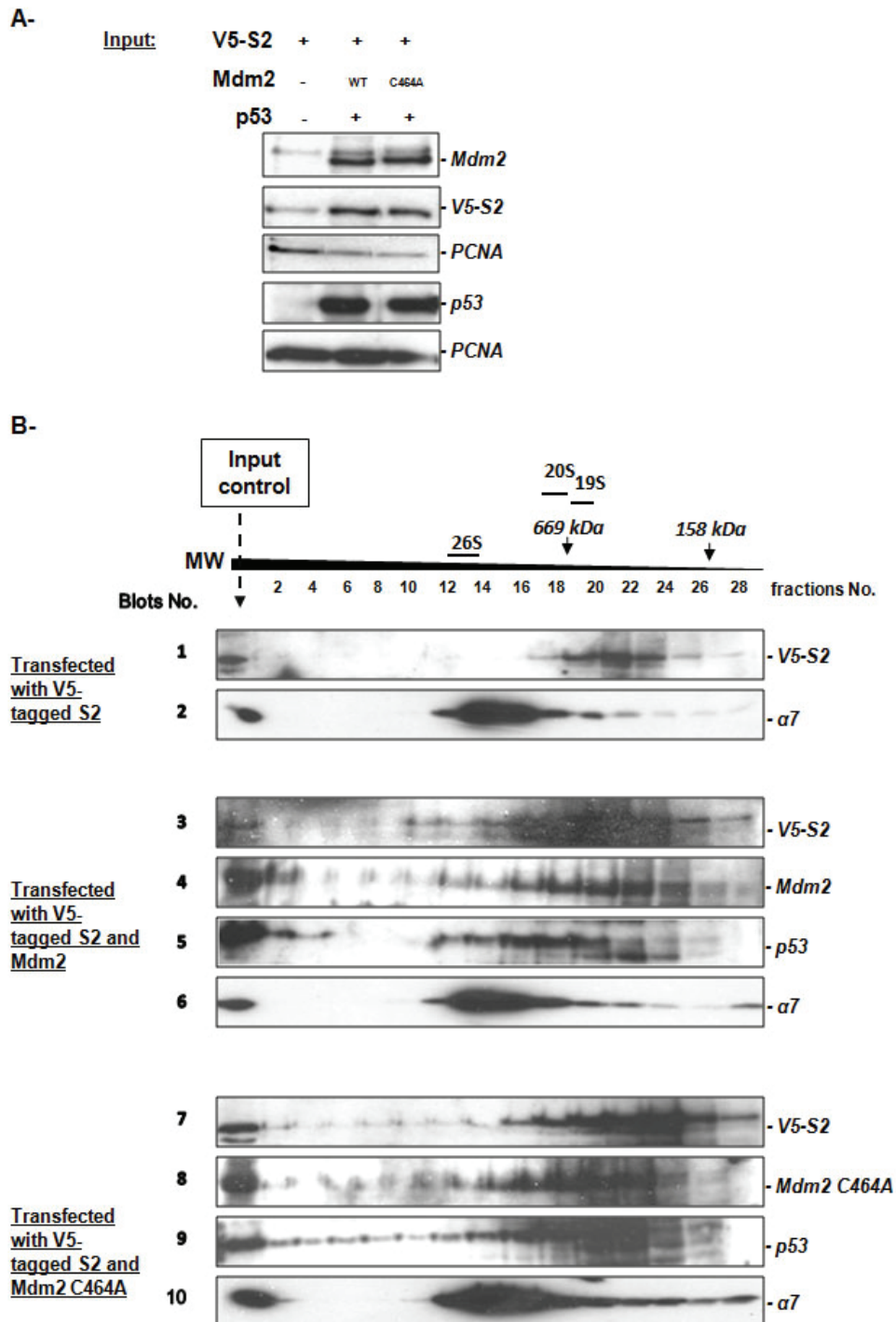


Figure 19: The “RING” mutant (C464A) behaves the same way on the distribution of S2 subunit as Mdm2 WT. H1299 cells were transfected with either 3 μg of a plasmid encoding V5-tagged S2 or with 3 μg of a plasmid encoding V5-tagged S2 together with 7 μg of a plasmid encoding WT or mutant (C464A) Mdm2 together with 2 μg of a plasmid encoding p53. **A-** Cells were lysed in NP-40 buffer containing ATP-Mg²⁺ and 40 μg was tested for expression of V5-tagged S2, Mdm2, p53 and PCNA, for control. **B-** The sucrose gradient was analysed as described in the legend to *Figure 10*. Fractions were collected, separated by SDS-PAGE and proteins were detected with 4B2 (Mdm2, C464A), V5, $\alpha 7$ and DO-1 (p53) antibodies. The upper part of the membranes (blots 3, 4, 7 and 8) were first tested for S2 expression, then the blots were stripped and reprobed for Mdm2. The input control normalized the signal of the proteins between all the gradients and was composed of 40 μg of cell lysate corresponding to each gradient. (MW: molecular weight)

Only the distribution of p53 was affected by the replacement of Mdm2 WT by the C464A mutant with an increase of p53 in fractions 2 to 12 (*compare blots: 5 and 9; Fig. 19 B*). In contrast to the input control (*Fig. 19 A*); the level of p53 seemed to remain the same in presence of WT or C464 mutant of Mdm2. However, the level of PCNA corresponding to this blot was not constant and was weaker for p53 in presence of the C464A mutant. After quantification of the ratio p53/PCNA with the ImageJ software, an increase of 41% of the p53 protein level could be detected with the C464 mutant in comparison to the WT Mdm2, that corroborated results obtained with the sucrose gradient analysis. These observations are in agreement with the literature data, because p53, not ubiquitinated by the C464A mutant and not degraded by the proteasome, accumulated in cells (Honda et al., 2000).

Thus, the C464A mutant of Mdm2 has no impact on the 19S proteins distribution. This support the hypothesis that ubiquitination of the 19S subunits is not required for the recruitment of proteasomal subunits during the assembly of the proteasome.

3.3.2 Deubiquitination of S2 favor its incorporation into the full-assembled proteasome complexes

To further explore if the ubiquitination of 19S subunits is associated with the assembly or with the disassembly of the proteasome, cell lysates were treated with the deubiquitinase USP2 (Ubiquitin-Specific Protease 2) prior sucrose gradient analysis. The ubiquitin-conjugates are extremely difficult to detect as explained before, even with NEM (inhibitor of the deubiquitinase) in the lysis buffer, probably due to their low quantity and their distribution among molecular weight. Hence, the benefit of USP2 which should remove the ubiquitin chains and, thus blunts the activity of E3s ligases as Mdm2. The non-ubiquitinated forms of 19S subunits should accumulate in cells.

USP2, a cysteine protease and a member of the ubiquitin specific protease family, is overexpressed in prostate cancer and stabilizes fatty acid synthase, which has been associated with the malignancy of some aggressive prostate cancers (Graner et al., 2004). Several articles related that the catalytic core domain of USP2 deubiquitinates ubiquitin-conjugates in cells or in tissue extracts (Lin et al., 2001; Ventadour et al., 2007). The GST-USP2-core protein was produced in bacteria and purified with Glutathione-sepharose beads. USP2-core was eluted with the factor Xa which cleaved the GST part. The efficiency of the purification of USP2-

core was checked by SDS-PAGE followed by staining with Coomassie blue (Fig. 20 A). The activity of the USP2-core to cleave polyubiquitin chains of proteins was confirmed for whole cell lysate of H1299 cells with the Fk1 antibody (Fig. 20 B). Ventadour and co-workers already demonstrated that USP2-core disassembles the K48-linked chains (Ventadour et al., 2007). However in our case, it remains important to test USP2-core for the disruption of the unconventional ubiquitin chains linked to the 19S subunits.

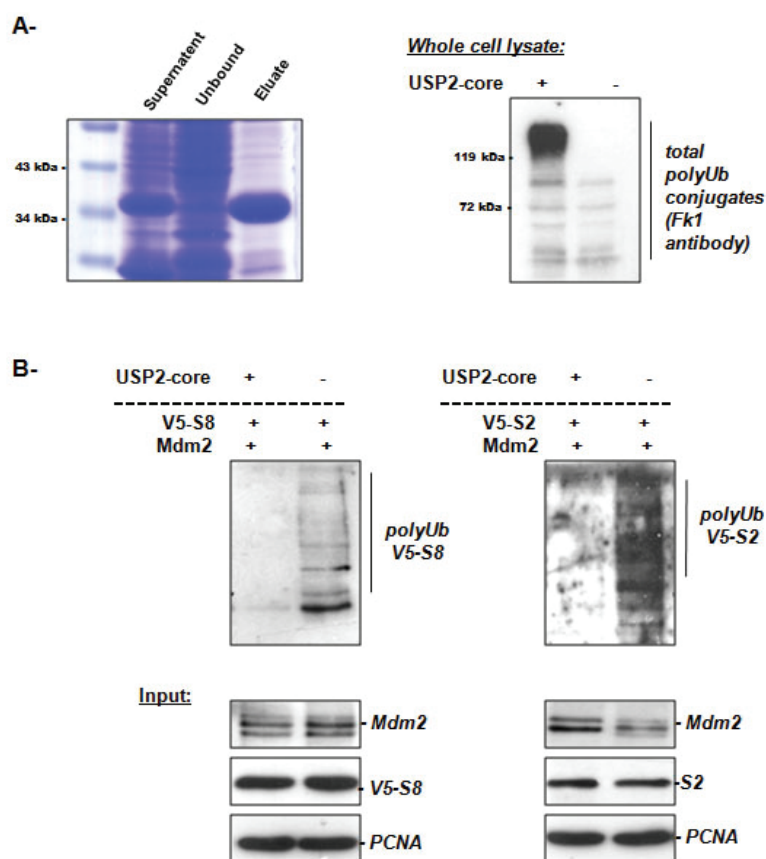


Figure 20: USP2-core disassembles ubiquitin chains. A- Purification of USP2-core. Bacteria were transformed with pGEX-5X1-USP2-core. The GST-USP2-core was purified using glutathione-sepharose 4B, and eluted from the glutathione sepharose by incubation with Xa factor, which cleaves between the GST and the USP2 part of the protein. Aliquots of the supernatant, the unbound fraction and the eluate were loaded onto SDS-PAGE and stained with Coomassie blue. To monitor the USP2-core activity, H1299 cell lysates (40 μ g) were incubated for 3h at 4°C with or without USP2-core (0.4 μ g/ μ l) in deubiquitination buffer. The reaction was stopped by addition of SDS-loading buffer. Samples were separated by SDS-PAGE and analysed by Western blotting using the Fk1 antibody that recognizes polyubiquitin-conjugates. **B-USP2 disassembles ubiquitin chains from S2 and S8 subunits.** H1299 cells were co-transfected with 3 μ g of a plasmids encoding V5-tagged S8 or S2 together with 7 μ g of a plasmid encoding Mdm2 and 1 μ g of a plasmid encoding His-tagged ubiquitin. 48h after transfection, cells were harvested in 100 μ l of NP-40 buffer and incubated or not with the USP2-core enzyme for 4 h at 4°C (USP2-core at 0.4 μ g/ μ l diluted in deubiquitination buffer). Aliquots of cells lysates (40 μ g) were analyzed for the presence of Mdm2 (4B2), V5-S2 or S8 and PCNA by Western blotting. Concerning the part B for the membrane V5-S2, the upper part was first incubated with an anti-V5 antibody, then the blots were stripped and reprobed for Mdm2 (Input). The remaining of cell lysate was diluted in guanidinium buffer. Ubiquitinated proteins were purified by adsorption to Ni²⁺-agarose beads and analyzed as described in the legend to Figure 11. (Ub: ubiquitin)

To analyze the efficiency of USP2-core on the polyubiquitin-chains linked to the 19S proteins, lysates of H1299 cells transfected with V5-tagged S2 or S8 and Mdm2 were treated with the deubiquitinase and an ubiquitination assay was performed. As shown in *Figure 20 B*, upon the action of USP2-core, the ubiquitin chains linked to S2 and S8 subunits were completely removed. USP2-core allows then a total disassembly of the mixed-ubiquitin chains of the 19S subunits.

Deubiquitinating proteases such as USP2 reverses protein ubiquitination. Herein the use of this approach which should discriminate about the role of ubiquitination of the 19S subunits with the comparison of their elution patterns in presence or not of USP2. To test the effect of USP2-core on the assembly of 19S subunits, H1299 cells were transfected with V5-tagged S2 or with V5-tagged S2, Mdm2 and p53 DNAs. The protein expression of Mdm2, S2, and p53 was analyzed by Western blotting (*Fig. 21 A*). Cell lysates were treated or not with USP2-core (0.4 $\mu\text{g}/\mu\text{l}$ for 4 h) prior to the loading onto sucrose gradients. The sucrose gradient experiment (*Fig. 21 B*) was carried out similarly to the *Figure 10*.

The elution pattern of Mdm2 was not modified by USP2-core treatment (*compare blots: 4 and 10; Fig. 21 B*), even if Mdm2 has been shown to be a substrate of this deubiquitinase (Stevenson et al., 2007). The bigger amount of S2 proteins was located in fractions 14 to 26 (*blot: 1; Fig. 21 B*). As already observed the presence of Mdm2-p53 complexes shifted the S2 protein in higher molecular weight from fraction 12 to 18 (*compare blots: 1 and 3; Fig. 21 B*). The S2 elution pattern was really disturbed in presence of USP2-core enzyme, S2 proteins increased in higher molecular weight complexes, fractions 2 to 12 (*compare blots: 1/3 and 7/9; Fig. 21 B*).

The deubiquitination of S2 seemed therefore favor the incorporation of this subunit in higher order complexes corresponding to the 19S and 26S proteasome. In the same extend than S2, the effect of USP2-core was also observed on the endogenous S8 subunits, the treatment of the cell lysate also enhanced its incorporation into native 26S proteasome (*data not shown*). These results confirmed that the ubiquitination of S2 and S8 by Mdm2 was not associated with the assembly of the proteasome.

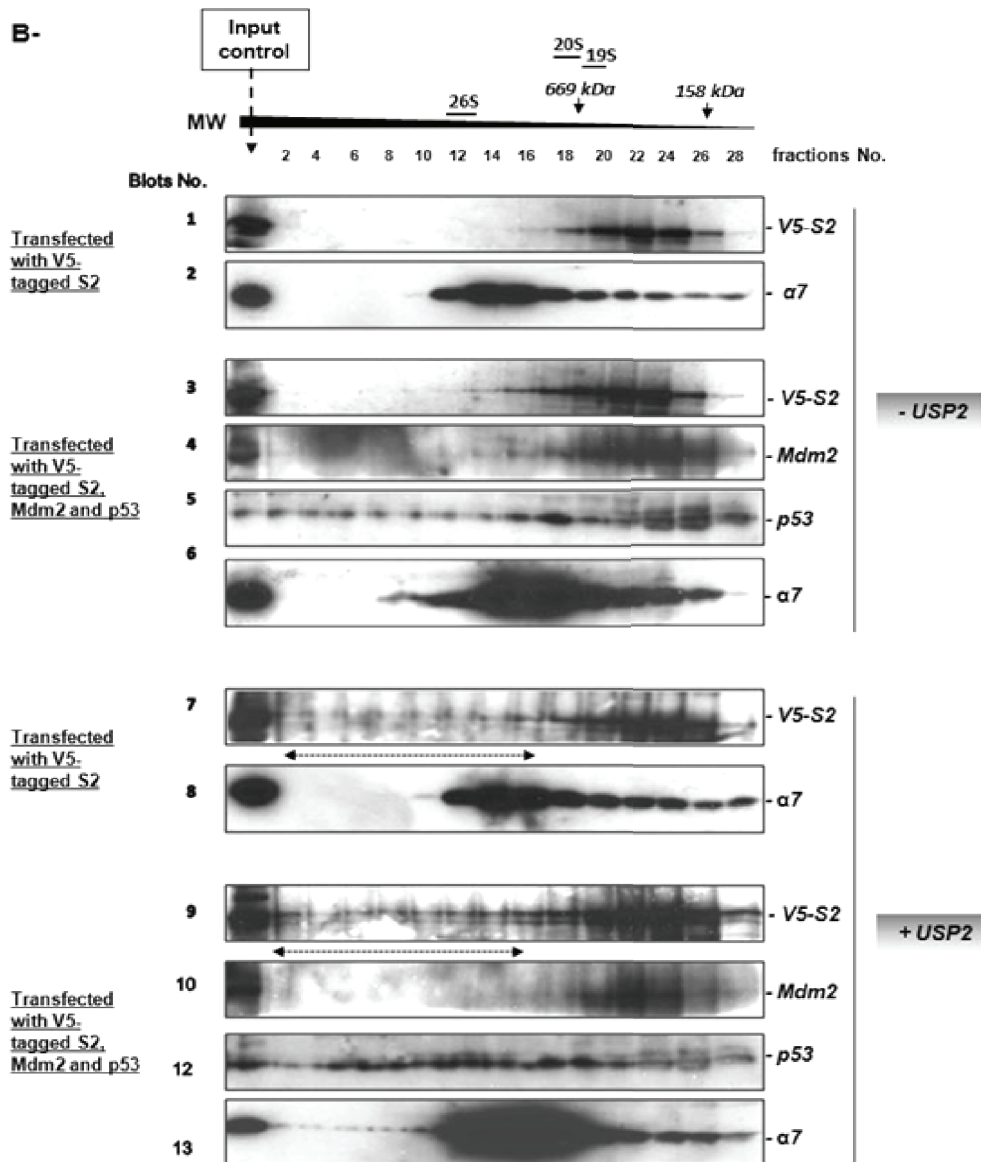
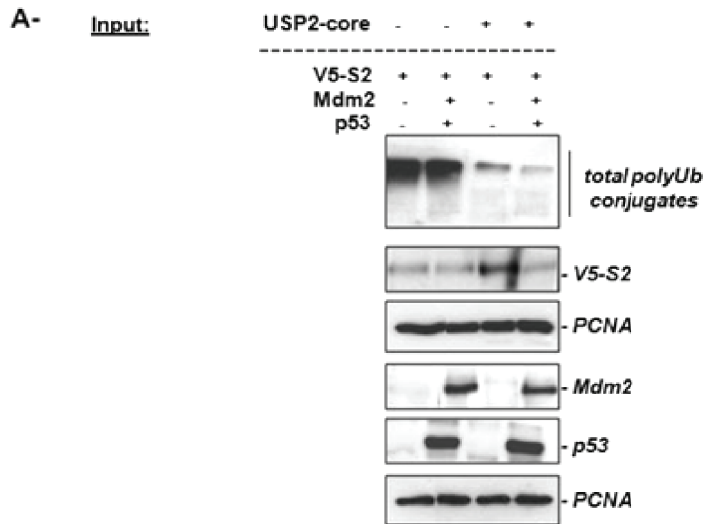


Figure legend p74

Figure 21: Treatment of cell lysate with USP2 leads to the accumulation of S2 in higher molecular weight complexes. H1299 cells were transfected either with 3 µg of a plasmid encoding V5-tagged S2 or with 3 µg of a plasmid encoding V5-tagged S2 together with 7 µg of plasmid encoding Mdm2 and 2 µg of plasmid encoding p53. Cells were lysed and incubated in the presence or absence of USP2-core (0.4 µg/µl) in deubiquitination buffer for 4 h before loading onto sucrose gradients. **A-** An aliquot of the cells was tested for expression of V5-tagged S2, Mdm2, p53 and PCNA which is used as a loading control (input). **B-** The sucrose gradients were analysed as described in the legend to *Figure 10*. Proteins were detected with 4B2 (Mdm2), V5, endogenous α_7 and DO-1 (p53) antibodies. The upper part of the membranes were first tested for S2 expression, then the blots were stripped and re probed for Mdm2 (blots 3, 4, 9 and 10). The input control normalized the signal of the proteins between all the gradients and was composed of 40 µg of cell lysate corresponding to each gradient. The arrows highlight the increase of S2 subunit in fractions of the 26S proteasome after treatment with the deubiquitinase USP2. (MW: molecular weight)

The distribution of endogenous α_7 proteins was tested as control (*compare blots: 2, 6, 8 and 13; Fig. 21 B*); the major amount was identified from fraction 12 to 20 validating that endogenous α_7 proteins effectively eluted with 26S and 20S complexes. Furthermore, the p53 elution pattern has been analyzed. P53 was found in all the fractions of the sucrose gradient for lysates treated or not with USP2-core (*blots: 5 and 13; Fig. 21 B*) and, in addition, the p53 protein level remained the same with Western blot analysis (*Fig. 21 A*). These observations confirmed the data from Stevenson and co-workers, demonstrating that USP2 did not affect the ubiquitination level of p53 (Stevenson et al., 2007) and thus, its degradation.

Another important point was to proof that the shift of S2 proteins was not due to the binding of USP2-core to the proteasome. A binding assay was performed; GST-USP2-core was produced in bacteria and purified. H1299 cells were co-transfected with plasmids encoding V5-tagged S2 and S8. GST-tagged USP2 bound on Glutathione sepharose beads was incubated with cell lysate or GST-USP2 was loading alone as control, to test the binding between USP2-core and 19S subunits (*Fig. 22*).

Interestingly, the binding assay did not show an interaction between USP2-core and V5-tagged S2 or S8 or with S8 endogenous proteins (*Fig. 22*) or even with 20S subunits (*data not shown*); the GST-USP2-core did not pull-down any of the tested proteasomal subunits. Surprisingly, the V5 antibody recognized a non-specific linked protein or USP2-core around 65-70 kDa.

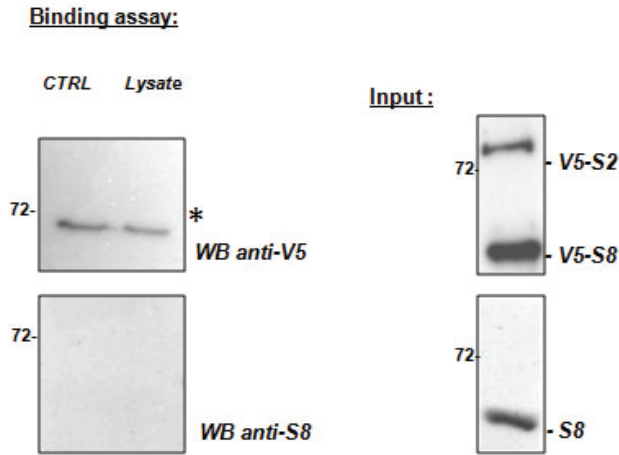


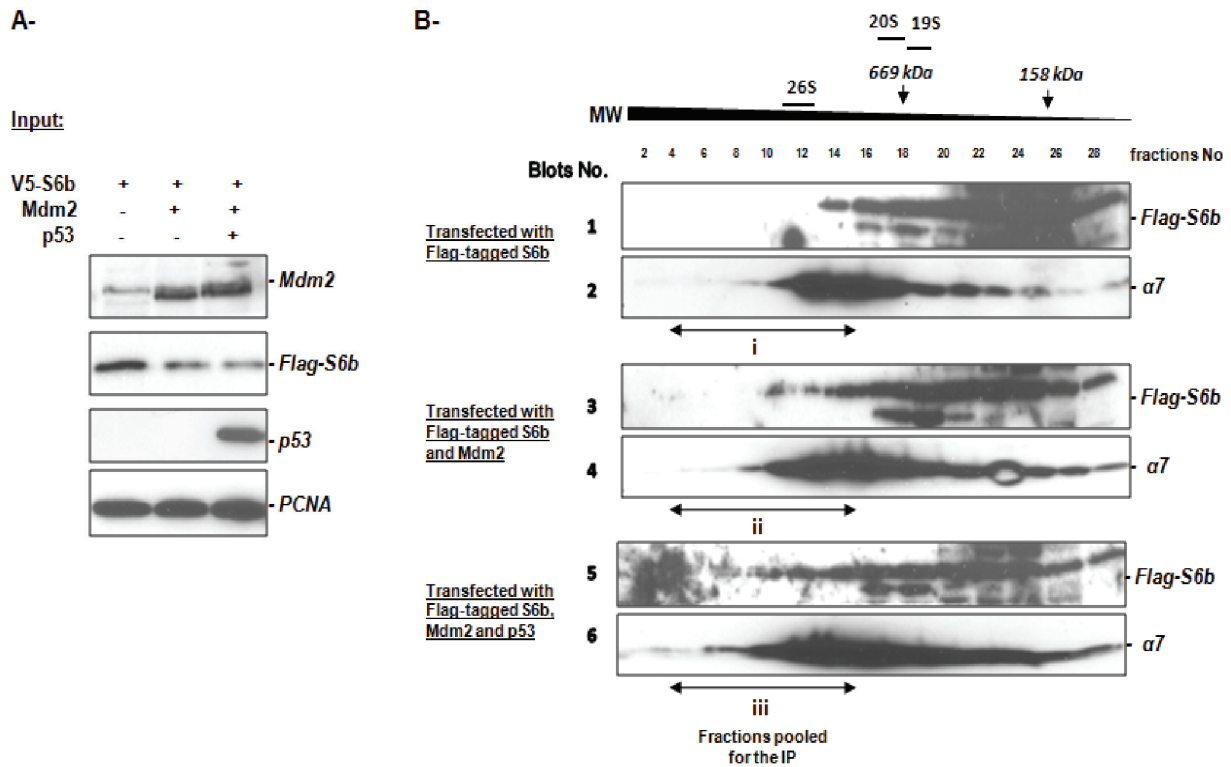
Figure 22: The USP2-core enzyme does not interact with S2 and S8 proteasomal proteins. H1299 cells were co-transfected with V5-tagged S2 and S8 and lysed in NP-40 buffer. Cell lysate (40 µg) was tested for expression of V5-tagged S2 or S8 and endogenous S8 (Input). GST-tagged USP2 bound to glutathione sepharose was incubated with 500 µg of cell lysate or with buffer for control. The samples were run on a SDS-PAGE gel and Western blot analysis was performed with the indicated antibodies. The asterisk marks to a non-specific protein which was recognized by the V5 antibody.

The deubiquitination of S2 subunit is thus associated with an increase of its incorporation into the proteasome. These results favor the previous hypothesis that the ubiquitination of 19S subunits is not correlated with the assembly of the proteasome but with its disassembly.

3.3.3 Deubiquitination increases the interactions between S6b and S8 subunits of 19S proteasome

To better understand the role of the ubiquitination of 19S proteasomal subunits on the proteasome disassembly, the association between two subunits of the 19S proteasome (Flag-tagged S6b and endogenous S8 proteins) was tested upon deubiquitinase USP2-core treatment. This strategy should discriminate whether the deubiquitination increases or decreases the association between the 19S proteins.

H1299 cells were transfected with either plasmids encoding Flag-S6b (i) or Flag-S6b with Mdm2 (ii) or Flag-S6b together with Mdm2 and p53 (iii) (*Fig. 23 A*), and complexes were separated by a sucrose gradient analysis (*Fig. 23 B*). The Flag-tagged S6b proteasome protein was precipitated with an antibody against Flag-tag, from the pool of fractions 4 to 14 where are supposed to be located the polyubiquitinated forms of the 19S subunits. The pool of fractions was then treated or not with the deubiquitinase USP2. Afterwards, the precipitate was separated by SDS-PAGE and the level of co-precipitated S8 proteins was determined by Western blotting (*Fig. 23 C*).



C- IP α -Flag from the pool of fractions 4 to 14

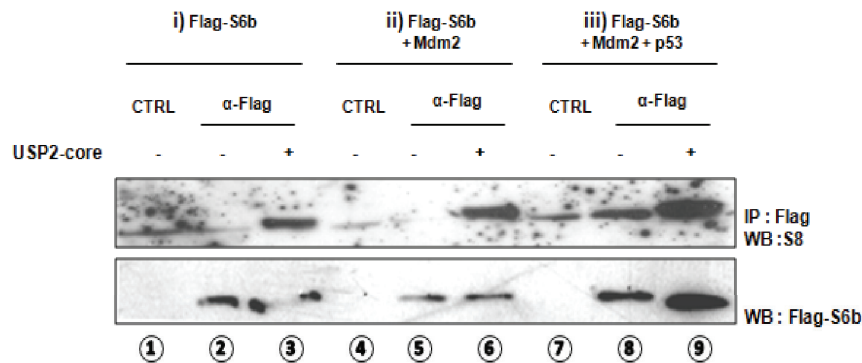


Figure 23: Treatment with USP2-core increases the interaction between S6b and S8 proteins. H1299 cells were transfected with Flag-tagged S6b (i) with or without Mdm2 (ii) and p53 (iii). **A-** Cells were lysed in NP-40 buffer containing ATP-Mg²⁺ and 40 μ g of cellular protein was tested for expression of Flag-tagged S6b, Mdm2 and p53. PCNA was used for loading control (Input). **B-** The sucrose gradients were analysed as described in the legend to *Figure 10*. Fractions were separated by SDS-PAGE and proteins were detected with anti- $\alpha 7$ and anti-Flag antibodies. **C-** Fractions 4 to 14 were pooled and the pool of fractions was divided into 3 parts: control, non-treated and treated with USP2-core in a deubiquitination buffer (final concentration 0.4 μ g/ μ l, during 4 h). Proteins A agarose beads were incubated with an anti-Flag antibody or with Ig G for control for 2 h at RT, by an incubation with 500 μ g of cell lysate overnight at 4°C. The agarose was washed and bound proteins were eluted in SDS-loading buffer. Eluates were loaded onto a SDS-PAGE gel and, analyzed by Western blotting using an anti-Flag and an anti-S8 antibody. (IP: immunoprecipitation, WB: Western blotting)

About the sucrose gradient analysis, analogous results were obtained with the Flag-tagged S6b proteins in comparison to the V5-tagged 19S proteins (*Fig. 23 B*). The distribution of S6b proteins was located from fraction 14 to 28 (*blot:1 ; Fig. 23 B*) and shifted likewise in higher molecular weight fractions in presence of Mdm2 (*compare blots: 1 and 3; Fig. 23 B*) or Mdm2-p53 complexes (*compare blots: 1 and 5; Fig. 23 B*). The major concentration of α_7 subunits was correctly detected around fractions corresponding to the 26S and 20S proteasome (fractions 10 to 20) (*compare blots: 2, 4 and 6; Fig. 23 B*).

As shown in *Figure 23 C*, S8 and S6b co-immunoprecipitated which supported the model of the base assembly defended by Kaneko and co-workers (Kaneko et al., 2009). The co-immunoprecipitation experiment revealed that deubiquitination of the 19S subunits S6b and S8 by USP2-core enhanced their association (*Fig. 23 C*). An enhancement of the interaction between S6b and S8 could be distinguished in presence of Mdm2, interaction which is potentialized in presence of p53 because an increase is already observed without USP2 treatment (*compare blots: 2, 5 and 8; Fig. 23 C*). These data correlated with results obtained with sucrose gradient experiment, more S6b proteins were found in the pool of fractions 4 to 14 (*compare blots: 1, 3 and 5; Fig. 23 B*), results which could be explained by the role of Mdm2 and its substrate p53 on the formation of the proteasome.

The deubiquitination of the 19S subunits by the deubiquitinase USP2-core increased the interaction between the S6b and S8 proteins. Therefore, the ubiquitination does not favor the interaction between 19S proteasomal subunits (S6b and S8) and is rather associated with the proteasome disassembly.

3.3.4 P53 enhances the interaction between Mdm2/S8 and the ubiquitination of S2 subunit of the 19S proteasome

In this last part of results, experiments were focused on the functions of the substrate p53 on the proteasome assembly and on the ubiquitination of the proteasomal subunits. The aim was to determine if the ubiquitination of some 19S subunits and the interaction of Mdm2 with 19S proteins might be substrate-specific.

An interesting point to settle was the effect of substrate on the ubiquitination of the 19S subunits by Mdm2; an ubiquitination assay was carried out in presence of p53. H1299 cells were transfected with His-tagged ubiquitin (His₆-Ub) together with V5-tagged S2 alone or

with Mdm2 or with Mdm2 and p53. Ubiquitinated proteins were purified by adsorption on Ni²⁺ beads, separated by SDS-PAGE and analyzed by Western blotting (Fig. 24).

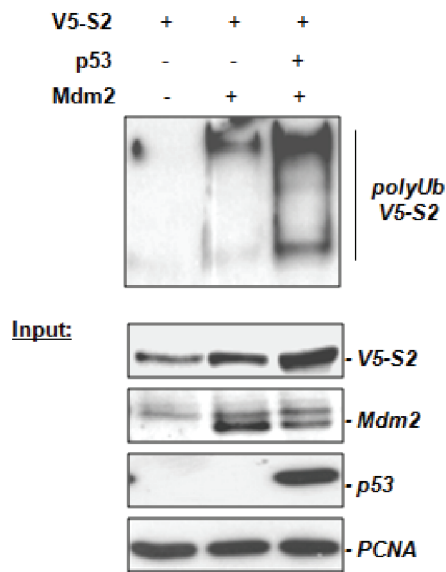


Figure 24: The presence of p53 increases ubiquitination of S2 by Mdm2. H1299 cells were co-transfected with V5-tagged S2 and His-tagged ubiquitin or together with His-tagged ubiquitin and Mdm2 or with His-tagged ubiquitin, Mdm2 and p53. Cells were harvested and lysed in NP-40 buffer. Expression of V5-S2, Mdm2, p53 and PCNA was determined by Western blotting. The upper part of the membrane of the input was first incubated with an anti-V5 antibody, then the blot was stripped and reprobed for Mdm2. Equal loading is confirmed by the analysis of PCNA. The ubiquitination assay was performed as described in the legend for Figure 11.

Surprisingly, the presence of the substrate p53 increased the ubiquitination of S2 proteins of the 19S proteasome, ubiquitination already detected with Mdm2 (*upper panel, Fig. 24*).

This result was not expected and suggested that the presence of high amount of p53 enhanced the ubiquitination of 19S subunits implicated in the proteasome disassembly.

4. DISCUSSION

In our group, it has been shown that Mdm2 promotes the formation of a ternary complex between p53, Mdm2 and the proteasome which is important to regulate the level of tumor suppressor p53. It was previously observed that Mdm2 interacts with a variety of 19S subunits of the proteasome, including S2, S4a, S5a, S6a, S6b, S8 and S10b (Kulikov et al., 2010).

Other authors have already reported the interaction of the Mdm2 ligase with proteasomal subunits such as the α_7 protein of the 20S proteasome, in order to promote the degradation of the pRb protein (Sdek et al., 2005). However, the association between E3 ligase and proteasomal subunits seemed not to be the only properties of Mdm2. A number of studies have previously described this kind of association. Interestingly, the E7 protein of the papillomavirus, an E3 ligase, has been shown to interact with the S4 subunit of the 19S regulatory complex of the 26S proteasome to target pRb for proteasomal degradation (Berezutskaya et al., 1997). Another example included the interaction of the E3 ligase VHL with S6a to degrade its substrate HIF1 α (Corn et al., 2003).

Some data from our laboratory validated that the interaction of RING E3 ligases with 19S proteins might be a general rule because two other RING E3 ligases Siah-1 and c-Cbl have been tested for their association with the proteasome. Both Siah-1 and c-Cbl interact with S8 and S10b proteins of the 19S proteasome. Nevertheless, one important question remains the functions of the interaction between E3 ligases and 19S proteasome subunits whether it might be only a general way for the ligases to shuttle their substrates for degradation or could have an additional role.

4.1 E3 ligases or E3 ligase-substrate have a direct impact on the proteasome assembly

4.1.1 Mdm2 and Mdm2-p53 enhance the recruitment of native proteasome

In the present study, results demonstrated that Mdm2 with its substrate p53 favors the recruitment of proteasomal proteins and thus, the assembly of the proteasome. Mdm2 is

associated with full 19S proteasomes which is confirmed by a common elution pattern shared by Mdm2 with 19S subunits (S1, S2, S6a and S8 subunits). However, this result also demonstrated that Mdm2 could be associated with 19S subcomplexes of lower molecular weight and thus, it could indicate a probable role of Mdm2 as chaperone for the formation of the 19S proteasome but this hypothesis should be further validated. According the elution pattern from the sucrose gradient assay, Mdm2 was also found in fractions that correspond in size to the 26S proteasome, indicating that Mdm2, 20S and 19S proteins are parts of the same huge complex.

Most interestingly, the majority of proteasomal proteins are not assembled into 26S proteasome, instead they are found in smaller subcomplexes raising the thought that the 26S proteasome may only assemble completely when protein degradation takes place.

Besides, in the presence of Mdm2 and even more in the presence of Mdm2-p53, the amount of 19S proteins is increased in higher molecular weight fractions which correspond in size to the 26S proteasome. These 19S subunits indeed interact directly with Mdm2 (S2, S8, S6b or S4 proteins) or not (S9 protein), raising the hypothesis that Mdm2 or Mdm2-p53 can affect the whole proteasome assembly.

My results also confirmed that Mdm2 or p53 did not alter the steady-state level of 19S proteasomal proteins validating the impact of Mdm2 and p53 in the recruitment of 19S subunits to form native proteasome. Furthermore, p53 is known as a transcription factor which under stress stimuli can activate a plethora of genes (Brooks and Gu; 2003). The capability of p53 to up-regulate 19S genes was as well investigated. A qRT-PCR analysis did not show an increase of S8 transcription in the presence of p53 (*data not shown*) and the promoter region of the S8 gene seemed not to have p53 response elements (Beckerman and Prives, 2010). These observations further confirm that p53 does not seem to regulate transcription of 19S subunits; it is rather another not-transcriptional activity of p53 involved in the formation of the proteasome.

Additionally to Mdm2 effects, the role of p53 on the recruitment of proteasomal proteins was confirmed through the observation that increased amount of p53 strengthens the association of Mdm2 with S8 subunits of the 19S proteasome. The presence of p53 promotes the interaction between two ATPases subunits of the 19S proteasome: S8 and S6b. Moreover, the presence of p53 raised the amount of proteasomal subunits in higher order complexes,

suggesting a direct impact of p53 on the formation of the proteasome. However, in presence of Mdm2 and Mdm2-p53 complexes, a weak shift of the α_7 subunit used as control could be detected in higher molecular weight. This shift could represent an internal variation of gradients. Otherwise, another hypothesis is that this shift is due to the enhancement of the formation of the full-length proteasome by Mdm2 or Mdm2-p53, in spite of only 30 to 40 % of cells were transfected with the calcium-phosphate method and if transfected proteins (Mdm2 and p53) constituted approximately 1% of the whole cellular proteins (in comparison 20S proteasome proteins represent around 0.6% of bulk cell protein, in HeLa cells (Hendil, 1988)).

Altogether, these findings suggest that, in addition to enhance the assembly of the 19S proteasome, Mdm2-p53 complex stimulates the subsequent association of the 19S and 20S subunits to form native 26S proteasome.

4.1.2 Does this general mechanism could be extended to other E3 ligases?

Two other RING E3 ubiquitin ligases Siah-1 and c-Cbl, similarly to Mdm2, can associate with some 19S proteins (S8 and S10b) (Kulikov et al., 2010). My findings demonstrated that the incorporation of 19S proteins (S6b subunit) is increased in 26S proteasome complexes when Siah-1 or c-Cbl is overexpressed. Thus, these two E3 ligases are able to increase the recruitment of full-assembled 26S proteasome.

Concerning Siah-1 and c-Cbl, the mechanism implicated in the recruitment of proteasomal subunits might be different from Mdm2. Siah-1 and c-Cbl were not encountered in fractions of the 26S proteasome. The major amount was detected in smaller weight complexes and these two proteins interact only with two 19S subunits (S8 and S10b) whereas Mdm2 interacts with several subunits (S2, S4a, S5a, S6a, S8 and S10b) (Kulikov et al., 2010) and co-eluted with proteins of the 26S proteasome. It is supposed that interactions of Siah-1 and c-Cbl with these two 19S subunits can be enough to induce the recruitment of proteasomal subunits. However, this recruitment can require some co-factors as multiubiquitin receptors or some sequence signal or post-translational modifications (acetylation, phosphorylation or neddylation...) of the 19S subunits.

To this extent, the recruitment of proteasomal subunits by E3 ligases is not limited to Mdm2 and seemed to be an intrinsic property of several monomeric RING E3 ligases (Mdm2,

Siah1 and c-Cbl). Studies in yeast have already described that E3 ubiquitin ligases are associated to some degree with the 26S proteasome, leading to the recruitment of the ubiquitination machinery (Verma et al., 2000). My data highlight a direct role of E3 ligases on the proteasome assembly in human cells, although the impact of the substrate still has to be investigated for both E3 ligases Siah-1 and c-Cbl. This kind of mechanism has been recently demonstrated for the E3 ligase Parkin which directly modulates the assembly and activity of the 26S proteasome (Um et al., 2010).

4.2 Characterization of the ubiquitinated 19S subunits

While the effect of the association between subunits of the proteasome and E3 ligases is well established, the role of post-translational modifications of proteasomal proteins by E3 ligases is poorly described. Only few authors reported ubiquitination of proteasomal subunits (Uchiki et al., 2009; Ventadour et al., 2007) and phosphorylation (Zhang et al., 2007).

Our results demonstrated the ubiquitination of S2, S4, S5a, S6a, S6b and S8 proteins of the 19S proteasome components by the Mdm2 ligase, in a cellular model. However, in parallel to my investigations, Uchiki and co-workers have shown with *in vitro* model that the S5a protein can be ubiquitinated by several E3 ligases belonging to different subclasses (Uchiki et al., 2009). They found out that S5a is ubiquitinated via its binding through its ubiquitin interacting domains (UIMs) to the growing polyubiquitin chains on the E3 ligases (during self-ubiquitination) or via its interaction with the substrate bound to the E3.

Additionally to Mdm2, the E3 ubiquitin ligases c-Cbl and Siah-1 also promoted ubiquitination of S5a, S6b and S8 subunits. The effect of Mdm2 was further validated using mutant of Mdm2 in its RING domain (C464A mutant). The mutation completely abrogates the ubiquitination of 19S subunits by Mdm2. Similar results were obtained with Siah-1 and c-Cbl RING mutants. (*Blattner C., unpublished data*)

Mdm2 associates directly to the ubiquitinated subunits (S2, S4, S5a, S6a, S6b and S8), whereas for c-Cbl and Siah-1, these two E3 ligases did not bind directly to S5a or S6b but remained able to ubiquitinate them. Thus, the direct associations between E3 ligase and 19S proteins do not represent a prerequisite for the ubiquitination of these subunits. Other co-factors might also be required to allow the ubiquitination of the 19S proteins by c-Cbl or Siah-1. Interestingly, this phenomenon was also observed with the enhancement of the proteasome assembly by these two E3 ligases, as explained in the previous paragraph.

4.2.1 Ubiquitination of some 19S subunits by E3 ligases is not a target signal for proteasomal degradation

The most essential question remains to settle the function of the polyubiquitination of proteasomal proteins. All these observations raised the hypothesis that the polyubiquitination could be signal for protein degradation by the proteasome.

Some data already shows that the ubiquitination of S5a (19S subunit) leads to its degradation by the proteasome (Uchiki et al., 2009). The S5a protein is a multiubiquitin receptor which takes part in the recognition of polyubiquitinated proteins for proteasomal degradation (Verma et al., 2004). This protein can exist as a free subunit or as a subunit of the 26S proteasome. In yeasts S5a or at least the free forms of S5a (Rpn10 in yeast) present in the cytosol are substrates of the proteasome (Rubin et al., 1997). Uchiki and co-worker described as well a short half-life of about 30 min for S5a in C2C12 (mouse) myoblasts. The short half-life of S5a is presumably explained by the presence of the UIM domain and reflects the ubiquitination of free S5a by many E3 ligases (Uchiki et al., 2009).

Although previous work indicated that S5a is targeted for proteasomal degradation, in this study, ubiquitinated 19S proteasomal proteins were not degraded via the proteasome pathway. In H1299 cells co-transfected with Mdm2, no decrease of S5a protein level was detected (*Blattner C., unpublished data*). Either, no accumulation of proteasomal proteins were noticed when U2OS cells were treated with an inhibitor of the proteasome MG132.

Nonetheless, Uchiki and co-workers' results concerned only the free pool of S5a proteins, while I focused on studies of the whole S5a protein ubiquitination without distinguished between free S5a and S5a included into proteasome complexes. Therefore, the distribution in cells of the V5-tagged S5a protein of the 19S proteasome should be elucidated. Probably, most of these V5-tagged proteins are associated with the proteasome and not with the free pool of S5a protein. Thus, it is interesting to study the distribution of the S5a protein by sucrose gradient analysis, to check if the V5-tagged S5a proteins are found in complexes and/or in free form.

As a summary, in our study, it appears that ubiquitination of proteasomal proteins is not a target signal for proteasomal degradation.

4.2.2 A non-conventional chain of ubiquitin molecules is linked to the 19S subunits

Unexpectedly, the MLG matrix did not associate with polyubiquitinated S8 protein of the 19S proteasome, while it clearly interacted with polyubiquitinated p53. Furthermore, some articles already related that recombinant S5a or MLG did not recognize monoubiquitin or di/tri-ubiquitin linked to a substrate which did not constitute a target signal for proteins degradation by the proteasome (Young et al., 1998; Wang et al., 2005).

This raised the hypothesis that the MLG-matrix may only recognises those ubiquitin chains that target proteins for degradation and that a non-conventional ubiquitin chain is linked to these proteasomal proteins. This idea should be tested and confirmed through further experiments.

However, other results validate already that ubiquitinated 19S proteins are not targeting for degradation and it seemed to be an uncommon ubiquitin chain linked to these subunits. The ubiquitination assay demonstrated that these polyubiquitin chains are not made of lysine 48-linked ubiquitin molecules, which is the classical linkage for proteasomal degradation (Ikeda et al., 2008). Instead, several lysines of the ubiquitin protein contributed to polyubiquitination of proteasomal proteins.

Despite both S2 and S5a subunits are linked to “mixed” polyubiquitin chains, the chains of these two proteasomal proteins are not identical. Eventually, the difference in the linkage of ubiquitin molecules of these two proteins can be explained by the distinct functions of these two subunits in cells. S2 is described as a protein of the proteasome base while S5a is localized between base and lid, where it connects these two parts of the 19S subunit of the proteasome with each other. In addition, S5a is a polyubiquitin receptor and S2 is not (Deveraux et al., 1994). It has been previously reported that monoubiquitination of S5a regulates the recruitment of substrates to the proteasome, which is not the case for S2 (Isasa et al., 2010).

4.3 Ubiquitination of 19S subunits, disassembly of the proteasome and role of p53

4.3.1 The disassembly requires the ubiquitination of some 19S subunits by Mdm2

It was not as yet elucidated why proteasomal proteins are ubiquitinated. Obviously, ubiquitination of 19S subunits are not associated with the recruitment of full-assembled 26S proteasome because a mutant of Mdm2 (C464A) depleted in its RING ubiquitin ligase activity is still able to increase the incorporation of 19S proteins (S2, S8 subunits) in higher order complexes.

A second hypothesis was that the ubiquitination of 19S subunits by Mdm2 is linked to the disassembly of the proteasome. This idea implicates that ubiquitination leads to the disruption of the proteasome and in contrary inhibition of the ubiquitination should favor its assembly.

This theory has been validated with the treatment of cell lysates by a deubiquitinase USP2 before to start the sucrose gradients analysis. Deubiquitinase which removes the ubiquitin chain linked to the 19S subunits. Interestingly, a higher amount of 19S proteasomal proteins (S2, S8 subunits) has been detected in fractions corresponding to the 26S proteasome, indicating the role of the 19S subunits ubiquitination in the dissociation of the proteasome.

Furthermore, the supposition that E3 ligases induced ubiquitination of proteasomal proteins to promote the proteasome disassembly was supported by the observations that deubiquitination of proteasomal proteins by the USP2 enzyme facilitates their interaction. The association between two ATPases subunits (S6b and S8) of the 19S proteasome has been facilitated after treatment with USP2.

This is the first time that results highlight the role of post-translational modifications of 19S subunits such as ubiquitination in the proteasome dissociation. Nevertheless, the localisation of ubiquitin conjugates of these 19S subunits have still to be elucidated, some preliminary results seemed to show that they are located around the fractions of the 19S proteasome with sucrose gradient experiments. Thus, many questions remain still open: Does

the 19S proteasome first dissociated from the 20S proteasome and then, fully disassembled during substrates degradation? It is transitory? Does the 20S also disassembled afterwards?

4.3.2 Role of p53 in the ubiquitination of 19S subunits and on the proteasome disassembly

The role of p53 was also investigated concerning the ubiquitination of 19S subunits and thus, the proteasome disassembly. The presence of p53 increased clearly the ubiquitination of S2, subunit of the 19S proteasome.

An hypothesis could be that high amount of p53 might enhance the recruitment of full-assembled proteasome to completely degrade the substrate. The proteasome can then simply dissociate when its activity is not required anymore.

A second possibility could be that the assembly/disassembly of the proteasome might be transient during substrates degradation. These observations can explain the high level of 19S ubiquitinated proteins. This principle is further confirmed by the major concentration of proteasomal proteins in subcomplex forms in cells. Some authors previously suggested that degradation of polyubiquitinated proteins is coupled to the dissociation of the 26S proteasome. As example, subunits of the 19S proteasome or perhaps even subcomplexes from yeast proteasome could be released during the substrate degradation (Babbitt et al., 2005). In contrary others demonstrated that mammalian 26S proteasome remains intact during protein degradation, but those findings were only demonstrated for few examples *in vitro* (Kriegenburg et al., 2008). Therefore, it is interesting to validate that this principle could be extended to all substrates and that some co-factors are not missing in those experiments such as ubiquitin, E2, E3...

Finally, the last hypothesis could be that the assembly/disassembly of the proteasome is dependent of the nature and the rate of substrate degradation.

4.4 Model of the proteasome regulation by Mdm2 and conclusion

Results obtained during my PhD underline a new and interesting aspect of the regulation of the proteasome by the E3 ligase Mdm2 and its subsequent substrate p53. This mechanism

leads to a model about the dual role of Mdm2 on the assembly and the disassembly of the 26S proteasome (Fig. 26).

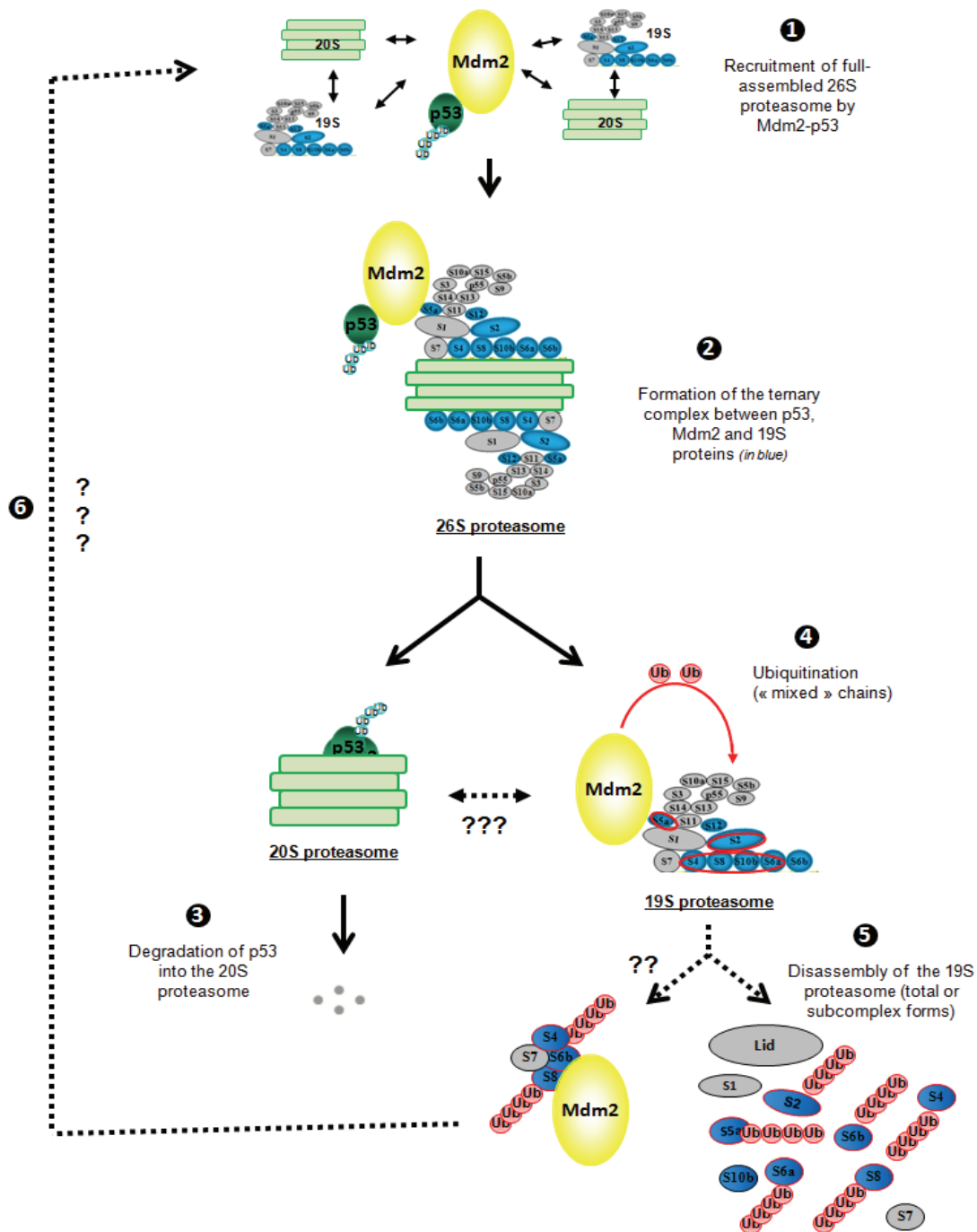


Figure 27: Model of the proteasome regulation by an E3 ligase Mdm2. Recruitment of full-assembled 26S proteasome by Mdm2-p53 (step 1), followed by the formation of a ternary complex (step 2) and the degradation of p53 (step 3). In parallel, 19S subunits are ubiquitinated by Mdm2 (step 4) which induced a total or incomplete dissociation of the 19S proteasome (step 5). This mechanism could be transient and native 26S proteasome could be formed *de novo* (step 6).

First, Mdm2 recruits native proteasome (step 1), phenomenon which is linked to the formation of a ternary complex (step 2) between Mdm2, p53 and some 19S subunits that leads to the degradation p53 (step 3). The recruitment of proteasomal subunits seemed to be further enhanced by the presence of the substrate p53. During the substrate degradation into the 20S proteasome, a dissociation of the 19S proteasome could occur (step 5), due to ubiquitination of some subunits (S2, S4, S5a, S6a, S6b and S8) by Mdm2 (step 4). The recruitment of full-assembled 26S proteasome could be transient and as works from Babbitt and co-workers suggest the full 26S proteasome could assembled *de novo* (step 6) (Babbitt et al., 2005).

Another interesting point to know is if this preliminary model of the proteasome regulation by Mdm2 could be extent to other E3 ligases. In this study, results already show a similar regulation of the proteasome for the two E3s siah1 and c-Cbl, including an impact on the recruitment of full-assembled 26S proteasome and the ubiquitination of some 19S subunits.

Therefore, this mechanism might represent a general way for the regulation of the 26S proteasome; it could be useful to design new drugs to modulate this pathway, because certain tumor cells are more sensitive to proteasome inhibitors than normal cells (Dick et al., 2010). Thus, the disruption of the interaction between proteasomal subunits and E3 ligases or the blocking of the dissociation of the proteasome might constitute a novel approach to regulate the degradation of specific factors.

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Publications

Review

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KIT PhD Symposium, 30th of September 2010.

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