

Identification and Functional Characterization of the Docking Sites of FAT10 and NUB1L at the 26S Proteasome

Dissertation

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*Dedicated to my family, whose unwavering support and
encouragement make every endeavor more fulfilling*

"The aim of science is to seek the simplest explanation of complex facts. We are apt to fall into the error of thinking that the facts are simple because simplicity is the goal of our quest. The guiding motto in the life of every natural philosopher should be "Seek simplicity and distrust it."

---- Alfred North Whitehead (1861-1947)

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Summary

FAT10 is a 18 kDa protein with two ubiquitin-like domains encoded in the major histocompatibility complex class I region close to the *HLA-F* gene and hence, the assignment of its name as, HLA-F locus adjacent transcript 10. It is the only known ubiquitin-like modifier that targets its substrates for proteasomal degradation independently of ubiquitin. It bears a diglycine motif at the C-terminus that is required for the covalent conjugation with the target proteins destined for destruction. The degradation of FAT10 and its conjugates is further accelerated by its non-covalent interaction with an UBL-UBA domain protein NEDD8 ultimate buster-1 long (NUB1L). This process requires the attachment of NUB1L to the proteasome through its UBL domain although its interaction with FAT10 is not essential. Several studies provide hints about the significance of FAT10 in immune responses, including the synergistic induction of FAT10 in the presence of the proinflammatory cytokines (TNF- α and IFN- γ), its up-regulation in several types of cancers, the hypersensitivity of the FAT10 knock-out mouse to lipopolysaccharide, high expression of FAT10 in the lymphoid organs such as thymus, lymph nodes and spleen, and its up-regulation upon maturation of dendritic cells.

The primary goal of this thesis was to attain a deep knowledge on the mechanism of the degradation of FAT10 and its conjugates by the 26S proteasome. The first step was the identification of the subunit(s) of the proteasome with which FAT10 and NUB1L can interact. Yeast two hybrid and GST-pull down assays revealed a direct interaction of the hRpn10 subunit with FAT10. Unexpectedly, the N-terminal von Willebrand A (VWA) domain of hRpn10 bound to both FAT10 and NUB1L in contrast to ubiquitin, which binds ubiquitin interacting motifs (UIM1 and UIM2) in hRpn10. This finding raised the following questions: why and how NUB1L accelerates the degradation of FAT10, which are dealt with to some extent in this thesis. In addition, NUB1L can interact with Rpn1 subunit, apart from the known hRpn10 subunit. To understand the mechanism of degradation, a heterologous yeast system was employed because of the fact that yeast, but not mammalian cells, can survive, and show only minimal sensitivity to canavanine, in the absence of Rpn10. The VWA domain of hRpn10 can functionally reconstitute the *rpn10 Δ* strain of yeast, as demonstrated by the degradation of FAT10. Moreover, it could

be shown that this degradation is indeed dependent on the proteasome and not on the free cytosolic hRpn10. The importance of the VWA domain in the degradation of FAT10 was further highlighted by identifying a single amino acid residue, Asp11, which was critical for the degradation of FAT10. Interestingly, NUB1L is incapable of accelerating the degradation of FAT10 in the absence of hRpn10, which revealed an essential role of hRpn10 in the regulation of this pathway. This led to the identification of the VWA domain as a novel interaction site for the ubiquitin-like proteins on the proteasome that assists in the degradation of FAT10.

Furthermore, by obtaining a three-dimensional structure of hRpn10 computationally, we tried to identify the docking site of FAT10 and NUB1L on the VWA domain of hRpn10. With the help of the three-dimensional structure of NUB1L generated by computational methods, a new putative UBA domain has been identified that was left unrecognized in earlier studies. The multiple sequence alignment of all the UBA domains of NUB1L and the UBA domains of a ubiquitin receptor, hHR23A, supported this finding. Furthermore, the VWA domain and the full-length hRpn10 proteins were purified for the future X-ray crystallography studies to understand more precisely the importance of the VWA domain in the context of protein degradation and prove our findings.

Unlike ubiquitin, FAT10 is not a very stable protein and is degraded along with the covalently bound substrates. The biological relevance of FAT10, in the context of its yet unknown substrates, still remains enigmatic although its function has been several times related to apoptosis and cell cycle regulation. FAT10 mRNA is highly expressed in the lymphatic organs like thymus, spleen and lymph nodes but barely expressed in the brain. It is also known that FAT10 localizes to aggresomes under proteasome inhibition and this process is mediated by its interaction with HDAC6, a dynein motor complex associated protein. This finding encouraged us to determine the expression and significance of FAT10 in neurodegenerative diseases, which are characterized by the presence of aggregates in the brain. In this thesis, the up-regulation of FAT10 mRNA and protein is determined in the brain, after infection with LCMV and also in certain mouse models of neurodegenerative diseases, like Parkinson's disease, Huntington's disease and Alzheimer's disease. Immunohistochemical stainings revealed the accumulation of FAT10 in aggregates in such diseases, which could suggest a protective role of FAT10

analogous to the function of ubiquitin in neurodegenerative diseases. Furthermore, to support this idea, monoclonal and polyclonal antibodies specific for mouse FAT10 were generated to study the FAT10 expression in the mouse models of different diseases by employing different proteomics approaches.

In addition, this thesis deals with the FAT10-conjugation pathway, which is analogous to the ubiquitin conjugation pathway represented by a cascade of three enzymes: activating enzyme (E1), conjugating enzyme (E2) and ligation enzyme (E3). A novel E2 enzyme USE1 was identified, which accepts FAT10 as well as ubiquitin as substrates and is specific for the UBA6 E1 enzyme. UBA6-activated FAT10 can be transferred to USE1 *in vitro* and *in vivo*. Moreover, catalytically inactive USE1 was unable to interact with FAT10, which supports the characterization of USE1 as an E2 enzyme for FAT10. The siRNA mediated knock-down of USE1 down-regulated the conjugates of FAT10. Moreover, USE1 was identified as the first substrate of FAT10 as it can be auto-FAT10ylated *in cis* by an isopeptide bond. This provided a regulatory mechanism for the FAT10-conjugation pathway.

In conclusion, this thesis provides detailed insights into the “FAT10-proteasome” and the “FAT10-conjugation” pathways. It could be shown that the expression of FAT10 is differentially regulated at several steps by hRpn10 and NUB1L. The VWA domain of hRpn10 is identified as a novel interaction site for ubiquitin-like modifiers and defined as a domain which can support the degradation of FAT10. This marks a major difference in the regulation of proteins controlled by the ubiquitin-proteasome and the “FAT10-proteasome” pathway. The conjugation studies provide further support to identify the covalently-modified substrates of FAT10, and the physiological role of FAT10. Moreover, the unexpected finding of the up-regulation and accumulation of FAT10 in aggregates in the brain of mouse models of neurodegenerative diseases would be beneficial in understanding these diseases by identifying the role of FAT10 and providing a significant link between FAT10 and the immune system.

Zusammenfassung

FAT10 ist ein 18 kDa großes Protein, das aus zwei Ubiquitin-ähnlichen Domänen aufgebaut ist. Es ist in der Haupthistokompatibilitätskomplex Klasse I-Region in der Nähe des HLA-F-Gens kodiert, woraus sich sein Name HLA-F Locus angrenzendes Transkript 10 ergibt. Es ist das einzig bekannte Ubiquitin-ähnliche Protein, das seine Substrate unabhängig von Ubiquitin für den proteasomalen Abbau markieren kann. FAT10 trägt ein di-Glycin Motiv am C-Terminus, welches für die kovalente Konjugation mit zum Abbau markierten Ziel-Proteinen benötigt wird. Der Abbau von FAT10 und seiner Konjugate wird zudem durch eine nicht-kovalente Wechselwirkung mit dem UBL-UBA-Domänen-Protein „NEDD8 ultimate buster-1 long“ (NUB1L) unterstützt. Dieser Prozess erfordert die Interaktion von NUB1L mit dem Proteasom via seiner UBL Domäne, wobei eine Wechselwirkung mit FAT10 dabei nicht essentiell ist. Eine Vielzahl an Studien geben Hinweise zu einer besonderen Bedeutung von FAT10 bei Immunantworten. Hierzu gehört eine synergistische Induktion von FAT10 in Anwesenheit der proinflammatorischen Zytokine TNF- α und IFN- γ , eine Hochregulation von FAT10 bei verschiedenen Arten von Krebs, die Überempfindlichkeit der FAT10 Knock-out-Maus bei einer Behandlung mit Lipopolysaccharid (LPS), eine hohe Expression von FAT10 in den lymphatischen Organen wie Thymus, Lymphknoten und Milz, sowie seine Hochregulation bei der Reifung von dendritischen Zellen.

Das primäre Ziel dieser Doktorarbeit war es, ein fundiertes Wissen über den Mechanismus des Abbaus von FAT10 und seiner Konjugate durch das 26S Proteasom zu erarbeiten. Der erste Schritt hierfür war die Identifizierung der Untereinheiten des Proteasoms mit denen FAT10 und NUB1L interagieren können. In einem „Yeast two Hybrid“ Ansatz und einem GST-Pulldown Experiment konnte eine direkte Interaktion der hRpn10 Untereinheit des Proteasoms mit FAT10 gezeigt werden. Interessant war dabei, dass die N-terminale von Willebrand A (VWA) Domäne von hRpn10 für die Bindung sowohl von FAT10 also auch von NUB1L nötig war. Im Gegensatz dazu bindet Ubiquitin hRpn10 über ein Ubiquitin interagierendes Motiv (UIM1 und UIM2). Dieser Befund hat daraufhin die folgende Frage aufgeworfen, deren Beantwortung Teil dieser Arbeit war: Warum und wie beschleunigt NUB1L den Abbau von FAT10? Darüber hinaus konnte

gezeigt werden, dass NUB1L auch mit Rpn1, einer weiteren Untereinheit des Proteasoms, interagieren kann. Um den Mechanismus des Abbaus besser zu verstehen, wurde ein heterologes Hefe-System verwendet. Dies hat den Vorteil, dass Hefezellen im Gegensatz zu Säugerzellen in Abwesenheit von Rpn10 überleben können und nur eingeschränkte Empfindlichkeit gegenüber Canavanin zeigen. Die VWA Domäne von hRpn10 konnte funktionell den *rpn10Δ* Hefestamm rekonstituieren, wie durch den Abbau von FAT10 gezeigt werden konnte. Darüber hinaus zeigten die Experimente, dass dieser Abbau in der Tat vom Proteasom abhängig ist und nicht auf eine Interaktion mit freiem, zytosolischen hRpn10 zurückzuführen ist. Die Bedeutung der VWA-Domäne für den Abbau von FAT10 wurde weiter durch die Identifizierung einer einzigen Aminosäure (Asp11) hervorgehoben, die essentiell für den Abbau von FAT10 ist. Interessanterweise ist NUB1L in Abwesenheit von hRpn10 unfähig den Abbau von FAT10 zu beschleunigen, was die Bedeutung von hRpn10 in der Regulation von FAT10 verdeutlicht. Dies führte zur Identifikation der VWA-Domäne als neuartige Interaktionsstelle für Ubiquitin-ähnliche Proteine auf dem Proteasom, die den Abbau von FAT10 unterstützt.

Darüber hinaus wurde in dieser Arbeit versucht, durch eine rechnerisch ermittelte dreidimensionale Struktur von hRpn10, die Interaktionsstelle von FAT10 und NUB1L mit der VWA Domäne von hRpn10 zu identifizieren. Mit Hilfe dieser dreidimensionalen Struktur von NUB1L konnte eine vermeintlich neue UBA-Domäne identifiziert werden, die in früheren Studien bisher unentdeckt blieb. Ein Sequenz-Vergleich aller UBA-Domänen von NUB1L und den UBA-Domänen eines Ubiquitin-Rezeptors, hHR23A, haben diese Feststellung bestätigt. Darüber hinaus wurden die VWA-Domäne und das gesamte hRpn10 Protein aufgereinigt, um in Zukunft durch Röntgenkristallographie untersucht zu werden. Hierdurch könnte die Bedeutung der VWA-Domäne im Kontext des Proteinabbaus bewiesen werden und unsere hier vorgestellten Ergebnisse bestätigen.

Im Gegensatz zu Ubiquitin wird FAT10 zusammen mit den kovalent gebundenen Zielproteinen abgebaut und somit nicht wiederverwertet. Die biologische Relevanz von FAT10 ist nach wie vor unklar, obwohl seine Funktion mehrmals im Zusammenhang mit Apoptose und Zellzyklus-Regulation gesetzt wurde. FAT10 mRNA ist in lymphatischen Organen wie Thymus, Milz und Lymphknoten stark exprimiert, jedoch im Gehirn kaum vorhanden. Es ist auch bekannt, dass FAT10 nach Proteasom-Hemmung mit Aggresomen

assoziiert, und dass dieser Prozess durch seine Wechselwirkung mit HDAC6, einem Dynein-Motor-Komplex assoziierten Protein, vermittelt wird. Dieser Befund hat uns ermutigt, die Expression und Bedeutung von FAT10 in neurodegenerativen Erkrankungen zu bestimmen, die durch die Anwesenheit von Aggregaten im Gehirn charakterisiert sind. In dieser Arbeit wurde die Hochregulation von FAT10 mRNA und Protein im Gehirn nach der Infektion mit LCMV und auch in bestimmten Mausmodellen neurodegenerativer Erkrankungen wie Morbus Parkinson, Morbus Huntington und Morbus Alzheimer bestimmt. Immunhistochemische Färbungen zeigten eine Anhäufung von FAT10 in Aggregaten solcher Krankheiten, die auf eine schützende Rolle von FAT10 analog zu der Funktion von Ubiquitin in neurodegenerativen Erkrankungen hindeuten könnten. Darüber hinaus, um diese Idee zu unterstützen, wurden spezifische monoklonale und polyklonale anti-Maus FAT10 Antikörper erzeugt, mit denen die Expression von FAT10 in verschiedenen Maus-Modellen getestet werden sollte.

Des Weiteren beschäftigt sich diese Arbeit mit dem FAT10-Konjugationsmechanismus, der analog zum Ubiquitin aus einer Kaskade von drei Enzymen besteht: das aktivierende Enzym (E1), das konjugierende Enzym (E2) und das ligierende Enzym (E3). Es wurde ein neuartiges E2-Enzym mit dem Namen USE1 identifiziert, welches beide, FAT10 und Ubiquitin, als Substrate verwendet und spezifisch für das E1-Enzym UBA6 ist. FAT10, was von UBA6 aktiviert wird, kann *in vitro* und *in vivo* auf USE1 übertragen werden. Darüber hinaus konnte katalytisch inaktives USE1 nicht mehr mit FAT10 interagieren, was die Charakterisierung von USE1 als E2-Enzym für FAT10 bestätigte. Eine USE1-spezifische siRNA Behandlung verringerte die Anwesenheit von FAT10 Konjugaten. Darüber hinaus wurde USE1 als das erste Substrat von FAT10 identifiziert, welches in cis-Stellung mit einer Isopeptidbindung auto-FAT10yliert wird. Dies zeigt einen möglichen Regulationsmechanismus für den FAT10-Konjugationsmechanismus auf.

Zusammenfassend bietet diese Doktorarbeit detaillierte Einblicke in die FAT10-Proteasom Interaktion und den FAT10-Konjugationsmechanismus. Es konnte gezeigt werden, dass die Expression von FAT10 an mehreren Stellen durch hRpn10 und NUB1L geregelt werden kann. Die VWA Domäne von hRpn10 ist als neuartige Interaktionsstelle für Ubiquitin-ähnliche Proteine identifiziert worden und ist definiert als eine Domäne, die den Abbau von FAT10 unterstützen kann. Dies ist ein wesentlicher Unterschied zur

Regulation von Proteinen durch das Ubiquitin-Proteasom-System. Die Konjugationsstudien lieferten weitere Möglichkeiten die kovalent modifizierten Substrate von FAT10 zu identifizieren, und die physiologische Rolle von FAT10 aufzuklären. Darüber hinaus könnte die unerwartete Entdeckung einer Akkumulation von FAT10 in Aggregaten des Gehirns von Mäusen mit neurodegenerativen Erkrankungen für das Verständnis dieser Krankheiten nützlich sein und einen signifikanten Zusammenhang zwischen FAT10 und dem Immunsystem aufzeigen.

Table of Contents

Acknowledgements	iv
Summary.....	vii
Zusammenfassung	x
Table of Contents	xiv
1. General Introduction	1
1.1 The 26S Proteasome	2
1.1.1 The Protease (20S Proteasome).....	4
1.1.2 The Regulators.....	6
1.1.2.1 The 11S Proteasome Activator (PA28)	6
1.1.2.2 The 19S Proteasome Activator (PA700)	7
1.2 Ubiquitin	9
1.3 Ubiquitin Receptors	14
1.3.1 Rpn10 subunit (S5a, Mbp1, Sun1, Mcb1, Pus1)	14
1.3.2 Rad23 (hHR23).....	18
1.4 Ubiquitin-like proteins	19
1.4.1 Ubiquitin-like modifiers (UBLs).....	19
1.4.1.1 FAT10 (HLA-F locus adjacent transcript 10).....	21
1.4.1.2 ISG15 (Interferon stimulated gene-15).....	24
1.4.1.3 SUMO (Small Ubiquitin-like Modifier)	25
1.4.1.4 NEDD8/RUB1 (Neural precursor cell expressed developmentally down-regulated protein 8/Related to ubiquitin-1).....	26
1.4.2 Ubiquitin domain proteins (UDPs).....	27
1.4.2.1 NUB1 (NEDD8 Ultimate Buster-1)	27
1.5 UPS and Neurodegeneration	29
1.5.1 Link between UPS, Autophagy and Neurodegeneration	29
1.5.2 Examples of Neurodegenerative Diseases	30
1.5.3 Neuroinflammation.....	32
1.6 Aim of the Thesis	33
1.7 Organization of the Thesis.....	33

2. RPN1 and the VWA domain of RPN10 bind the UBL-UBA protein NUB1L and the ubiquitin like modifier FAT10 for proteolytic targeting to the 26S proteasome.....	35
2.1 Introduction	36
2.2 Results	38
2.2.1 Identification of 19S RP subunits interacting with FAT10 and NUB1L	38
2.2.2 FAT10 directly interacts with hRpn10 and NUB1L binds both hRpn10 and hRpn1	39
2.2.3 The C-terminal UBL domain of FAT10 is sufficient to interact with hRpn10, <i>in vitro</i>	42
2.2.4 FAT10 and NUB1L interact with the VWA domain of hRpn10 <i>in vitro</i>	42
2.2.5 Reconstitution of Rpn10 deficient yeast with full length human Rpn10 or the VWA domain alone	43
2.2.6 FAT10 migrates in the high molecular weight fractions of glycerol gradients of wild type but not <i>rpn10Δ</i> yeast	44
2.2.7 The VWA domain of hRpn10 is required for the degradation of FAT10, <i>in vivo</i>	45
2.2.8 NUB1L relies on Rpn10 for promoting the degradation of FAT10.....	48
2.2.9 Asp11 residue in the VWA domain is crucial for the degradation of FAT10	48
2.2.10 hRpn10 VWA domain mediated degradation of FAT10 is proteasome dependent.....	49
2.3 Discussion.....	51
2.4 Methods.....	56
2.4.1 Strains and Plasmids.....	56
2.4.2 Yeast Two Hybrid Analysis	56
2.4.3 GST-Pull Down Assay	56
2.4.4 Transfection of HEK 293T Cells and Co-immunoprecipitation	56
2.4.5 Yeast Strain Construction.....	57
2.4.6 Canavanine Sensitivity Assay	57
2.4.7 Glycerol Gradient Centrifugation.....	58
2.4.8 Cycloheximide Chase	58
2.4.9 Site directed mutagenesis	58
2.4.10 Proteasome Inhibition.....	59
2.5 Acknowledgements.....	59
2.6 Supplementary Information.....	60
2.6.1 Supplementary Figures	60
2.6.2 Supplementary Methods	64
2.6.2.1 Yeast two hybrid analysis	64
2.6.2.2 Multiple protein sequence alignment.....	65

3. Studies on the Structure of the hRpn10 Subunit of the 26S Proteasome and the UBL-UBA Domain Protein NUB1L	68
3.1 Introduction	69
3.2 Results	73
3.2.1 Predicted secondary and tertiary structure of the hRpn10 subunit.....	73
3.2.2 Site-directed mutagenesis in the VWA domain of hRpn10 to study its interaction with FAT10 and NUB1L	74
3.2.3 Characterization of the VWA protein.....	78
3.2.4 CD spectra of the purified VWA protein	78
3.2.5 Thermofluor	81
3.2.6 Purification of the full-length hRpn10	81
3.2.7 Predicted secondary and tertiary structure of NUB1L	82
3.3 Discussion.....	84
3.4 Methods.....	89
3.4.1 Site-directed mutagenesis.....	89
3.4.2 GST-pull down assay	89
3.4.3 Purification of VWA protein	89
3.4.4 CD spectra analysis	90
3.4.5 Thermofluor procedure.....	90
3.4.6 Purification of hRpn10 protein.....	90
3.4.7 Sequence retrieval.....	90
3.4.8 Primary amino acid sequence alignment.....	90
3.4.9 Secondary structure prediction	91
3.4.10 Three-dimensional structure prediction.....	91
4. Investigating the Role of FAT10 in Neurodegenerative Diseases.....	92
4.1 Introduction	93
4.2 Results	95
4.2.1 Expression of FAT10 mRNA in different organs of C57BL/6 mouse after LCMV infection	95
4.2.2 Induction of mRNA expression level of FAT10 in the brain of C57BL/6 mouse after intracranial infection with LCMV	97
4.2.3 Induction of FAT10 mRNA in the brain of transgenic Huntington's disease mouse model...	97
4.2.4 Induction of FAT10 mRNA in the brain of alpha-synuclein transgenic mouse	99
4.2.5 FAT10 protein is the component of aggregates in the brain of transgenic Huntington's disease mouse	101
4.2.6 FAT10-positive aggregates detected in the human HD and PD transgenic mouse brain	102
4.2.7 FAT10 induction is not dependent on misfolded protein stress conditions.....	105

4.3	Discussion.....	105
4.4	Methods.....	109
4.4.1	Mice.....	109
4.4.2	Virus infection.....	109
4.4.3	RNA preparation and real-time RT-PCR.....	109
4.4.4	PolyGln aggregates dissociation from Htt transgenic mice.....	110
4.4.5	Proteasome inhibition, canavanine, puromycin and thapsigargin treatment.....	110
4.4.6	Western blot analysis.....	111
4.4.7	Immunohistochemistry.....	111
4.5	Acknowledgements.....	111
5.	USE1 is a Bispecific Conjugating Enzyme for Ubiquitin and FAT10 which FAT10ylates itself in <i>cis</i>.....	112
5.1	Introduction.....	113
5.2	Results.....	115
5.2.1	Identification USE1 as an interaction partner of FAT10.....	115
5.2.2	USE1 serves as a conjugating enzyme for FAT10 <i>in vitro</i> and <i>in vivo</i>	117
5.2.3	Formation of a USE1-FAT10 conjugate in cytokine stimulated cells.....	118
5.2.4	Silencing of USE1 leads to a reduction of FAT10 conjugates.....	122
5.2.5	USE1 auto-FAT10ylates itself in <i>cis</i> but not in <i>trans</i>	127
5.3	Discussion.....	130
5.4	Methods.....	134
5.4.1	Primers and constructs.....	134
5.4.2	Yeast two hybrid Screen.....	136
5.4.3	Immunoprecipitation.....	137
5.4.4	<i>in vitro</i> conjugation assay.....	137
5.4.5	USE1 knockdown and real-time PCR.....	138
5.4.6	Expression and purification of proteins.....	138
5.4.7	Generation of polyclonal USE1 and UBA6 antibodies.....	139
5.4.8	Generation of a monoclonal FAT10-reactive antibody.....	139
5.4.9	Immunofluorescence and confocal microscopy.....	140
5.5	Acknowledgements.....	140
5.6	Notes.....	140
6.	Generation of Monoclonal and Polyclonal Antibodies Specific for Mouse FAT10	142

6.1	Introduction	143
6.2	Results and Discussion	143
6.2.1	Monoclonal and polyclonal antibodies recognizes human as well as mouse FAT10, irrespective of the tag	144
6.2.2	Monoclonal and polyclonal antibodies detect human as well as mouse FAT10 in transiently transfected HEK293T cells	146
6.2.3	Monoclonal and polyclonal antibodies display cross-reactivity with ubiquitin-like modifier, ISG15	146
6.2.4	The isotype of the FAT10-reactive monoclonal antibody is IgG2a with kappa light chain..	149
6.2.5	Monoclonal as well as polyclonal antibodies recognizes FAT10 in the thymus of the mouse	149
6.3	Materials and Methods	151
6.3.1	Expression constructs	151
6.3.2	Purification of human and mouse recombinant FAT10 protein.....	151
6.3.3	Generation of monoclonal antibody in the mouse.....	152
6.3.4	Generation of polyclonal antibody in rabbits	152
6.3.5	ELISA	152
6.3.6	Tissue culture and transfection	153
6.3.7	Preparation of lysates and immunoprecipitation	153
6.3.8	Multiple protein sequence alignment	153
6.4	Acknowledgements.....	153
7.	Discussion.....	154
	Appendix.....	164
	References.....	167
	Abbreviations	190
	Record of Contributions.....	194

1. General Introduction

1.1 THE 26S PROTEASOME

Proteolysis is an essential physiological process involved in achieving protein homeostasis in all eukaryotes. It is highly controlled and plays a critical role in various biological processes, including cell development, DNA repair, apoptosis, signal transduction, metabolism, transcription, protein quality control and regulation of immune responses. The half-life of an individual protein may range from minutes to years. A basic question concerned with the protein degradation is how cells should decide which protein has to be degraded? The degradation signal, or “degron” is defined as the minimal amino acid sequence required for the recognition and degradation of a protein by the proteolytic machinery (Varshavsky, 1991). Proteins can be degraded by two major pathways in the body. The first is lysosomal degradation, which accounts for approximately 20% of normal protein turnover. Lysosomal proteases are nonspecific and can degrade all proteins at the same rate. The specific inhibitors of lysosomal proteases have minimal effect on the proteolysis of intracellular proteins and hence, it is considered that extracellular proteins are degraded by this pathway. These observations prompted researchers to look for a more selective system that targets cellular proteins. This led to the discovery of another pathway called the ubiquitin-proteasome system (Reinstein and Ciechanover, 2006).

The 26S proteasome executes the degradation of intracellular proteins in a highly selective energy dependent manner. The 26S proteasome is a highly conserved large 2.5 MDa protein complex which consists of a 20S core particle (CP) and a 19S regulatory particle(s) (RP) (Gallastegui and Groll, 2010; Voges et al., 1999) as shown in Figure 1.1. The selection of proteins to enter the proteasome is “usually” mediated by the covalent modification of target proteins by ubiquitin. Exceptions to this mode of selection include proteins like ornithine decarboxylase (ODC) and thymidylate synthase (Forsthoefel et al., 2004; Hoyt and Coffino, 2004). The processing of proteins by the proteasome involves several steps including recognition, unfolding, translocation and deubiquitylation of the substrate. The peptides generated from the cytosolic pool of proteins by the 26S proteasomes are transported into the endoplasmic reticulum and are presented on MHC class I molecules where they are displayed to cytotoxic T lymphocytes (Coux et al., 1996; Finley, 2009). The majority of these peptides are derived from proteins encoded by the cell but exogenous proteins (introduced artificially or by bacterial or viral invasion) can

also contribute to class I binding peptides. The major source of antigenic peptides is the proteasomal degradation of defective products of protein synthesis, or DRiPs (defective ribosomal products). DRiPs are polypeptides that result due to the error in translational or post-translational processes, which are necessary for the proper protein folding. The delayed degradation of DRiPs can contribute to several diseases (Schubert et al., 2000; Yewdell et al., 1996).

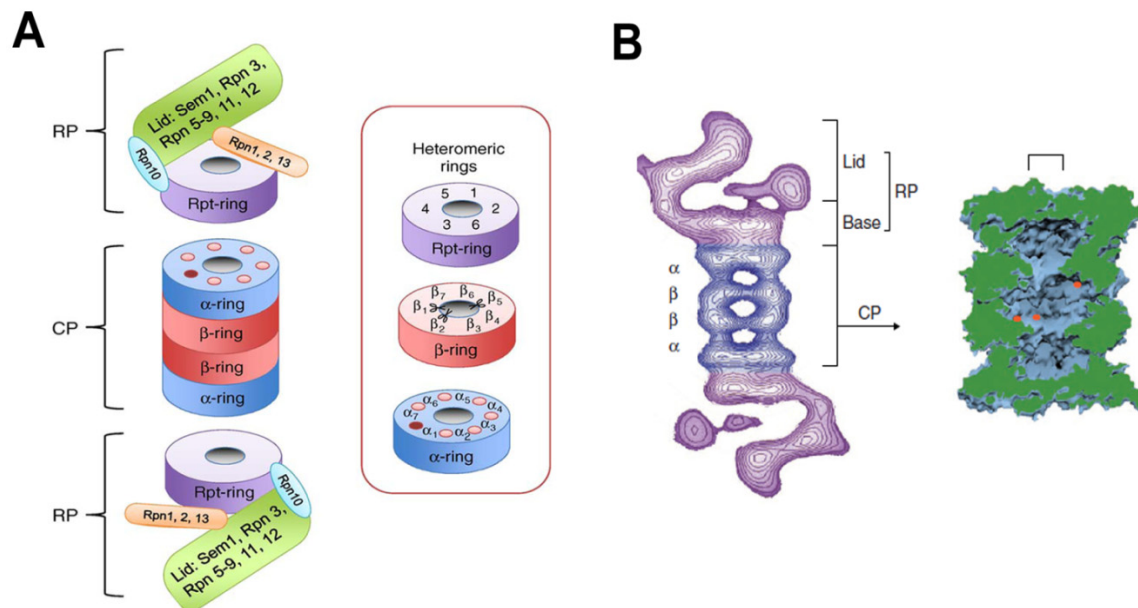


Figure 1.1 The 26S proteasome. The proteasome is formed by two 19S RPs on both sides of a cylindrical 20S CP. The CP is formed by two α -rings and two β -rings. The RP comprises of a base and a lid. Rpn10 is at the interface between these complexes (shown in light blue). The lid (green) consists of nine subunits as indicated and its function is not well understood, with the exception of Rpn11, which functions as a deubiquitylating enzyme. The base comprises of a ring formed by six ATPases, Rpt1–6 (purple ring), and the non-ATPase subunits Rpn1, Rpn2 and Rpn13 (orange box). In all, three heteromeric rings are present (twice each) in the proteasome (shown on the right side). The α - and β -rings in the 20S CP are formed of seven subunits; scissors indicate the catalytically active sites in β 1, β 2 and β 5 subunits and the dots in the α -ring represent the binding pockets for the Rpt tails. The pocket between α 7 and α 1 lacks a Lys typically found in the pocket and might not harbor an Rpt C-terminus (shown as dark red dot). (Modified from Bedford et al., 2010). (B) Left: Electron micrograph of a eukaryotic proteasome, with its major subassemblies (the CP, RP, lid, and base) indicated. Right: Medial cut-away view of the *S. cerevisiae* CP in the closed state. Green color represents slice surface. Proteolytic sites are marked with red dots. A bracket marks the site of the channel when the CP is open (Modified from Finley, 2002).

1.1.1 The Protease (20S Proteasome)

The 20S proteasome (also called core particle) was first discovered in 1968 in electron micrographs of human erythrocytes lysates. Initially it was presumed that 20S proteasome is the characteristic of eukaryotes but later, this was identified in the archaebacterium, *Thermoplasma acidophilum*, and the bacterium, *Rhodococcus erythropolis*. It is a barrel-shaped protein complex consisting of 28 subunits, arranged into four heptameric rings – two outer α -rings and two inner β -rings. β -rings constitute the proteolytic chamber and α -rings function as a gate for the entry of proteins into the chamber. β -rings are flanked on top and bottom by α -rings and both types of rings are composed of 7 distinct subunits $[(\alpha1-\alpha7)(\beta1-\beta7)(\beta1-\beta7)(\alpha1-\alpha7)]$. Of these 14 subunits, $\beta1$ /Pre3, $\beta2$ /Pup1 and $\beta5$ /Pre2 are the proteolytic subunits having caspase-like activity (cleaves after acidic residues), trypsin-like activity (cleaves after basic residues) and chymotrypsin-like activity (cleaves after hydrophobic residues), respectively. This complex is known as **constitutive proteasome** (Voges et al., 1999) (Figure 1.2). The 20S proteasome can be classified into two more different types based on the difference in the incorporation of β -subunits:

Immunoproteasome: Three additional β -subunits were identified later, designated as $\beta1i$ (also known as LMP2), $\beta2i$ (also known as MECL1) and $\beta5i$ (also known as LMP7) (Figure 1.2). These subunits are encoded by genes in the MHC class II region in the higher vertebrates and are inducible by proinflammatory cytokines, IFN- γ and TNF- α . These subunits replace the corresponding “constitutive subunits” after stimulation with cytokines. These immunoproteasomes are more efficient in producing antigenic peptides than constitutive proteasomes and hence, play an important role in shaping the cytotoxic T lymphocytes (CTL) responses at the level of antigen presentation and pathogen clearance (Groettrup et al., 2010).

Thymoproteasome: A gene product homologous to the $\beta5$ and $\beta5i$ subunits was identified specific for thymus and this was termed $\beta5t$ (Figure 1.2). ~20% of the 20S proteasomes in the thymus contain $\beta5t$. It is specifically expressed in cortical thymic epithelial cells (cTECs), which catalyze positive selection of developing immature thymocytes. Thymoproteasomes exhibit weak chymotrypsin-like activity but comparable trypsin- and caspase-like activity and they are suggested to be responsible for the positive selection of CD8⁺ T cells in the thymus (Groettrup et al., 2010; Murata et al., 2007).

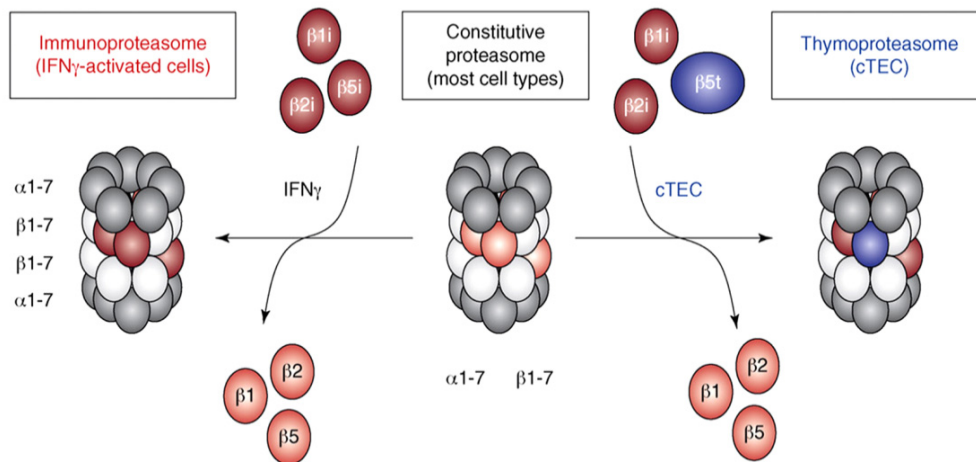


Figure 1.2 Constitutive, immuno- and thymo-proteasomes. (A) Constitutive proteasome expressed by the majority of cells in the body is shown in the middle. In vertebrates, three subunits, $\beta 1i$, $\beta 2i$, and $\beta 5i$, are induced by $IFN\gamma$ and preferentially incorporated into proteasomes, generating immunoproteasomes (left). A newly identified catalytic subunit of 20S proteasomes, $\beta 5t$, is incorporated substituting $\beta 5$ or $\beta 5i$, and together with $\beta 1i$ and $\beta 2i$ forms the so-called thymoproteasome, which is specifically expressed in cortical epithelial cells (right). (Modified from Takahama et al., 2008).

The α - and β -type subunits share similar folds consisting of two antiparallel five-stranded β -sheets flanked by two helices on one side and three helices on the other side. They are properly assembled with the help of proteasome-assembling chaperones 1-4 (PAC) and maturation factors like ubiquitin maturation protein 1 (UMP1) (Murata et al., 2009). Proteins entering the 20S proteasome are degraded in a sequential manner. It traps proteins until they are degraded to peptides of a certain length which can range from 4-25 residues. The catalytic sites in the 20S proteasome are mature and therefore, to protect the cell from unregulated protein degradation, the N-termini of the α -subunits form a gate that can be opened in a regulated manner by the interaction with regulatory particles. The peptide bonds of the substrate are hydrolyzed by the N-terminal threonine residue, Thr1, which is buried in the β -subunits (Gallastegui and Groll, 2010; Voges et al., 1999). There is a controversy about the fate of the 26S proteasome after the degradation of proteins. One report proposed that the 19S RP disassembles from the 20S CP after protein degradation (Babbitt et al., 2005) whereas another report stated that the proteasome remains intact (Kriegenburg et al., 2008).

1.1.2 The Regulators

The protein degradation by 26S proteasome is strictly under the control of regulators which acts like “gatekeepers” for the entry of proteins into the 20S proteasome. The activation of the 20S proteasome is controlled by the regulators and hence, also referred to as proteasome activators (Figure 1.3).

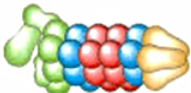
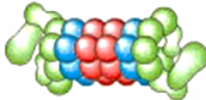
Type of Complexes	Model of Proteasome, PA700 PA28 and Their Complexes		Relative Content (%)
20S Proteasome	A		31 ± 4
PA700	B		10 ± 1
PA28	C		15 ± 10
PA28-20S -PA28	D		15 ± 7
PA700-20S -PA28	E		18 ± 7
PA700-20S -PA700	F		11 ± 2
Total			100

Figure 1.3 The relative distributions of different complexes of proteasomes in HeLa cells. The relative content of various types of proteasome complexes in HeLa cell cytosol were calculated as described by Tanahashi, N. et al., 2000, based on a combination analyses by immuno-precipitation and western blotting. (Modified from Tanahashi et al., 2000).

1.1.2.1 The 11S Proteasome Activator (PA28)

This is a heteroheptameric complex of 28 kDa subunits which binds to the cylinder of the CP, thus opening the CP channel. It consists of two different subunits, α - and β -subunits. PA28 α/β either exists as a free oligomer or can bind to one or both ends of the 20S CP.

PA28 lacks ATPase activity and the ability to bind ubiquitin conjugates. It lacks the ability to unfold complex substrates and can only enhance hydrolysis of small peptides. It is encoded within the MHC locus, inducible by IFN- γ , and is required for efficient cell surface presentation of some peptide antigens on MHC class I molecules. It has been proposed, but not confirmed, that PA28 could facilitate the exit of peptides from chimeric PA700-CP-PA28 proteasomes providing an exit channel in *trans* from that being used by the PA700 for substrate entry. It is assumed that peptides entering or exiting the CP pass through the channel of PA28 which is aligned with the CP (Groettrup et al., 1995; Voges et al., 1999).

1.1.2.2 The 19S Proteasome Activator (PA700)

Two nomenclatures are used to designate the subunits of 19S proteasome, the mammalian by “S”, and the *S. cerevisiae* by “Rp”. The Rp nomenclature differentiates between Rp triphosphatases (Rpt) subunits and Rp non-ATPase (Rpn) subunits (Voges et al., 1999). The 19S RP has been subdivided into the lid and the base, which are distal and proximal to the CP, respectively. The **base** complex consists of six ATPases, Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6' and Rpt6/S8, and the two largest subunits, Rpn1/S2 and Rpn2/S1, as well as Rpn13/Adrm1 and Rpn10/S5a (assumed to occupy a position between base and the lid). The gate opening of the 20S proteasome results directly from engagement of the C-termini of Rpts of the 19S regulator with the α -pockets. A distinct sequence motif HbYX, encoding a hydrophobic residue, followed by a tyrosine and an unspecified C-terminal residue, is sufficient for gate opening (Finley, 2009). Other functions of ATPases include hydrolysis of ATP, which promotes the association of 19S regulator and 20S CP to form 26S proteasome, and unfolding of the substrate proteins.

The **lid** complex is comprised of nine subunits, Rpn3/S3, Rpn5/p55, Rpn6/S9, Rpn7/S10a, Rpn8/S12, Rpn9/S11, Rpn11/S13, Rpn12/S14 and Rpn15/S15. Rpn11 (known as Poh1 in humans) is the only subunit with a known function among the lid subunits. It acts like a deubiquitylating enzyme (DUB), which removes ubiquitin groups from substrates before they enter the 20S proteasome. The subunits of lid correspond to the subunits of the COP9 signalosome and the translation initiation factor eIF3 (Finley, 2009; Voges et al., 1999).

Table 1.1 Subunits of the regulatory particle of the proteasome (Modified from Finley, 2009).

Base-	Activity or domain type	Budding yeast	kDa (humans)	Human	Human (Rechsteiner)	Bovine (DeMartino)	Comments (other names)
	AAA ATPase	Rpt1	48.6	PSMC2	S7	p48	(Cim5)
	AAA ATPase	Rpt2	49.2	PSMC1	S4	p56	
	AAA ATPase	Rpt3	47.3	PSMC4	S6 (or S6b)	p47	
	AAA ATPase	Rpt4	44.1	PSMC6	S10b	p42	(Sug2)
	AAA ATPase	Rpt5	49.1	PSMC3	S6' (or S6a)	p50	
	AAA ATPase	Rpt6	45.7	PSMC5	S8	p45	(Sug1, Cim3)
	Scaffold	Rpn1	100.2	PSMD2	S2	p112	α -Helical toroid
	Scaffold	Rpn2	105.9	PSMD1	S1	p97	α -Helical toroid
	Ub receptor	Rpn10	40.7	PSMD4	S5a	p54	UIM domain
	Ub receptor; Uch37 receptor	Rpn13	42.1	ADRM1	—	—	Pru domain
Lid	PCI	Rpn3	61.0	PSMD3	S3	p58	
	PCI	Rpn5	52.9	PSMD12	—	p55	
	PCI	Rpn6	47.4	PSMD11	S9	p44.5	
	PCI	Rpn7	45.5	PSMD6	S10a	p44	
	MPN	Rpn8	37.1	PSMD7	S12	p40	(Mov34)
	PCI	Rpn9	42.9	PSMD4	S11	p40.5	
	MPN; deubiquitination	Rpn11	34.6	PSMD14	S13	—	(Pad1, Pohl)
	PCI	Rpn12	30.0	PSMD8	S14	p31	
	—	Rpn15	24.6	PSMD9	S15	p27L	(Sem1, Dss1)

1.2 UBIQUITIN

Ubiquitin was first called APF-1 (ATP-dependent proteolysis factor-1) but later, after its discovery as a ligatable protein by Hershko and colleagues, it was called “ubiquitin” by Wilkinson and co-workers. It was first identified in 1975 with an unknown function. Ubiquitin was first thought to be a thymic hormone, but later it was found to be present in many tissues and organisms, and hence its name (Ferrell et al., 1996; Hershko et al., 2000). Ubiquitin is a highly conserved 76-amino acid polypeptide encoded on multiple genes and expressed in all eukaryotes. In yeast, it is encoded by four genes: *Ubi1*, *Ubi2* and *Ubi3* encode ubiquitin fused to ribosomal subunits, which are involved in various cellular activities under basal metabolic conditions, whereas *Ubi4* encodes a tandem head-to-tail repeat of five ubiquitin moieties and is important during stress. Mammalian ubiquitin is also encoded by four genes: *Uba52* and *Uba80* code for ubiquitin fusion proteins with ribosomal subunits, whereas *Ubb* and *Ubc* code for tandem repeat, head-to-tail spacer-less ubiquitin units. The generation of an active single ubiquitin moiety requires posttranslational cleavage of the ubiquitin precursor at the C-terminus essentially by the ubiquitin-specific proteases (usually deubiquitylating enzymes (DUBs)) (Shabek and Ciechanover, 2010).

Ubiquitin is the building block of a repertoire of molecular signals spanning from a single ubiquitin to multiple ubiquitin molecules forming chains of different linkages, involved in the posttranslational modification of cellular proteins (Hershko and Ciechanover, 1998). It modifies several proteins at a lysine residue in the target protein forming an isopeptide bond (ubiquitylation). This modification has drastic effects on the fate and function of proteins. Different types of ubiquitin chains are formed by the attachment of multiple ubiquitin moieties via the internal lysine residues (K6, K11, K27, K29, K33, K48, and K63) or the amino-terminal methionine (M1). Different linkages are associated with different functions. The formation of heterogeneous as well as homogenous chain of ubiquitin has been reported (Ikeda et al., 2010; Shabek and Ciechanover, 2010). Among these, K48- and K64-linkages are the most widely studied (Figure 1.4). K48-linked chains are involved in the protein degradation via the proteasome, K63-linked chains are usually involved in several non-proteolytic events such as DNA repair and protein trafficking via endocytosis and lysosomal targeting, K11-linked chains function in the endoplasmic reticulum associated degradation (ERAD) and mitosis, whereas K29-linked chain might

be a signal to recruit a novel chain assembly factor before proteasomal proteolysis (Xu et al., 2009).

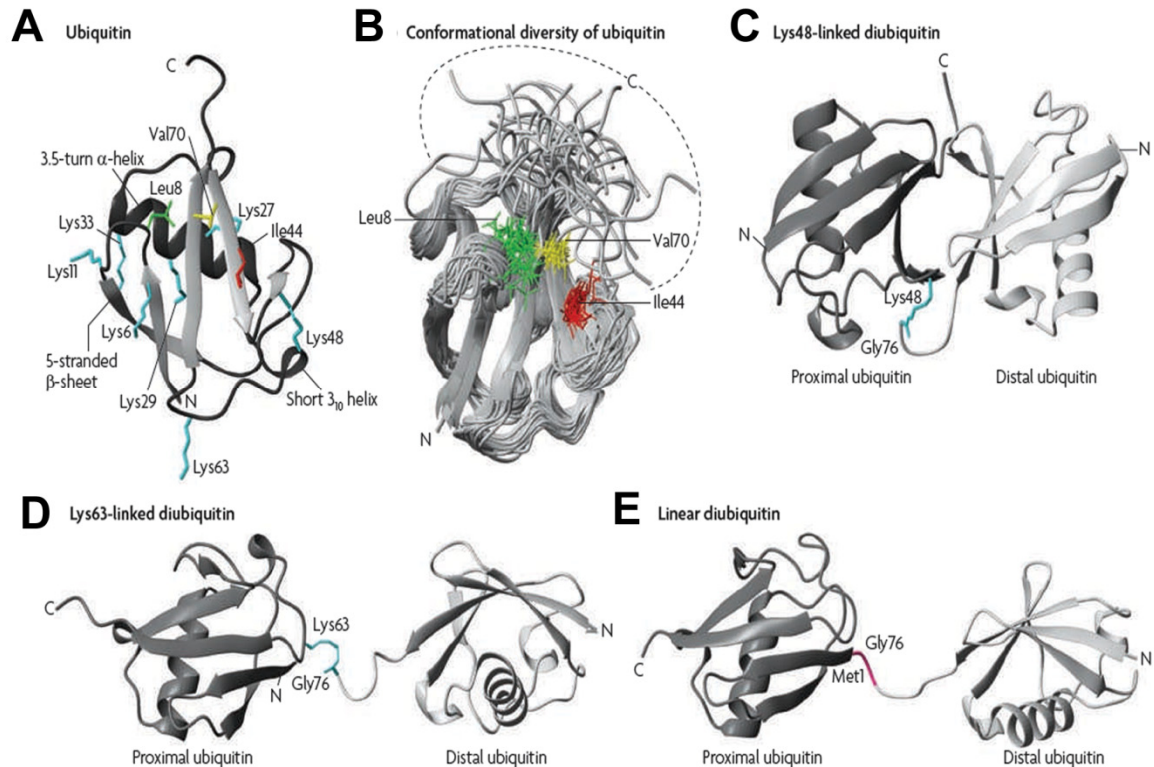


Figure 1.4 Structure of ubiquitin and its modifications. (A) A ribbon representation of monoubiquitin. Ubiquitin contains a five-stranded β -sheet, a 3.5-turn α -helix and a short 3_{10} helix. Seven solvent-exposed Lys residues (blue) are available to assemble ubiquitin chains, and the hydrophobic residues Leu8 (green), Ile44 (red) and Val70 (yellow) are critical for many ubiquitin-binding domain (UBD) interactions. (B) Free ubiquitin in solution is a dynamic molecule with conformational diversity. Different conformations are required by individual UBDs and several are shown here to highlight the dynamic range of motions that ubiquitin displays in solution. (C) Ribbon representation of Lys48-linked diubiquitin. The isopeptide bond linkage is shown in cyan. (D) Ribbon representation of Lys63-linked diubiquitin. The isopeptide bond linkage is shown in cyan. (E) Ribbon representation of linear diubiquitin, forming a peptide bond (magenta) between Met1 and Gly76. (Modified from Dikic et al., 2009).

Ubiquitylation is mediated by a cascade of three enzymes: the ubiquitin activating enzyme (E1), a conjugating enzyme (E2) and a ligase (E3). E1 forms a thiolester bond between its active site cysteine and the carboxyl-terminal glycine of ubiquitin. The activated ubiquitin is then transferred to the active site cysteine of an E2 by transesterification. Subsequently, E3 binds ubiquitin-charged E2 and also the substrate and thereby, facilitates the formation of an isopeptide linkage between the carboxyl-

terminal glycine of ubiquitin and an ϵ -amino group of a lysine on the substrate. Ubiquitin can also be conjugated to the substrate via the serine, threonine or cysteine residue to generate an ester or thiolester bond, or to the N-terminal residue to form a linear isopeptide bond. Successively, the degradation of the substrate is mediated by the 26S proteasome. This pathway is known as ubiquitin-proteasome system (UPS) (Ciechanover, 1998; Hershko and Ciechanover, 1998) (Figure 1.5). The UPS appears to be hierarchical: mammalian cells express two E1 enzymes (UBE1 and UBA6) (Chiu et al., 2007; Groettrup et al., 2008) that transfer ubiquitin to approximately 50 distinct E2 enzymes and each E2 enzyme is associated with several E3 ligases (approximately 1000).

Substrate specificity is conferred by E3 ligases which recognize specific target protein. E3 ligases are broadly classified into two families: HECT (Homologous to E6-AP C-terminus) domain proteins (~350 amino acid highly conserved motif) and RING (Really Interesting New Gene) finger domain proteins (eight conserved cysteines and histidines that together coordinate two zinc ions in a cross-braced manner). The HECT domain protein E6-AP was first identified to be involved in the ubiquitylation of p53 (Scheffner et al., 1994). RING finger domain proteins became apparent with the identification of Rbx1/Roc1/Hrt1, essential for multi-subunit SCF (SKp1-Cul1-F-box) complex E3 activity. Two other motifs, possessing the E3 ligase activity, related to the RING finger have also been identified: the PHD domain and the U-box (Fang and Weissman, 2004).

An important step in this pathway involves the release of ubiquitin from its substrates. This is a critical step in two ways: first, 'proofread' mistakenly ubiquitylated proteins, and secondly, it is a ubiquitin biosynthesis process (Ciechanover, 1998). In this way, this process can either accelerate the proteolysis or inhibit it. This category of recycling enzymes is classified into two classes: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin-specific proteases (UBPs; isopeptidases). UCHs are involved in the co-translational processing of precursors of ubiquitin, and in the release of ubiquitin from adducts with small molecules, such as amines and thiol groups. UBPs catalyze release of ubiquitin from cellular protein conjugates. Some UBPs are free, while others are associated with the 19S RP (Ubp6 and Doa4 in yeast) (Borodovsky et al., 2001; Fang and Weissman, 2004; Shabek and Ciechanover, 2010).

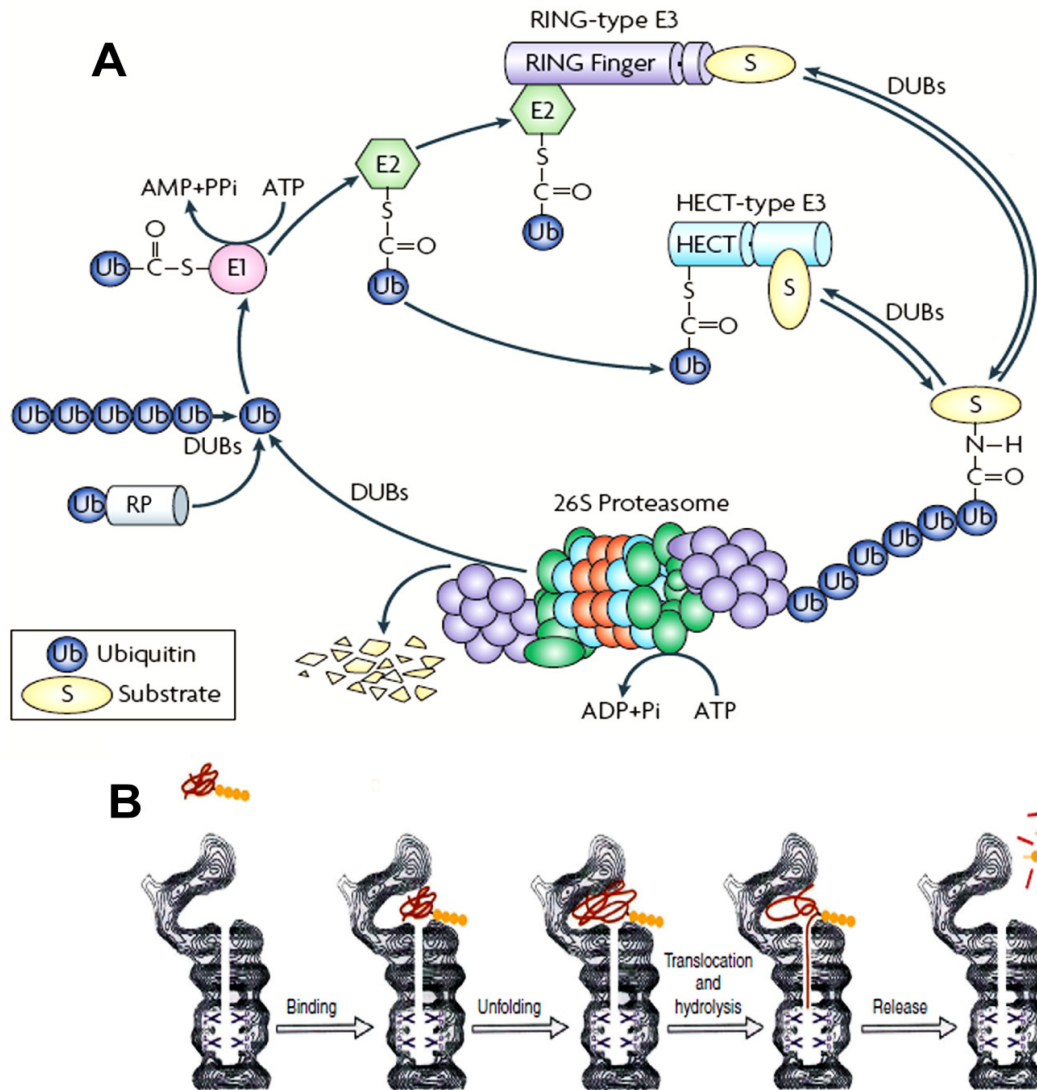


Figure 1.5 The ubiquitin-proteasome pathway. (A) Ubiquitin (Ub) is translated either as a tandemly fused polyubiquitin or as a fusion protein with the ribosomal protein (RP). De-ubiquitylating enzymes (DUBs) hydrolyze the isopeptide bond between Ub molecules or Ub and the RP to produce free Ub. The Ub-conjugation pathway consists of three kinds of enzymes: E1 (Ub-activation enzyme), E2 (Ub-conjugation enzyme) and E3 (Ub ligase). E1 activates Ub by forming a high-energy thiolester bond between the carboxy-terminal glycine residue of Ub and the active site cysteine of E1 in an ATP-dependent reaction. The activated Ub is transferred to E2, and then E3 ligates Ub to a specific substrate protein. Two classes of the E3 enzymes are represented: RING finger domain E3s bind to both E2 and a substrate and help E2 to transfer Ub to a substrate, whereas HECT-type E3s form a thiolester bond with the activated Ub from E2 before transfer of Ub to a substrate. Ub is covalently attached to internal lysine residues of a substrate. DUBs remove polyubiquitin from proteasome substrates before substrates are translocated into the 20S proteasome and regenerate free Ub from unanchored polyubiquitin chains. (Modified from Murata et al., 2009). (B) A model depicting ubiquitin-conjugate degradation by the proteasome. Multiple steps, including substrate binding, unfolding, translocation, and hydrolysis during ubiquitin-substrate degradation are represented. The substrate is shown in red, the polyubiquitin chain in yellow. Proteolytic sites are represented as scissors. For simplicity, the proteasome is shown with a single RP (Modified from Finley, 2002).

Despite the exceptional stability of ubiquitin, several studies have demonstrated the degradation of ubiquitin by the 26S proteasome via three different routes: as a monomer, as a part of its conjugated substrate, and as a fusion protein with a C-terminal tail (Shabek and Ciechanover, 2010). Polyubiquitin chains conjugated to substrates are recognized primarily by two subunits of the 19S RP: Rpn10/S5a and Rpn13/ADRM1. Ubiquitin signals are recognized and processed by a specific class of proteins called ubiquitin-binding domain proteins (UBDs). Approximately 200 intracellular proteins have been recognized to contain one or more UBDs. UBDs bind non-covalently to mono- or polyubiquitin chains and this interaction is vital in various aspects of cellular physiology including protein degradation, receptor trafficking, DNA repair, autophagy and apoptosis (Ikeda et al., 2010). A hydrophobic patch on ubiquitin, comprising Leu8, Ile44 and Val70, is required for its interaction with different UBDs: UBA, UIM, MIU, DUIM, CUE, PAZ, NZF, GLUE, UEV, GAT, BUZ and VHS (Dikic et al., 2009; Hicke et al., 2005).

The covalent attachment of ubiquitin to the target proteins could have diverse, non-degradative function as well including, DNA repair, transcription regulation, signal transduction, endocytosis, cell cycle and division, response to stress and extracellular modulators, regulation of the immune and inflammatory responses, biogenesis of organelles and apoptosis. Some years ago, several studies indicated the possible role of ubiquitin system in recognizing and eliminating intracellular bacteria (Welchman et al., 2005).

The association of the UPS with the pathogenesis has been studied during the past several years. It is classified into two mechanism-based groups: first, pathogenesis resulting from the mutations in a UPS enzyme or a target substrate (loss of function), and secondly, pathogenesis resulting from an accelerated degradation of the target protein (gain of function). Modulating the activity of UPS has the potential for the treatment of various diseases. Proteasome inhibition has been the target to treat several diseases. The first drug synthesized for inhibiting the UPS was bortezomib, which blocks the entire UPS. The major mechanism of action of bortezomib appears to be the induction of apoptosis, which is dose-dependent and cell-type specific. It appears to be efficient in a few cases, but it cannot be considered for long-term treatment due to several side effects (e.g., thrombocytopenia and peripheral neuropathy). The challenge is to develop new, efficient

and highly specific drugs that target only a single or a few proteins without affecting others. Targeting the E3 enzymes has far fewer side effects, e.g., attempts have been made to inhibit Hdm2 to stabilize p53 protein (Reinstein and Ciechanover, 2006).

1.3 UBIQUITIN RECEPTORS

The recognition of ubiquitin conjugated to the substrate is mediated by “ubiquitin receptors” associated with the proteasome. The currently known receptors include two proteasome subunits, Rpn10 and Rpn13, and three “shuttle factors”, Rad23/hHR23, Dsk2/PLIC, and Ddi1. These proteins are conserved among eukaryotes. The nonessential behavior of these proteins in yeast suggests that probably additional ubiquitin receptors remain to be identified. Some intrinsic ubiquitin receptors in the proteasome as suggested by *in vitro* chemical cross linking studies include Rpt5, Rpt1, and Rpn1 (Figure 1.6).

1.3.1 Rpn10 subunit (S5a, Mbp1, Sun1, Mcb1, Pus1)

The first ubiquitin receptor identified was the Rpn10 subunit of the proteasome (Deveraux et al., 1995a). This subunit is also present in free monomeric form in significant amounts in yeast. This suggests the possibility that Rpn10 is in a dynamic equilibrium between a free and a complex-bound form. Diverse forms of Rpn10 are found in vertebrates (five isoforms Rpn10a to Rpn10e), accomplished by developmentally regulated alternative splicing (Kawahara et al., 2000). Rpn10c was reported to associate with Scythe/BAG-6 and regulate apoptosis in *Xenopus laevis* (Kikukawa et al., 2005).

The Rpn10 subunit of yeast (scRpn10) and human (hRpn10) has one and two ubiquitin interacting motifs (UIM), respectively. UIM-1 and UIM-2 of hRpn10, located at its C-terminus, recognizes ubiquitin, whereas the von Willebrand A (VWA) domain, located at its N-terminus, is known to be important for maintaining the integrity of 26S proteasome by supporting the association of the lid and the base of the 19S RP. LALAL and IAYAM motifs in UIM-1 and UIM-2, respectively, were identified as small hydrophobic patches critical for ubiquitin binding. The hydrophobic stretch, comprised of Leu8, Ile44 and Val70 in ubiquitin, is exposed on the surface as identified by the crystal structure of the

monomeric, Lys48-linked dimeric, and Lys48-linked tetrameric ubiquitin (Beal et al., 1998; Narasimhan et al., 2005; Zhang et al., 2009).

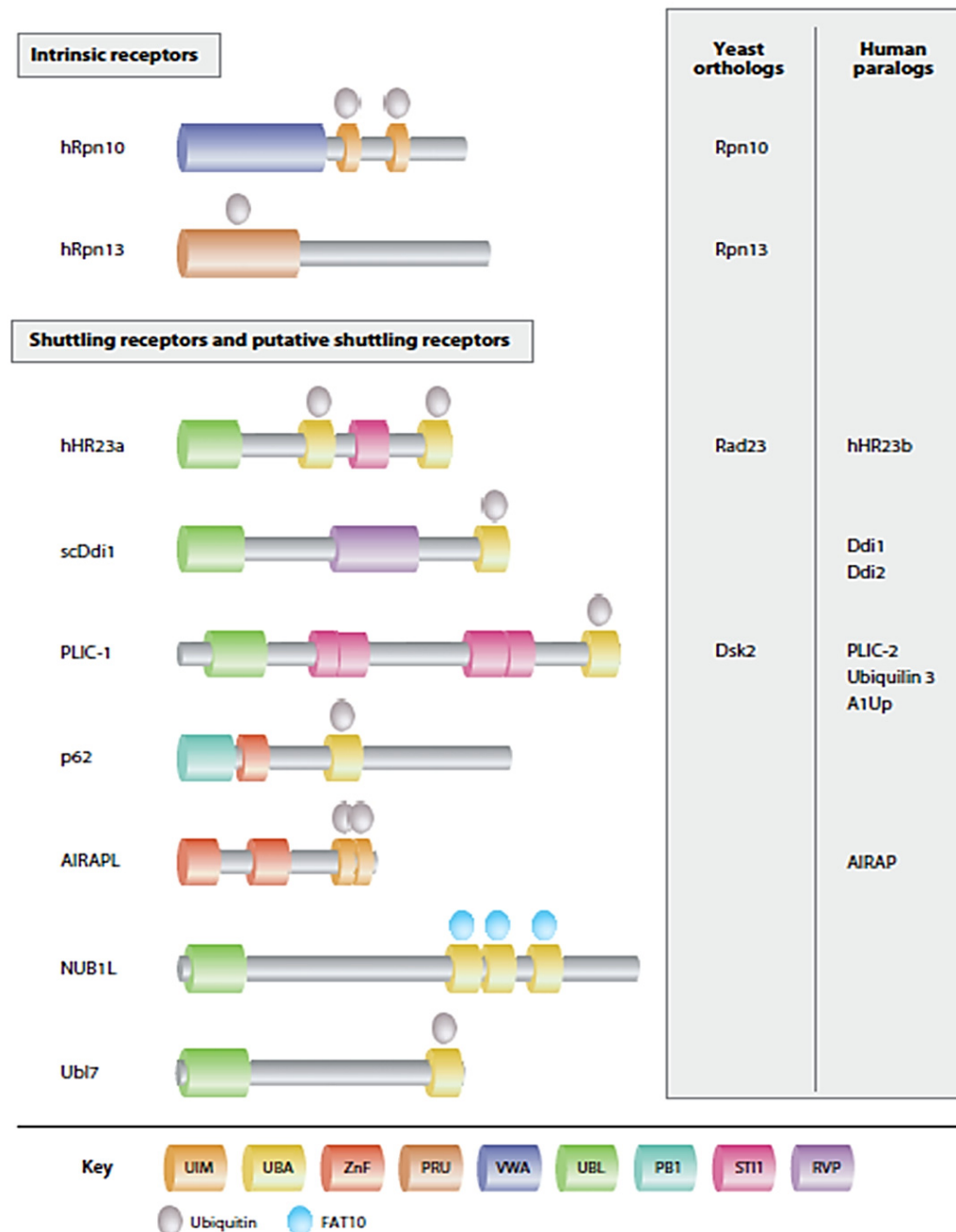


Figure 1.6 Ubiquitin receptors of *Saccharomyces cerevisiae* and humans. Ubiquitin receptors are classified as intrinsic receptors or shuttling receptors. The domains in the receptor proteins are shown as seen in humans, except for Ddi1, whose human homolog has no ubiquitin-associated (UBA) domain. The canonical members of each class are shown, and apart from ubiquitin, all proteins and domains are drawn to scale. UIM, UBA, STI1, VWA, and PB1 are SMART domains. RVP is a Pfam domain found in Ddi protein and is encompassed by the slightly larger aspartyl protease Pfam domain. In all cases but two, ubiquitin-like (UBL) domains are located at the N-terminus in the shuttling receptors. The ZnF domain refers to two different types of zinc finger domains: ZnF(AN1) in AIRAPL and ZnF(ZZ) in p62. The PRU domain is present in Rpn13 which is responsible for the binding to ubiquitin. (Modified from Finley, 2009).

hRpn10 can receive ubiquitylated proteins from extraproteasomal receptors, including hHR23 and PLIC proteins, which bind to them with ubiquitin-like domains (Hiyama et al., 1999; Husnjak et al., 2008; Walters et al., 2002). In budding yeast, the free form of Rpn10 (UIM domain) is complexed to the UBL domain of Dsk2, thus preventing the binding of Dsk2 to the proteasome, and thereby, inhibiting the substrate degradation (Matiuhin et al., 2008; Walters and Zhang, 2008). Although not essential in budding yeast (Ferrell et al., 1996), Rpn10 is essential in mice (Hamazaki et al., 2007) and *Drosophila melanogaster* (Szlanka et al., 2003). This suggests that either Rpn10 is required for the turnover of only a small subset of ubiquitylated substrates, or it is required for the turnover of a large number of proteins, but in its absence such proteins are targeted by other ubiquitin receptors. The two ubiquitin receptors, Rpn10 and Rpn13 can bind the distal and proximal region of K48-linked diubiquitin simulatenously with subunit specificity (Zhang et al., 2009).

Recently, it was observed that monoubiquitylation of Rpn10 strongly inhibits its capacity to interact with substrates, and it was proposed that this acts as a stress sensitive mechanism. Rsp5 (ubiquitin ligase) and Ubp2 (deubiquitylating enzyme) control the levels of monoubiquitylation at K71, K84 and K99, located within the VWA domain, and in K268 located within the C-terminus of the protein (Isasa et al., 2010). Another study reported that hRpn10 can be ubiquitylated by two very different self-ubiquitylated E3 ligases, muRF1 and CHIP, along with an E2, UbcH5, which suggests its short half-life when present in the free cytosolic form (Uchiki et al., 2009). During this ubiquitylation process, hRpn10 prevents the formation of non-degradable forked ubiquitin chains on the substrate probably by shielding the lysine residues on the proximal ubiquitin of the growing chain from reacting with the ubiquitin released from an E2 enzyme. Therefore, this process can stimulate the degradation of several substrates (Kim et al., 2009).

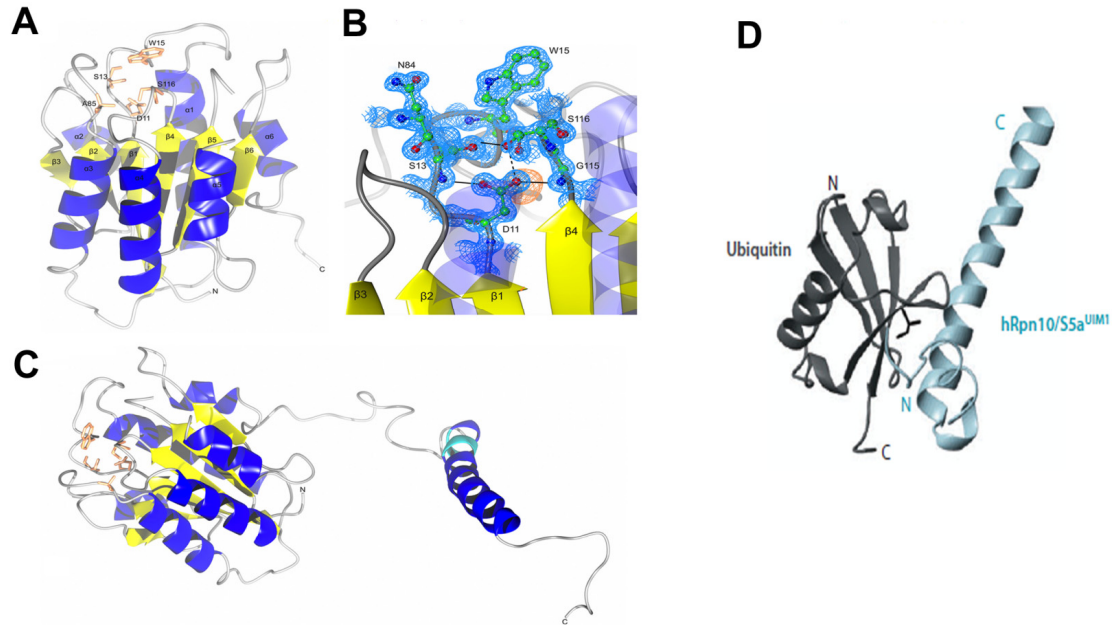


Figure 1.7 Structure of SpRpn10. (A) Secondary structure of SpRpn10 VWA domain representing β -sheets (β 1– β 6) in yellow and α -helices (α 1– α 6) in blue color. Amino acids that constitute the pseudo-MIDAS motif at the end of β 1 are shown as cylinders in coral color. (B) Structure showing the pseudo-MIDAS motif in VWA domain. The MIDAS motif is characterized by the sequence DXSXS together with threonine and aspartate residues in the second (between α 2 and α 3) and third (between β 4 and β 4) loops, respectively. In the SpRpn10 VWA domain these residues are replaced by the sequence DNSEW (Asp11, Ser13, and Trp15 are drawn) and amino acids Ala85 and Ser116. The hydrogen bonds formed by Asp11 show the importance of in maintain the structural integrity. The electron density map is drawn in blue. (C) Model of full-length SpRpn10 derived from the VWA crystal structure and the homology-modeled C-terminus. The ubiquitin binding LALAL motif is highlighted in cyan. (Modified from Riedinger et al., 2010). (D) Complex formation between ubiquitin and hRpn10 mediating substrate recognition by the proteasome. (Modified from Finley, 2009).

The UIM domains form α -helices. The two UIM domains seem to act co-operatively, though UIM-2 binds ubiquitin with an approximately five-fold higher affinity than UIM-1. Interestingly, the UIMs of hRpn10 have distinct specificity for UBL domain proteins (Finley, 2009). The VWA domain of *Shizosaccharomyces pombe* forms six-stranded β -sheet of five parallel stands and one antiparallel strand sandwiched between two triplets of α -helices (Riedinger et al., 2010) (Figure 1.7). The fold and topology are typical of VWA domain in other proteins as well. The majority of VWA domains are found in cell adhesion proteins and extracellular matrix proteins although VWA domains in intracellular proteins are most widely distributed phylogenetically (Whittaker and Hynes, 2002). The VWA domain docks Rpn10 to the proteasome. Paradoxically, the removal of UIM domain by itself has no discernable effect on the turnover of some substrates, like

cell cycle regulator, Sic1 and a UPS substrate, Ub-proline- β -galactosidase (Ub-Pro- β -gal), suggesting that the function of Rpn10 may rely on the VWA domain (Verma et al., 2004). All VWA domains contain a conserved aspartate in the DXSXS motif that appears to be buried within the protein and required to maintain the folding of the protein by electrostatic interactions (Emsley et al., 1998; Matsushita and Sadler, 1995). Asp11 in the VWA domain of Rpn10 was identified as important for the amino acid analog sensitivity as well as for the degradation of ubiquitin fusion degradation (UFD) pathway substrates. It was postulated that the salt bridge formed by Asp11 assists in maintaining the structural integrity of VWA in Rpn10 (Borodovsky et al., 2001).

1.3.2 Rad23 (hHR23)

Rpn10 assembled in the 26S proteasome does not react to a chemically reactive tetraubiquitin chain (Lam et al., 2002), and recombinant Rpn10 inhibits proteolysis in frog extracts (Deveraux et al., 1995b). These findings diverted the attention to another group of proteins, Rad23 and Dsk2. These proteins belong to the UBA-UBL family proteins and are considered as “shuttle factors” that regulates the delivery of proteolytic substrates to the proteasome. Several genetic evidences predict an overlapping proteolytic function for Rad23, Dsk2 and Ddi1. They are involved in UFD, ER-associated degradation (ERAD), spindle pole body duplication, and the delivery of polyubiquitylated substrates to the proteasome (Biggins et al., 1996; Lambertson et al., 1999; Medicherla et al., 2004; Saeki et al., 2002). Rad23 was initially identified as having a role in nucleotide excision-repair (NER) by dimerizing with Rad4, a DNA repair protein, and localizing in the nucleus. Initially, it was identified in *S. cerevisiae*, followed by its discovery in *S. pombe* (Rhp23) and higher eukaryotes (HR23A and HR23B) (Su and Lau, 2009).

hHR23A comprises of one UBL domain at the N-terminus and two UBA domains at the C-terminus. The UBA-1 domain binds tetraubiquitin as well as ubiquitin-conjugated proteins more efficiently as compared to UBA-2 domain. The UBL domain of hHR23A is important for its interaction with the proteasome either via Rpn10 or Rpn1 subunit (Su and Lau, 2009).

Rad23 may have different roles in proteasomal degradation depending on the substrates. Overexpression of Rad23 causes partial stabilization of ubiquitylated substrates in the presence of Rpn10, suggesting that its interaction with multi-ubiquitin chains could

inhibit further elongation of the ubiquitin chain or probably it regulates the delivery of ubiquitylated substrates to Rpn10 incorporated in the proteasome (Chen and Madura, 2002; Ortolan et al., 2000). Loss of Rad23 in conjunction with Rpn10 lacking UIM domain stabilizes the CDK inhibitor, Sic1 (Verma et al., 2004). On the other hand, in higher eukaryotes, the UBA-2 domain of hHR23A/B shields polyubiquitylated p53 from deubiquitylation, thereby inhibiting the degradation of p53 (Glockzin et al., 2003). Rad23 and deubiquitylating enzyme Ubp6 both bind the proteasome subunit, Rpn1, through the UBL domain. Instead of competing, these two proteins appear to be brought into proximity by Rpn1, which suggests a scaffolding function of Rpn1 (Elsasser et al., 2002).

One could question why would an interaction of a substrate with UBA/UBL domain proteins required, if a polyubiquitylated substrate was sufficient to be targeted to the proteasome by itself? A possible answer to this could be that such “shuttle factors” might contribute to the regulation and transient stabilization of proteins that are constitutively degraded, e.g., during DNA damage certain DNA repair factors require stabilization, which could be accomplished by Rad23.

1.4 UBIQUITIN-LIKE PROTEINS

Several proteins have been discovered during the last ~20 years that are related to ubiquitin either structurally or functionally. The broad class of such ubiquitin-like proteins was subdivided into two: ubiquitin-like modifiers (UBLs) and ubiquitin domain proteins (UDPs). UBLs act as modifiers in a manner similar to ubiquitin. UDPs bear ubiquitin-like domains but the sequence and function are unrelated to each other and these proteins cannot be conjugated to other proteins.

1.4.1 Ubiquitin-like modifiers (UBLs)

These modifiers are involved in the protein-protein interactions or alter protein conformation by acting as flexible adaptors. UBLs are more plastic evolutionarily: duplication and diversification of UBLs can give rise to several related molecules with new functions involved in distinct cell regulatory mechanisms (Schwartz and Hochstrasser, 2003). UBLs participate in various biological processes such as DNA

repair, autophagy and signal transduction. At least 10 UBLs (ubiquitin, ISG15, AUT7/APG8, APG12, NEDD8/RUB1, SUMO1/SMT3, HUB1, FAT10, URM1, MNSF) share similar ligation pathways like that of ubiquitin by transferring small protein moieties onto various substrates (Figure 1.8). But each of these UBLs has a distinct cellular function. Some of these, like SUMO and RUB, have been identified in all eukaryotes, whereas others, such as ISG15, have restricted phylogenetic distribution. Except APG12 and URM1, all the UBLs have sequence homology to ubiquitin. APG12, URM1 and FAT10 are expressed as mature proteins and do not require any processing but others are expressed as inactive precursors which are processed rapidly endoproteolytically by specific proteases known as UBL specific proteases (ULPs), thereby releasing the active modifiers. Such modifiers initially exist as fusions with C-terminal extensions which prevent conjugation.

The first clue for the antecedents of UBL-protein conjugation came from the ubiquitin-activating enzyme E1 of yeast. E1 has weak, but significant similarity with an *E. coli* protein, MoeB, required for the biosynthesis of molybdenum cofactor (Moco) (Hochstrasser, 2009). Despite similarities in UBLs, considerable variation exists among different UBL conjugation pathways. Many of the UBLs have very few or no identified E3 ligases. There is an exception where two UBLs share a common E1 enzyme. Atg8 and Atg12, involved in autophagosome formation, share a single E1, Atg7. On the other hand, two different E1s specific for different UBLs can share the same E2, e.g., UBE1, the E1 for ISG15, and Uba1, the E1 for ubiquitin, both can use Ubch8 E2. Moreover, the ISG15 E3s are also able to function with ubiquitin. The modification of a substrate by ubiquitin or a particular UBL, sharing the same E3s, may be determined by the relative cellular levels of the activated modifiers or by additional factors (or both). Another well-studied example of E3 shared by ubiquitin and NEDD8 is Mdm2, which mediates the modification of p53, a tumor suppressor protein. Among all these exceptions, there exists one more where no separate E2 enzyme is required. The E1 Uba4 for Urm1 has a kind of in-built E2 present in a rhodanese-homology domain (RHD) in the protein (Kerscher et al., 2006; Welchman et al., 2005).

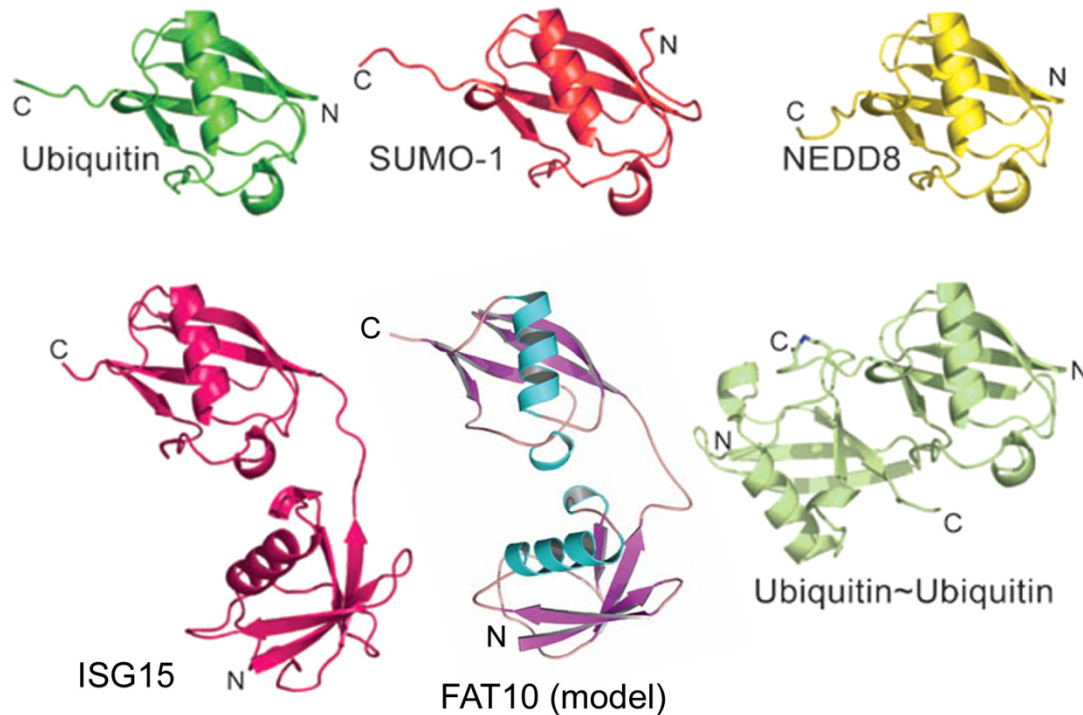


Figure 1.8 Ubiquitin-like protein three-dimensional structures. Structures of (A) Ubiquitin, (B) SUMO-1, (C) NEDD8, (D) ISG15, (E) FAT10 (model structure) and (F) Lys48-linked diubiquitin. The structures show similar folds like ubiquitin (Modified from Dye and Schulman, 2007 and Groettrup et al., 2008).

1.4.1.1 FAT10 (HLA-F locus adjacent transcript 10)

FAT10 was first identified as a gene located in the MHC class I region close to the HLA-F locus (Fan et al., 1996). It consists of two ubiquitin-like domains, and is hence also called di-ubiquitin or ubiquitin D, with an identity of 29% and 36% to its N- and C-terminal region, respectively (Bates et al., 1997). The lysine residues involved in the formation of polyubiquitin chains, K27, K33, K48 and K63, are conserved in both domains of FAT10. Initially, the mRNA of FAT10 was identified as highly expressed in mature dendritic cells and B cells (Bates et al., 1997) but later, it was found to be expressed in several organs, in particular, lymphatic organs like thymus, spleen and lymph nodes (Liu et al., 1999; Lukasiak et al., 2008). Thus, unlike ubiquitin, its expression is limited to certain organs of the immune system, however, it is inducible by proinflammatory cytokines, IFN- γ and TNF- α in almost every cell type (Raasi et al., 1999). FAT10 has a free diglycine motif at its C-terminus, which is important for its conjugation to target proteins (Chiu et al., 2007; Raasi et al., 2001).

Ubiquitin and FAT10 share the same E1 enzyme, UBA6, which can be transferred to different E2s (Chiu et al., 2007; Groettrup et al., 2008; Pelzer et al., 2007). Analogous to ubiquitin, FAT10 can target proteins for proteasomal degradation but, on the contrary, FAT10 itself is also degraded together with its substrate by the proteasome. Moreover, the degradation of FAT10 and its conjugates is independent of ubiquitin as implied by the instability of FAT10 lacking lysines (Hipp et al., 2005). In addition, the degradation of FAT10 could be accelerated in the presence of a non-covalent interacting UBL-UBA domain protein NUB1L (Hipp et al., 2004). Further studies revealed that all the three UBA domains of NUB1L bind FAT10, whereas NUB1L lacking the UBL domain does not interact with FAT10. In spite of this fact, NUB1L lacking UBA domains is capable of binding to the proteasome and accelerates the degradation of FAT10 (Schmidtke et al., 2006).

The degradation of FAT10 and its conjugates was also demonstrated in another study where purified 26S proteasome was shown to be able to degrade the model substrate FAT10-dihydrofolate reductase (DHFR) but only in the presence of NUB1L. This study revealed that mere binding of FAT10 to the proteasome is not sufficient for the degradation of this model substrate, in the NUB1L knockdown cells, *in vivo* (Schmidtke et al., 2009).

Several studies show that FAT10 might be involved in cancer. FAT10 interacts non-covalently with MAD2 (mitotic arrest deficient 2), a spindle checkpoint protein, and hence, may modulate the cell growth during B cell and dendritic cell development (Liu et al., 1999). Ovarian, breast and nasopharyngeal cancers are associated with reduced expression of MAD2 and colorectal and gastric cancers are associated with increased expression of MAD2. Another study reported the interaction of FAT10 with MAD2 during mitosis, which could reduce the efficiency of MAD2 to bind to the unattached kinetochore. Upon treatment with nocodazole, more FAT10-overexpressing cells escaped mitotic arrest and exhibited abnormal and multinuclear morphology compared to the parental cells. This suggested the role of FAT10 in mitosis dysfunction, resulting in aneuploidy and chromosomal instability which is commonly observed in several cancers but no direct evidence was demonstrated (Liu et al., 1999; Ren et al., 2006). FAT10 mRNA expression and promoter activity was significantly reduced in the presence of wild-type p53 (Zhang et al., 2006) but up-regulated by mutant p53 (Ji et al., 2009) and

hence, it was proposed that FAT10 might be involved in tumorigenesis. FAT10 overexpression was determined as the epigenetic marker for liver neoplasia (Oliva et al., 2008). Other studies demonstrated the up-regulation of FAT10 in hepatocellular, gastrointestinal and gynecological tumors and it was postulated that FAT10 might be important in cancer development through the dysregulation of MAD2 (Lee et al., 2003; Lim et al., 2006). However, further study identified that FAT10 has no transforming capability and the expression of FAT10 is up-regulated in such tumors due to the up-regulation of proinflammatory cytokines (Lukasiak et al., 2008). Recently, it was shown that FAT10 expression began at the serrated adenoma stage and continued in the villous and villotubular stages and the invasive adenocarcinoma stage (Qing et al., 2011).

Knockout of FAT10 in mice causes minimal phenotypic changes with no major pathological or histological differences. Lymphocytes from FAT10 deficient mice are susceptible to apoptosis and sensitive to even low doses of endotoxin, and hence may function as a cellular survival factor (Canaan et al., 2006). The enhanced expression of FAT10 resulted in apoptosis as demonstrated in the stable cell line expressing FAT10 and this is dependent of the caspase activity (Raasi et al., 2001). The down-regulation of FAT10 in renal tubular epithelial cells infected with human immunodeficiency virus, show reduced apoptosis (Ross et al., 2006). FAT10 suppression prevents *Vpr*-induced apoptosis in human and murine RTEC and these proteins interact non-covalently and co-localize to mitochondria (Snyder et al., 2009). However, it is still unknown whether FAT10-mediated apoptosis is harmful to the host or it is an adaptive response that limits the spread of virus in the host.

Some studies indicate the link of FAT10 to aggregates. FAT10 interacts and co-localizes with histone deacetylase 6 (HDAC6) under conditions of proteasome inhibition. FAT10 as well as a model conjugate, FAT10-GFP, localizes to the aggresome in a microtubule dependent manner, which suggests the possible role of HDAC6 in transporting FAT10 and its conjugates to aggresomes when the proteasomal degradation pathway is non-functional (Kalveram et al., 2008). FAT10 also co-localizes with LMP2 and LMP7 and its expression increases in liver cells forming Mallory-Denk bodies (characterized by accumulation and aggregation of ubiquitylated cytokeratins) (Bardag-Gorce et al., 2010; Qing et al., 2011). Recently, two proteins have been identified as the covalent conjugates of FAT10: p53 (a role of FAT10 in regulating the transcriptional activity of p53) (Li et

al., 2011) and huntingtin (a role of FAT10 in modulating the solubility of aggregates) (Nagashima et al., 2011), but these findings require some more extensive work in the future.

1.4.1.2 ISG15 (Interferon stimulated gene-15)

ISG15, also termed as ubiquitin cross reactive protein (UCRP), was identified in the late 1980s. Despite being the first UBLs to be identified, the function of ISG15 is still poorly understood. It consists of two ubiquitin-like domains. Its expression is induced by type I interferons (IFN- α and IFN- β), lipopolysaccharides and viral infections. It is the processed product of its precursor ISG17, which is cleaved by specific protease UBP43/USP18 to reveal a free glycine that can function to modify proteins when activated by the E1 UBE1L (ubiquitin-activating enzyme E1-like). ISG15 has not been identified in lower eukaryotes, such as yeast, nematodes, insects and plants, which indicate its role in specialized functions in vertebrates. The expression of UBP43 and conjugation of ISG15 are strongly induced by interferon- α , indicating that this conjugation pathway is tightly controlled. It is involved in specific signaling pathways that have a role in innate immunity.

More than 300 cellular proteins have been identified that are targeted for ISGylation (Giannakopoulos et al., 2005; Malakhov et al., 2003; Takeuchi et al., 2006; Zhao et al., 2005). The conjugation of ISG15 to JAK and STAT proteins suggests its role in the JAK-STAT signaling pathway (Malakhova et al., 2003). The lack of UBP43 results in an increase in IFN signaling and in the level of protein ISGylation (Malakhova et al., 2003). Several antiviral effector proteins like MxA, protein kinase R (PKR) and RNaseL also interact with ISG15 (Zhao et al., 2005). It was confirmed by several studies that ISG15 has antiviral activity against many viruses, including influenza, sindbis, herpes, human immunodeficiency and ebola (Hsiang et al., 2009; Lenschow et al., 2005; Okumura et al., 2006). But several viruses have evolved mechanisms to interfere with ISG15 conjugation, e.g., the NS1 protein of influenza B blocks the ISG15 conjugation pathway (Chang et al., 2008; Yuan and Krug, 2001) and the vaccinia virus E3 protein binds to ISG15 and blocks its antiviral activity (Guerra et al., 2008). Recently, it was reported that ISG15 can target a broad range of proteins and this system is designed to target newly synthesized proteins in the context of viral proteins or the interferon response (Durfee et al., 2010).

1.4.1.3 SUMO (Small Ubiquitin-like Modifier)

SUMO has been the most extensively studied UBL in the past few years. The SUMO conjugation pathway is involved in several cellular activities including cell-cycle control, nuclear transport and response to viral infections. It is essential for the viability of budding yeast where it is designated as SMT3 (suppressor of MIF2 mutations-3). Three isoforms of SUMO are identified in humans (SUMO-1, -2 and -3). SUMO-2 and -3 function distinctly from SUMO-1, which have the capability to form polySUMO chains due to the presence of a SUMOylation motif surrounding K11 (ψ KXE, where ψ represents a large hydrophobic amino acid and X represents any amino acid). SUMO specific proteases (SENPs) process the precursor of SUMO to generate the mature C-terminus, thereby exposing the diglycine motif for conjugation. SUMOylation is reversible and, in *S. cerevisiae*, deSUMOylation is mediated by Ulp1 (ubiquitin-like protein-specific proteases) (Jentsch and Pyrowolakis, 2000; Welchman et al., 2005).

RanGAP1, the GTPase-activating protein for the Ran GTPase, was the first identified target for SUMOylation. It is required for nucleocytoplasmic trafficking. Promyelocytic leukaemia proteins (PML) and Sp100 also require modification by SUMO for their localization to the PML bodies in the nucleus (Schwartz and Hochstrasser, 2003).

Some studies show that SUMO-1 and ubiquitin might compete to modify the same site, e.g., ubiquitylation of K21 of I κ B is required for its degradation, whereas SUMOylation of K21 stabilizes I κ B. NEMO, the regulatory subunit of the cytoplasmic I κ B kinase (IKK) complex, is SUMOylated which mediates NF- κ B activation by genotoxic stress (Huang et al., 2003). Recently, it was shown that SENP2 can efficiently associate with NEMO, deSUMOylate SUMO and inhibit NF- κ B activation induced by DNA damage, thus acting as a negative feedback loop to attenuate the cell survival response to genotoxic stress (Lee et al., 2011). SUMO-2 and -3 can be conjugated to the transcriptional factor C/EBP β 1 (CCAAT/enhancer-binding protein β -1) and topoisomerase II, which repress the ability of C/EBP β 1 to activate the transcription from the cyclin-D promoter and reposition topoisomerase II on mitotic chromosomes at the metaphase-anaphase transition, respectively. SUMO modification can also be involved in signaling pathways, e.g., SUMOylation of a *Dictyostelium* MAP kinase in response to differentiation signal (Schwartz and Hochstrasser, 2003; Welchman et al., 2005).

1.4.1.4 NEDD8/RUB1 (Neural precursor cell expressed developmentally down-regulated protein 8/Related to ubiquitin-1)

Among all the UBLs, NEDD8 is most similar to ubiquitin in sequence (~80% homology). The precursor of NEDD8 is processed to expose the diglycine motif for conjugation, which is attained by NEDD8-specific deNEDDylase 1 (DEN1)/NEDD8 protease (NEDP1) and ubiquitin C-terminal hydrolase isozyme L3 (UCH-L3). The dissociation of NEDD8 from protein substrates is dependent on ubiquitin-specific protease-21 (USP21), DEN/NEDP1 and the COP9 signalosome. NEDDylation plays a role in cell cycle progression and morphogenesis in mammals and is associated with the ubiquitylation of p27, I κ B α and the NF- κ B precursor by SCF (SKP1-CUL1-F-box) E3s (Schwartz and Hochstrasser, 2003; Welchman et al., 2005). Recently, it has been shown that BPLF1, the Epstein-Barr virus encoded protein acts as a deNEDDylase that induces virus replication by modulating the activity of cullin-RING ligases (CRLs) (Gastaldello et al., 2010).

The well characterized substrates of NEDD8 include all the members of the cullin (CUL) family except APC2 (anaphase promoting complex-2). Cullins, the scaffolding subunits of E3s such as SCF and CBC (CUL2-elongin-BC), interact with RING-finger proteins to enable recruitment of E2s. CAND1 (cullin-associated and NEDDylation-associated-1) has been identified as a protein that binds to Cul1-Rbx1, preventing recruitment of Skp1. This CAND1-Cul1 complex can be NEDDylated, which may promote formation of an active SCF. Another example of NEDDylation includes the modification of p53 mediated by Mdm2 E3 ligase, which led to an inhibitory effect in the transcriptional activity of p53. NEDD8 can also modify MDM2 itself during this process resembling auto-ubiquitylation of E3s (Welchman et al., 2005). Later, another E3 ligase, F-box protein FBXO11, was also found to mediate p53 NEDDylation (Abida et al., 2007). Apart from these substrates, von Hippel-Lindau (VHL) and breast cancer-associated protein 3 (BCA3) were identified as NEDD8 substrates (Gao et al., 2006; Russell and Ohh, 2008). Recent advances in the field of NEDD8 include the development of NEDD8-activating enzyme (NAE) inhibitor. NAE is essential for the NEDD8 conjugation pathway controlling the activity of cullin-RING ligases. The inhibitor represses the turnover of protein mediated by cullin-RING ligases leading to apoptotic death in human tumor cells (Soucy et al., 2009).

1.4.2 Ubiquitin domain proteins (UDPs)

Ubiquitin domain proteins (UDPs) constitute a broad range of proteins, which differ structurally and functionally but are only similar in the ubiquitin-like domain (UBL) region. UDPs are neither processed nor conjugated to other cellular proteins. Several studies have shown that UDPs share the ability to interact with the 19S regulatory particle of the 26S proteasome, which depends on the integral UBL domain in UDPs, e.g., Rad23, Dsk2, BAG-1 and NUB1L.

1.4.2.1 NUB1 (NEDD8 Ultimate Buster-1)

NUB1 was first identified as an interaction partner of NEDD8 which down-regulates the protein expression of NEDD8 and its conjugates via proteasomal degradation. It is a 601 amino acid long protein, inducible by interferon and predominantly localized in the nucleus (Kamitani et al., 2001). It is composed of a UBL domain at the N-terminus and two UBA domains at the C-terminus. An alternative splicing variant of NUB1 has one more UBA domain of 14 residues sandwiched between the two UBA domains of NUB1 and it is designated as NUB1L (NUB1 long) (Tanaka et al., 2003). The NEDD8-binding motif on NUB1 was localized to the C-terminus and the UBA2 domain. NUB1L interacts with UbC1, a ubiquitin precursor composed of nine tandem repeats of a ubiquitin unit linked by α -peptide bonds. However, it does not interact with isopeptide bond-linked polyubiquitin chain (Tanaka et al., 2004).

According to a report by Tanji et al. (2005), the C-terminus of NUB1 interacts with the hRpn10 subunit of the proteasome but the down-regulation of NEDD8 and its conjugates by NUB1 depends on the N-terminal UBL domain. Recently, a study demonstrated the involvement of NUB1 in the localization of p53 in the cytoplasm. It down-regulates the NEDDylation of p53 but promotes p53 ubiquitylation and this activity requires the E3 ligase Mdm2. This provides an example of co-operation between the NEDD8 and ubiquitin pathways in protein function regulation (Liu and Xirodimas, 2010; Tanji et al., 2005).

NUB1 has some implications in neurodegenerative diseases but the evidences are not very substantial. NUB1 was found to be associated with synphilin-1 protein, which is involved in the pathogenesis of Parkinson's disease (PD), dementia with Lewy bodies

(DLB) and multiple system atrophy (MSA). Endogenous NUB1 localizes to the synphilin-1 positive inclusions in HEK293 cells, and Lewy bodies in PD and DLB. This interaction initiates the proteasomal degradation of synphilin-1 and down-regulates the formation of synphilin-1 positive inclusions. NUB1 is highly accumulated in the inclusion bodies of the brain of patients with α -synucleopathies (Tanji et al., 2007; Tanji et al., 2006).

Other interaction partners of NUB1L include FAT10 and AIPL1 (aryl hydrocarbon receptor-interacting protein-like 1). NUB1L interacts with the N-terminal UBL domain of FAT10 through its UBA domains. This interaction is involved in accelerating the degradation of FAT10 via the proteasome (Hipp et al., 2004; Schmidtke et al., 2009; Schmidtke et al., 2006). AIPL1 is a photoreceptor-specific protein and mutations in AIPL1 are associated with Leber congenital amaurosis (LCA), a severe early-onset form of retinal degeneration. The NUB1-binding site on AIPL1 is located between amino acid residues 181 and 330 within which most of the mutations of AIPL1 reside (Akey et al., 2002). AIPL1 promotes the translocation of NUB1 from the nucleus to the cytoplasm, thereby modulating the downstream effects of NUB1 on cell signaling and cell growth. AIPL1 also suppresses the formation of inclusions by NUB1, perhaps by affecting the conformation of the proposed NUB1-binding site within which the mutation resides (van der Spuy and Cheetham, 2004).

Recently, a study indicated the role of NUB1 in cancer. NUB1 mRNA and protein was found to be up-regulated in the renal carcinoma cell lines (RCC) by IFN- α . NUB1 overexpression induces apoptosis in these cells and suppresses the inhibition of cell growth induced by IFN- α . Furthermore, NUB1 overexpression increased the cell population in S phase and up-regulated two proteins of cell cycle regulation: cyclin E and p27 (p27 mainly inhibits the activity of cyclin E-CDK2 complexes and thereby blocks progression from the G1 to the S phase), which indicates the role of NUB1 in cell-cycle arrest and apoptosis in RCC cells (Hosono et al., 2010).

1.5 UPS AND NEURODEGENERATION

1.5.1 Link between UPS, Autophagy and Neurodegeneration

The pathogenesis of neurodegeneration is associated with aberrations in the UPS. However, in some disorders, it appears that the aggregation of disease-specific proteins results in the inhibition of UPS activity. A defect in one of the enzymes of the system or an alteration in one of the protein substrates renders it resistant to proteolysis. Neurodegenerative diseases, including Parkinson's and Alzheimer's diseases, prion infection, amyotrophic lateral sclerosis, and polyglutamine expansion disorders, are associated with the accumulation of intracellular proteins conjugated to ubiquitin. The first evidence for the accumulation of ubiquitin-protein conjugates was in neurofibrillary tangles in Alzheimer's disease (Mori et al., 1987) and later it was observed in several other diseases as well (Lowe et al., 1988). Protein aggregation occurs due to unfolding during oxidative stress and by alterations in primary structure caused by mutation, RNA modification or translational misincorporation (Figure 1.9). In normal unstressed cells, these aggregates do not accumulate due to protein 'quality control' mechanisms, which involve the selective degradation of misfolded proteins before they accumulate. Initially, it was considered that inclusion bodies are generated as a result of association of abnormal proteins to one another, but now it is believed that the process may be more complex and involves active cellular machineries such as aggregation of reversible aggregate concentrates (aggresomes) (Johnston et al., 1998) or movement of the proteasome to specific sites where proteolysis of abnormal proteins may occur (Ciechanover and Brundin, 2003). Molecular chaperons play an essential role in the recruitment of misfolded proteins to the conjugation machinery (Bercovich et al., 1997). The aggregation is usually related to the toxicity in cells although this has been a subject of controversy.

The autophagy-lysosome system is associated with the pathogenesis of neurodegenerative diseases. Autophagy is a degradative process involving the formation of a double-membraned vesicle around the cytoplasm resulting in an autophagosome which fuses with the lysosome (autophagolysosome) where its contents are hydrolyzed. It is considered a beneficial process, protecting against neurodegeneration but the mechanism is not completely understood. Autophagy is also involved in the development and

homeostasis of the immune system by eliminating the autoreactive T cells in the thymus. Autophagy levels are high in thymic epithelial cells where it participates in the delivery of self-antigens to MHC class II loading compartments. Genetic disruption of *Atg5* is associated with autoimmunity. Autophagy proteins may also be involved in the delivery of endogenous antigens for MHC class II presentation to CD4⁺ T cells, the enhancement of antigen cross-presentation to CD8⁺ T cells, cross-presentation by dendritic cells to CD4⁺ T cells and MHC class I presentation of intracellular antigens to CD8⁺ T cells (Levine et al., 2011). Almost all aggregated proteins are decorated with ubiquitin. An example of protein linking the ubiquitylated aggregates with the autophagosome is p62 (SQSTM1). p62 has both the LC3-binding site (a protein in the membrane of the autophagosome) and a ubiquitin-binding site. p62 acts as an adaptor to direct aggregates to the autophagosome (Bjorkoy et al., 2005; Pankiv et al., 2007). p62 is considered as a link between UPS and autophagy. On the one hand, it has the capability to target tau protein for proteasomal degradation (Babu et al., 2005), while on the other, it could form a shell surrounding aggregates of mutant huntingtin leading to autophagy (Bjorkoy et al., 2005).

1.5.2 Examples of Neurodegenerative Diseases

Amongst all the neurodegenerative disorders, Parkinson's disease is the second most frequent after Alzheimer's disease with a prevalence of 0.1% of the global population. The major feature of this disease is the progressive death of neurons in the substantia nigra pars compacta and the presence of aggregated proteins in the form of cytoplasmic Lewy bodies. Lewy bodies are eosinophilic inclusions, which consists of intracytoplasmic aggregates of α -synuclein. This results in the gradual development of akinesia, rigidity and tremor. Some of the main possible contributory factors in Parkinson's disease include oxidative stress, environmental toxins, genetic factors and mitochondrial dysfunction (Whitton, 2007). Several genes are related to the pathogenesis: *α -synuclein*, *uchl1*, *parkin*, *dj1* and *pink1*. Most patients with familial parkinsonism are associated with mutations in the *parkin* gene (an E3 ligase). All mutations in the *parkin* gene result in a loss of its enzymatic activity, which probably is the cause of this disease. Interestingly, most patients with autosomal recessive juvenile parkinsonism lack Lewy bodies (Reinstein and Ciechanover, 2006). Several substrates for Parkin have been identified, but it is not clearly understood whether the accumulation of one or more of these substrates causes the

disease. Parkin ubiquitylates synphilin-1 (an α -synuclein-binding protein) and probably targets ubiquitylated-synphilin-1 to the inclusion bodies and removes it from the cytosol where it could be toxic.

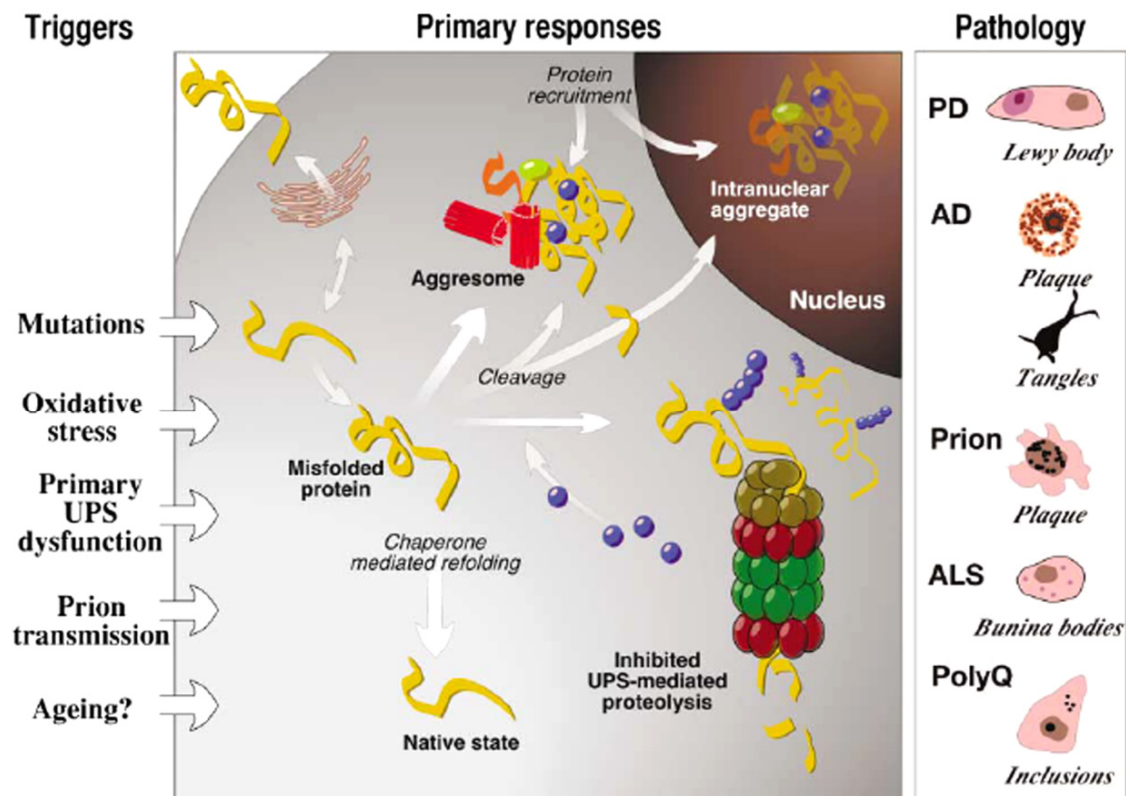


Figure 1.9 The UPS and neurodegeneration. The four different aspects related to protein misfolding and neurodegenerative diseases are represented. (1) *Triggers*, including mutations, oxidative stress, UPS dysfunction and prion transmission, leads to the accumulation of misfolded proteins. (2) The *primary responses* to the accumulating misfolded proteins which are related to the inhibition of the UPS that is the consequence of protein overload/inhibition by aggregated substrates. The chaperones may refold the accumulated misfolded proteins or else aggregates are accumulated in the cytoplasm, nucleus, or extracellular space. The aggregates may sequester several other proteins. (3) Types of *neuropathologies*. The accumulation of extracellular and intracellular protein that can be found in the central nervous system of patients with PD, AD, Prion disease, ALS, and polyglutamine (PolyQ) disorders are shown. (Modified from Ciechanover and Brundin, 2003).

Huntington's disease is associated with mutations involving a CAG triplet repeat expansion in the *huntingtin* gene. The precise function of huntingtin (Htt) is not clear but its role is implicated in interfering with basic cellular function such as transcription, signaling, transport and endocytosis which ultimately causes neuronal degeneration.

Mutant Htt can be either ubiquitylated or SUMOylated. The protein encoding this gene accumulates in ubiquitin- and proteasome-positive intranuclear inclusion bodies. p62 is known to associate with the polyubiquitylated Htt aggregates and hence, may drive the selective engulfment of the aggregates by binding to LC3 in autophagosomes. Ubiquitin and LC3 help to eliminate aggregated Htt but on the other hand, SUMO has the potential to increase the levels of toxic forms of Htt in the cell (Kerscher et al., 2006).

1.5.3 Neuroinflammation

Despite the presence of the blood-brain barrier and the lack of a lymphatic system, the brain is fully capable of mounting an inflammatory response. The pathogenic infection, trauma or stroke can trigger activation of microglia (resident macrophages of the brain), local invasion of circulating immune cells, and the production of reactive oxygen and nitrogen species (ROS/RNS), cytokines and chemokines. Overactivation and dysregulation of microglia might be disastrous and neurotoxic to the cells. Microglial activation leads to elevated levels of microglial immunoglobulin reactivity, upregulation of CD1 and cell adhesion molecules, such as lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18), intercellular adhesion molecule 1 (ICAM-1) (CD54) and vascular adhesion molecule 1 (VCAM-1) (CD106). Activated microglia cluster around dopaminergic neurons and become phagocytic (Tansey et al., 2007; Whitton, 2007).

Neuroinflammation in Parkinson's disease was initially suggested by McGeer et al. (1988), who described the upregulation of MHC molecules in such patients (McGeer et al., 1988). The upregulation of proinflammatory cytokines, like TNF- α , IL-1 β and IL-6 in the striatum as well as in the cerebrospinal fluid, during such disease have been demonstrated (Brodacki et al., 2008; Whitton, 2007). These cytokines seem to initiate the immune responses and maintain the neuroinflammation leading to a potentially lethal descent into irreversible destruction of dopaminergic neurons. Upon pathogenic events the central nervous system releases some other factors as well, including reactive oxygen species (ROS), reactive nitrogen species (RNS), prostaglandins and chemokines. Some of these factors are neuroprotective and provide support in the repair process, whereas others enhance oxidative stress and trigger apoptotic cascades. Therefore, targeted inhibition of microglial activity and inflammatory processes may provide a potential therapy to slow down or delay progression of neurodegenerative diseases.

1.6 AIM OF THE THESIS

FAT10, a small ubiquitin-like modifier, acts as an additional signal for the proteasomal degradation of its substrates in a ubiquitin-independent manner. FAT10 interacts non-covalently with an UBA-UBL domain protein NUB1L, which enhances the degradation of FAT10. Earlier reports show that FAT10 directly binds to the purified 26S proteasome, and NUB1L binds to the hRpn10 subunit of the proteasome. The thesis work started with some major questions. How is the degradation of FAT10 accomplished by the proteasome? Which subunit of the proteasome is involved in the degradation of FAT10 and its conjugates? Is this degradation only dependent on NUB1L or some other factor(s)? How does NUB1L influence and accelerate the degradation of FAT10? Is the “FAT10-proteasome” pathway similar to the ubiquitin-proteasome pathway?

This thesis deals with the mechanism of degradation of FAT10 by the 26S proteasome in the presence and absence of NUB1L. The goal was achieved by identifying a specific domain responsible for the docking of FAT10 and NUB1L on the proteasome and its functional relevance using different approaches. Furthermore, the role of FAT10 in neurodegenerative diseases was investigated in this thesis.

1.7 ORGANIZATION OF THE THESIS

The second chapter deals with the mechanistic insights into the docking of FAT10 and NUB1L on the 26S proteasome. The identification of a novel receptor (within the 26S proteasome) for ubiquitin-like proteins is demonstrated. Different approaches are shown to identify the interaction site(s) in these proteins and the functional relevance of the interaction. The third chapter demonstrates the approaches utilized to identify the amino acids involved in the interaction of hRpn10 with FAT10 and NUB1L. This includes obtaining a three-dimensional structure model of hRpn10 and NUB1L computationally, GST-pull down assays and protein purification for X-ray crystallography. Furthermore, a new domain was identified in NUB1L, which is displayed by the amino acid sequence alignments and the three-dimensional structure of NUB1L obtained computationally. In the fourth chapter of the thesis, the identification of FAT10 in aggregates in neurodegenerative diseases has been demonstrated. Up-regulation of FAT10 mRNA and

protein in the brain of different mouse models is described. The fifth chapter deals with the identification of an E2 enzyme in the cascade of FAT10-conjugation pathway. The auto-FAT10ylation of this enzyme, in addition to the enzymatic activity, is described. The last part of the thesis deals with the generation of monoclonal and polyclonal antibodies specific for mouse FAT10 to support the research on FAT10.

2. RPN1 and the VWA domain of RPN10 bind the UBL-UBA protein NUB1L and the ubiquitin like modifier FAT10 for proteolytic targeting to the 26S proteasome

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2.1 INTRODUCTION

A central proteolytic pathway in eukaryotic cells is the ATP-dependent ubiquitin-proteasome system (Finley, 2009). The 26S proteasome is a multi-protein complex of 2.5 MDa comprising the 19S regulatory particle (RP, also called PA700) of approximately 900 kDa and a 20S cylindrical core particle (CP) of approximately 700 kDa. The RP is required for the binding and unfolding of poly-ubiquitylated proteins to enable their degradation into peptides. The RP can be subdivided into a base composed of six ATPases (Rpt 1-6) and four non-ATPases Rpn2/S1, Rpn1/S2, Rpn13, and Rpn10 and a lid composed of nine structurally diverse subunits. The major known ubiquitin receptors on the proteasome are the RP subunits Rpn10 (designated hRpn10 or S5a in humans (Deveraux et al., 1994; Ferrell et al., 1996)) and Rpn13 (Adrm1 in humans (Husnjak et al., 2008)) while the ATPase subunit Rpt5/S6' has been cross-linked to poly-ubiquitin chains but evidence for its functional involvement in proteolysis is lacking (Lam et al., 2002). Rpn10 is assumed to lie at the interface of lid and base (Fu et al., 1998). This subunit comprises the N-terminal von Willebrand A (VWA) domain (Whittaker and Hynes, 2002) and C-terminal ubiquitin interacting motifs (UIM1 and UIM2, together referred as UIMs) (Hofmann and Falquet, 2001; Riedinger et al., 2010; Soucy et al., 2009). Polyubiquitin chains which are covalently attached to the lysine of substrates interact with Rpn10 via the ubiquitin interacting motifs (UIMs) (Beal et al., 1998; Fu et al., 1998; Soucy et al., 2009) and with Rpn13 via the pleckstrin-like receptor for ubiquitin (Pru) domain (Husnjak et al., 2008; Schreiner et al., 2008). In addition, there are further receptors for ubiquitin conjugates belonging to the UBL-UBA family of proteins, such as Rad23, Dsk2, and Ddi1, which only temporarily associate with the proteasome (Schauber et al., 1998). This indirect pathway involves interaction of ubiquitin chains with their C-terminal UBA domains while their N-terminal UBL domains interact with the Rpn1, Rpn2 or Rpn10 subunits of the proteasome (Elsasser et al., 2004; Funakoshi et al., 2002; Saeki et al., 2002; Verma et al., 2004). Rpn1 and Rpn2 are the two largest subunits (approximately 100 kDa) of the proteasome serving as a scaffold.

FAT10 (HLA-F locus adjacent transcript 10) is a 18 kDa protein which has two ubiquitin-like domains with an identity of 29% and 36% to ubiquitin in its N- and C-terminal parts, respectively (Fan et al., 1996). Several ubiquitin-like modifiers have been described but FAT10 is the only one with functions similar to poly-ubiquitin as a tag for proteasome

targeting (Hipp et al., 2005; Schmidtke et al., 2009). Recently, we have shown that FAT10 serves as a transferable signal for the proteasomal degradation independently of the ubiquitin system (Aichem et al., 2010; Hipp et al., 2005; Schmidtke et al., 2009). The expression of FAT10 is strongly and synergistically induced in the presence of the proinflammatory cytokines interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) (Liu et al., 1999; Raasi et al., 1999). Cytokine treated cells show an extensive high molecular weight smear of FAT10 conjugates reminiscent of ubiquitin conjugates and proteasome inhibition leads to the accumulation of FAT10 conjugates (Aichem et al., 2010; Chiu et al., 2007; Raasi et al., 2001). The E1 and E2 enzymes which mediate FAT10 conjugation have recently been identified as UBA6 and USE1, respectively (Aichem et al., 2010; Chiu et al., 2007; Groettrup et al., 2008; Jin et al., 2007; Pelzer et al., 2007).

The degradation of FAT10 and FAT10 conjugates is strongly accelerated by the scaffold protein NEDD8 ultimate buster-1 long (NUB1L), which is inducible by interferons and contains an N-terminal ubiquitin-like (UBL) domain and three C-terminal ubiquitin-associated (UBA) domains (Hipp et al., 2004; Kamitani et al., 2001; Schmidtke et al., 2009). NUB1L binds FAT10 via its three UBA domains and the 26S proteasome via hRpn10 (Tanji et al., 2005). NUB1L may hence serve as a linker between FAT10 and the proteasome. However, FAT10 can also directly bind to the proteasome and the deletion of the UBA domains of NUB1L interferes with FAT10 binding but not with the acceleration of FAT10 degradation (Schmidtke et al., 2006). The latter effect requires the UBL domain of NUB1L which is needed for binding to the proteasome suggesting a 'facilitator' function of NUB1L in FAT10 mediated degradation, reminiscent of the function of the Rpn10 VWA domain in Rad23-mediated degradation of poly-ubiquitylated substrates.

In this study we aimed at understanding the mechanism of accelerated degradation of FAT10 by NUB1L by finding the interacting subunits of the proteasome for FAT10 and NUB1L. We show that hRpn10 directly interacts with FAT10 and confirmed its interaction with NUB1L. Unexpectedly, this interaction was mediated solely by the VWA domain of hRpn10 unlike the interaction of hRpn10 with ubiquitin which occurs via its UIM domains. The VWA domain of hRpn10 sufficed to reconstitute FAT10 degradation in *rpn10 Δ* *S. cerevisiae* cells as well as its acceleration by NUB1L. We identify an

unexpected novel function of the VWA domain of hRpn10 as a receptor domain for UBL proteins, and we show that NUB1L also binds the juxtaposed scaffold subunit Rpn1/S2. Together, these findings allow to build a model of FAT10 and NUB1L

2.2 RESULTS

2.2.1 Identification of 19S RP subunits interacting with FAT10 and NUB1L

FAT10 can interact with the 26S proteasome and targets itself and conjugated proteins for proteasomal degradation (Schmidtke et al., 2009). Furthermore, NUB1 has been shown to bind to the proteasome via hRpn10 (Tanji et al., 2005). To identify the subunit(s) within the 19S RP that mediate FAT10 and NUB1L binding to the proteasome, we analyzed the interaction of each individual 19S RP subunit with FAT10 and NUB1L in a yeast two-hybrid assay. Human FAT10 or NUB1L was fused to the GAL4 activation domain (AD) and subunits of the human 19S RP were fused to the GAL4 binding domain (BD). BD-hRpn10 was the only subunit for which an interaction with AD-FAT10 could be detected (as evidenced by white colony color on selective plates and blue color in the X-gal filter assay (Figure 2.1a). BD-hRpn10 also interacted with the AD-NUB1L fusion protein (Figure 2.1b), confirming the previously described interaction between NUB1L and hRpn10 (Tanji et al., 2005). The previously established interaction of FAT10 with NUB1L was used as a positive control (Hipp et al., 2004). Taken together, these results suggest that FAT10 and NUB1L binding to the proteasome occurs via a common 19S RP subunit, hRpn10.

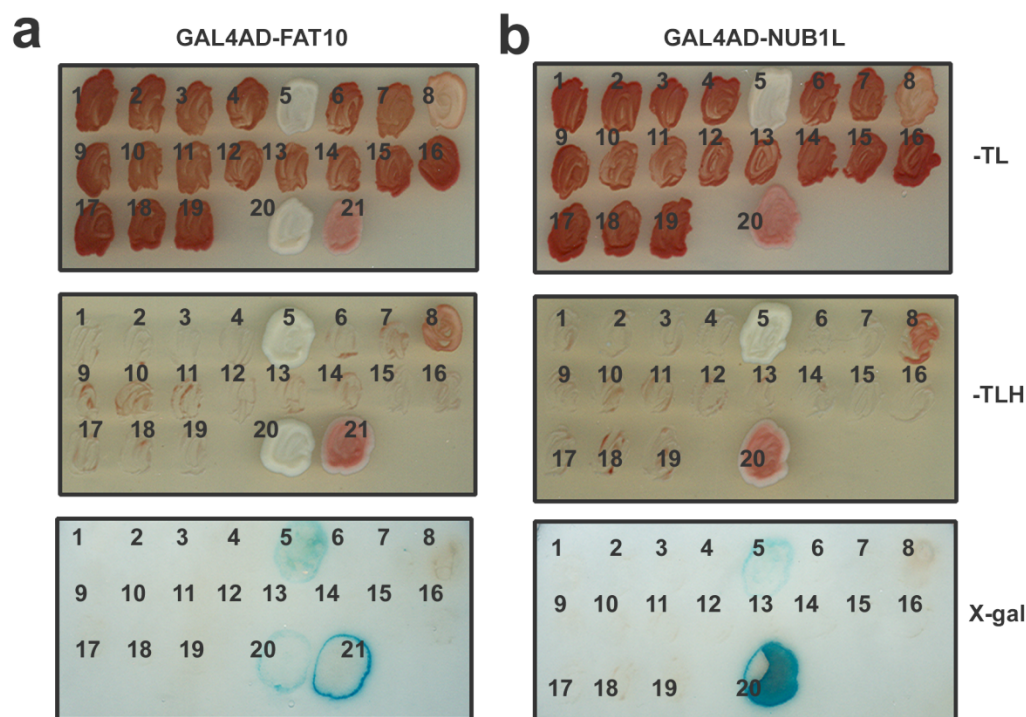


Figure 2.1 hRpn10 interacts with FAT10 and NUB1L in a yeast two-hybrid assay. (a) Yeast two-hybrid analysis of the interaction of FAT10 with individual subunits of the human 19S RP. AH109 cells were co-transformed with pGADT7-FAT10 and a pGBD vector encoding one of the human 19S RP subunits fused to GAL4-BD. The colonies represent co-transformants with pGADT7-FAT10 and the indicated pGBD construct: (1) S1/Rpn2, (2) S2/Rpn1, (3) S3/Rpn3, (4) S4/Rpt2, (5) S5a/Rpn10, (6) S5b, (7) S6a/Rpt5, (8) S6b/Rpt3, (9) S7/Rpt1, (10) S8/Rpt6, (11) S9/Rpn6, (12) S10a/Rpn7, (13) S10b/Rpt4, (14) S11/Rpn9, (15) S12/Rpn8, (16) S13/Rpn11, (17) S14/Rpn12, (18) S15, (19) p55/Rpn5. (20) As positive control we co-transformed the NMY51 cells with pGADT7-NUB1L and pLexA-FAT10. A second positive control (21) was the known interaction of NUB1L and hRpn10. Transformants were grown on selection plates containing 3-AT but lacking tryptophan and leucine (-TL) or tryptophan, leucine and histidine (-TLH), respectively (upper and middle panels). Confirmation of these results was obtained by X-gal filter assay (lower panel). The experiments have been repeated two times with similar outcomes. (b) Yeast two-hybrid analysis of the interaction of NUB1L with individual subunits of the human 19S RP. The yeast two-hybrid analysis was performed as in (a) except that plasmid pGADT7-NUB1L was used instead of pGADT7-FAT10.

2.2.2 FAT10 directly interacts with hRpn10 and NUB1L binds both hRpn10 and hRpn1

To test whether the interactions of FAT10 and NUB1L with hRpn10 can be attributed to a direct binding of the proteins, we performed GST-pull-down experiments. Bacterially expressed His-hRpn10 showed specific binding to GST-FAT10 and GST-NUB1L, but not to the GST control (Figure 2.2a) confirming a direct interaction of FAT10 and NUB1L with hRpn10. Moreover, we also found specific binding of the yeast Rpn10 protein (scRpn10) to GST-FAT10 and GST-NUB1L (Supplementary Figure 2.1).

Recently, it was shown that a conserved amino-terminal region of Rpn13, the Pru domain, binds ubiquitin and also binds ubiquitin-like (UBL) domains of UBL-ubiquitin-associated (UBA) proteins like hHR23a and hPLIC2 (Husnjak et al., 2008). Although, FAT10 failed to bind to the Pru domain of Rpn13 in an earlier study (Husnjak et al., 2008), we re-investigated this possibility with full-length hRpn13 in GST pull down assay and we likewise observed that hRpn13-myc was pulled down neither by GST-FAT10 nor GST-NUB1L (Figure 2.2b, left panel).

Taking into consideration that the UBL domain of Rad23 and Dsk2 interact with the leucine-rich-repeat-like (LRR-like) domain of the Rpn1 subunit of the 19S RP (Elsasser et al., 2002), we hypothesized that FAT10 or NUB1L might also interact with hRpn1. As no interaction between hRpn1 and FAT10 or NUB1L was detected in our initial yeast two-hybrid analysis (Figure 2.2a, b), we performed further binding experiments with recombinant GST-FAT10, GST-NUB1L or GST alone and radiolabeled *in vitro* translated His-hRpn1. Remarkably, His-hRpn1 bound robustly to GST-NUB1L but did not bind to GST-FAT10 or GST alone (Figure 2.2b, middle panel).

To test whether NUB1L binding to hRpn1 depended on the UBL domain or the two UBA domains of NUB1L, GST-pull down experiments with NUB1L deletion variants were carried out. The deletion of the UBL domain (GST-NUB1L Δ UBL) resulted in a strong reduction of His-hRpn1 binding (Figure 2.2c) indicating a pivotal role of the UBL domain of NUB1L in the binding of His-hRpn1. Moreover, we also investigated if the UBL domain of NUB1L is essential for the interaction with hRpn10 and, as observed in GST-pull down assay, the interaction was very weak if UBL domain was missing as compared to the interaction when the UBA domains were missing (Figure 2.2c). Together, our data show that NUB1L binds hRpn1 and hRpn10 predominantly via its UBL domain.

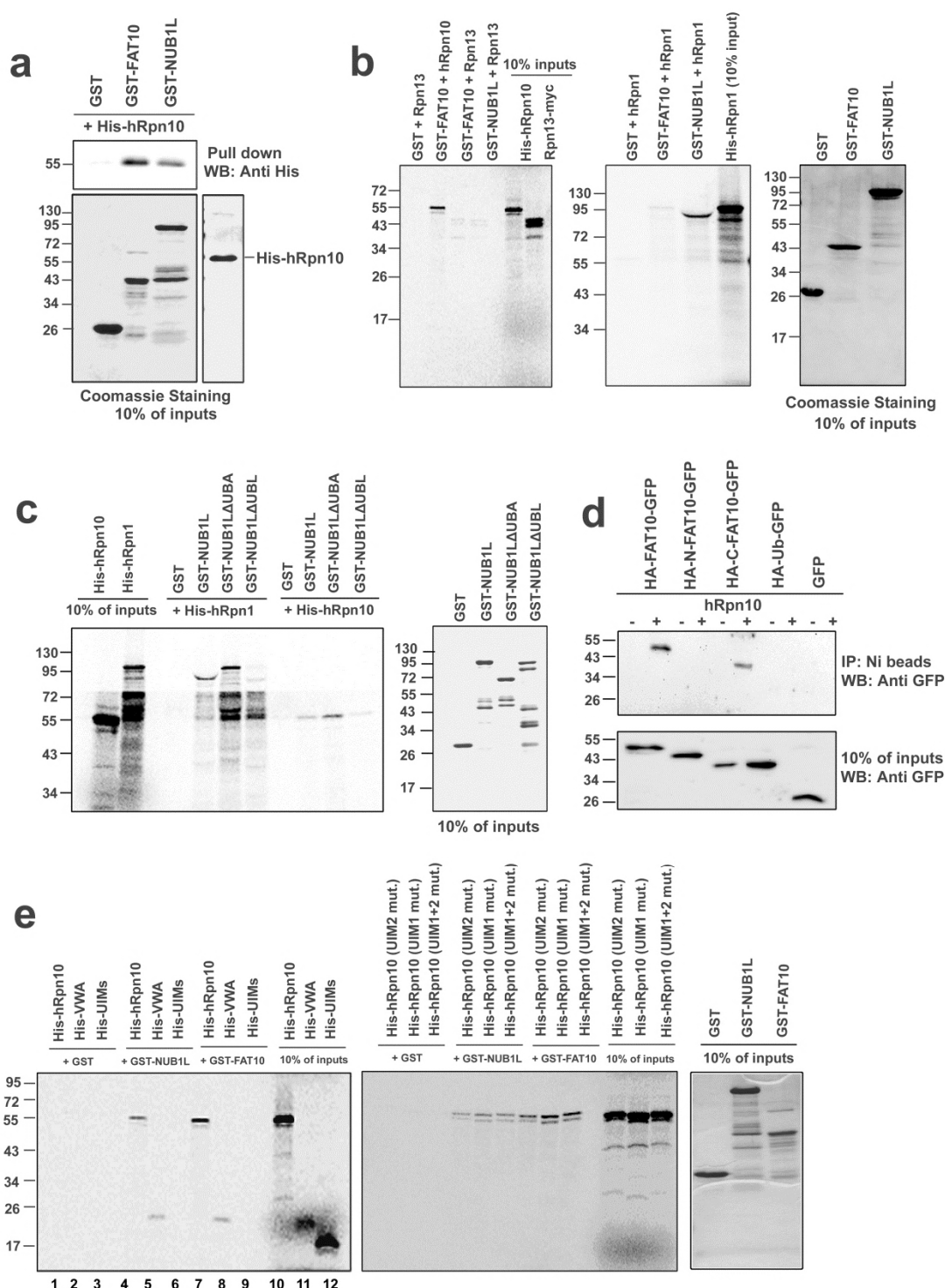


Figure 2.2 Direct interaction of FAT10 and NUB1L with the VWA domain of hRpn10 and binding of hRpn1 by NUB1L. (a) Direct interaction of FAT10 and NUB1L with hRpn10. Recombinant GST, GST-FAT10 or GST-NUB1L was incubated with recombinant His-hRpn10 and GSH beads. The interaction was analyzed by western blotting with anti-His antibody. 10% of the recombinant proteins are shown (see also Supplementary Fig. 1). (b) Left panel: No interaction of FAT10 or NUB1L with hRpn13. *In vitro* translated ³⁵S-labelled His-hRpn13 was incubated with recombinant GST, GST-FAT10 or GST-NUB1L on GSH beads and the possible interaction was observed by autoradiography. Middle panel: Binding of NUB1L to hRpn1. Same as stated above with ³⁵S-labelled His-hRpn1 instead of His-Rpn13. Right panel: 10% of input of the indicated GST-tagged proteins. (c) Binding of NUB1L to hRpn1 requires the UBL domain of

NUB1L. Left panel: GST-pull-down experiment as described in (b) with GST-NUB1LAUBL or GST-NUB1LAUBA. Right Panel: 10% of input of the indicated GST-fusion proteins. (d) The C-terminal UBL domain of FAT10 docks to hRpn10. Upper panel: Co-immunoprecipitation of recombinant His-hRpn10 on Ni-beads with the lysates from HEK 293T cells transfected with the plasmids shown. Positive interaction was determined by immunoblotting with anti-GFP antibody. Lower panel: 10% of the input of proteins. (e) FAT10 and NUB1L bind the VWA domain of hRpn10. Left panel: GST-pull-down experiments with GST-FAT10 or GST-NUB1L or GST alone and with *in vitro* translated radiolabeled full-length hRpn10, the VWA domain or the two UIMs. Proteins were visualized as in (b). Middle panel: GST-pull-down experiments with His-tagged hRpn10 mutants with mutated UIMs. Right panel: 10% of the input of GST-tagged proteins. Experiments were repeated three times with the similar outcome.

2.2.3 The C-terminal UBL domain of FAT10 is sufficient to interact with hRpn10, *in vitro*

FAT10 consists of two ubiquitin-like domains (Supplementary Figure 2.2b, c) (Fan et al., 1996). In order to determine the FAT10 domain that could interact with hRpn10, recombinant His-Rpn10 was incubated with lysates from HEK 293T cells transiently transfected with the plasmid carrying HA-FAT10-GFP, HA-N-FAT10-GFP, HA-C-FAT10-GFP, HA-Ub-GFP and GFP alone. Interestingly, only the C-terminal UBL domain of FAT10 bound to the hRpn10 but not the N-terminal FAT10 (Figure 2.2d). The full-length HA-FAT10-GFP served as a positive control and HA-Ub-GFP as well as GFP alone did not show any binding to the hRpn10 which served as negative controls. Whereas the N-terminal UBL domain of FAT10 binds NUB1L (Schmidtke et al., 2006), the C-terminal UBL domain binds the hRpn10 subunit of the proteasome.

2.2.4 FAT10 and NUB1L interact with the VWA domain of hRpn10 *in vitro*

As mentioned above, human Rpn10 contains three domains, the VWA domain and two ubiquitin interaction motifs (UIM1 and UIM2). The UIMs are the primary binding site for polyubiquitin chains (Fu et al., 1998; Soucy et al., 2009). In order to identify the domain within the hRpn10 protein mediating FAT10 and NUB1L binding, GST-pull down binding assays were performed. *In vitro* translated full-length His-hRpn10 as well as the His-tagged VWA domain alone (His-VWA) bound to GST-FAT10 and GST-NUB1L whereas no binding was detectable for the two UIMs of hRpn10 (Figure 2.2e).

Polyubiquitin chains bind preferably to UIM2 and to a lesser extent to UIM1. The amino acid sequence motifs crucial for the binding of polyubiquitin to UIM1 and UIM2 are

designated LALAL and IAYAM, respectively (Beal et al., 1998). In order to corroborate the above finding, we performed the same experiment with full-length hRpn10 bearing a mutated LALAL motif in UIM1 (mutated to AAAAA) and a mutated IAYAM motif in UIM2 (mutated to AAAAA; hereafter referred to as mutated UIMs) and observed that FAT10 and NUB1L can still interact with these mutated proteins (Figure 2.2e, middle panel).

Upon comparing the amino acid sequences of FAT10 and ubiquitin, we realized that the key residues within ubiquitin required for binding to the hRpn10 UIM domains, Leu8, Ile44 and Val70, do neither align with FAT10's N-terminal nor its C-terminal UBL domain (Supplementary Figure 2.2b, c). This observation is in accordance with separate binding sites for FAT10 and ubiquitin within hRpn10. Collectively, our results show that FAT10 and NUB1L bind to the VWA domain of hRpn10 in contrast to polyubiquitin chains which bind the UIM domains of hRpn10.

2.2.5 Reconstitution of Rpn10 deficient yeast with full length human Rpn10 or the VWA domain alone

The role of hRpn10, in particular that of its VWA domain, in the degradation of FAT10 was investigated in *S. cerevisiae* as Rpn10-deficient yeast is viable. First, we tested whether hRpn10, which has 50% identity with yeast Rpn10, can functionally substitute for scRpn10 in *S. cerevisiae*. Yeast cells lacking Rpn10 (*rpn10Δ*) show a pronounced sensitivity toward the amino acid analog canavanine (Ferrell et al., 1996). Canavanine is an arginine analog causing misfolding of proteins which then become poly-ubiquitylated and are targeted for proteasomal degradation. Rpn10 deficient proteasomes cannot cope with an increased load of such proteins, ultimately resulting in retarded growth and cell death. Consistent with an earlier study (Ferrell et al., 1996), N-terminally FLAG-tagged scRpn10 expressed from a high-copy plasmid led to a suppression of the canavanine sensitivity of *rpn10Δ* (NRY5) cells restoring wt-like growth on plates containing 3 μg/ml canavanine whereas the growth of *rpn10Δ* cells transformed with an empty plasmid was strongly retarded (Figure 2.3). In the same experiment, *rpn10Δ* cells transformed with the FLAG-hRpn10 expression construct showed growth on a canavanine containing plate comparable to that of *rpn10Δ* cells reconstituted with scRpn10 (Figure 2.3), indicating that hRpn10 can functionally substitute for scRpn10 in *S. cerevisiae*. Earlier study demonstrated that the *rpn10-uim* mutant show wild-type level of sensitivity to canavanine

(Elsasser et al., 2004). We also tested whether the VWA domain of hRpn10 alone or hRpn10 with mutated UIMs would be able to suppress the canavanine sensitivity of *rpn10Δ* cells. Interestingly, expression of both, the FLAG-tagged VWA domain as well as the hRpn10 with mutated UIMs, led to a partial suppression of the canavanine sensitivity (Figure 2.3) indicating that already the VWA domain alone is able to functionally reconstitute the Rpn10 deficient yeast proteasome.

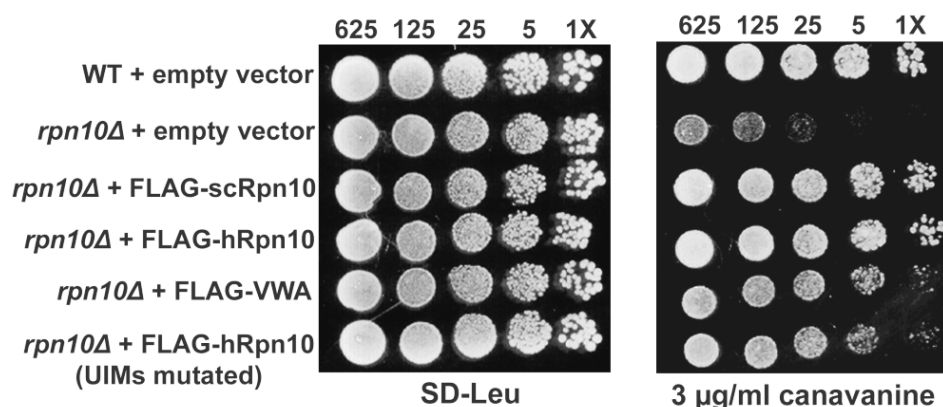


Figure 2.3 Reconstitution of canavanine resistant growth of scRpn10-deficient *S. cerevisiae* with full length hRpn10 and the VWA domain of hRpn10. Canavanine sensitivity plate growth assay with wild type (WT) and *rpn10Δ* *S. cerevisiae* transformants. WT or *rpn10Δ* cells transformed with high-copy expression plasmids for FLAG-scRpn10, FLAG-hRpn10, FLAG-VWA or FLAG-hRpn10-UIMs mutated or with the empty plasmid were spotted on plates with synthetic medium lacking leucine (SD-Leu) or on plates lacking leucine and arginine that contain 3 µg/ml canavanine. The growth assay has been repeated three times with similar outcome.

2.2.6 FAT10 migrates in the high molecular weight fractions of glycerol gradients of wild type but not *rpn10Δ* yeast

A potential binding of FAT10 with proteasome-associated scRpn10 in yeast was further analyzed by glycerol gradient centrifugation. Lysates of either WT or *rpn10Δ* cells expressing N-terminally HA-tagged FAT10 were separated by glycerol gradient centrifugation and collected fractions were analyzed by immunoblotting (Figure 2.4a, b). Western blots probed with anti-pre6, anti-scRpn10, anti-Rpt1 antibodies show the presence of 20S and 19S regulatory particle in fractions between 10-16 and 10-15 in wild type and *rpn10Δ* strain, respectively. Aminopeptidase I (~600 kDa decamer, AP1) also served as a size marker for the fractions containing 20S proteasome. In the extract derived from WT cells, HA-FAT10 migrated in the lower and the higher molecular weight

fractions (fractions 1-12 and 15-20; Figure 2.4a) whereas it was only found in the lower molecular weight fractions in extracts from cells lacking Rpn10 (fractions 1-8; Figure 2.4b) suggesting the association of FAT10 with proteasome-associated Rpn10. The presence of 20S proteasome in these high molecular weight fractions (fractions 10-15) was further corroborated by the presence of hydrolytic activity towards the fluorogenic substrate Suc-LLVY-AMC (Figure 2.4c). The peptidase activity drastically increased in some of the fractions in the presence of 0.02% SDS representing the presence of 20S proteasome in these fractions whereas the activity was reduced to minimal level in the presence of 5 μ M MG132 (Figure 2.4c).

2.2.7 The VWA domain of hRpn10 is required for the degradation of FAT10, *in vivo*

As FAT10 was found to interact with the VWA domain of hRpn10 (Figure 2.2d), we next investigated if this interaction would have functional relevance for the degradation of FAT10 *in vivo*. First, we determined the degradation rate of ectopically expressed HA-FAT10 in WT and *rpn10 Δ* yeast cells by cycloheximide chase analysis. HA-FAT10 was readily degraded in WT cells with a half-life of approx. 2 h, whereas it was stable over the 6 h chase period in *rpn10 Δ* cells (Figure 2.5a, f). HA-FAT10 degradation was almost completely restored in *rpn10 Δ* cells upon ectopic expression of either scRpn10 or hRpn10 from a low-copy plasmid (Figure 2.5b, f). Remarkably, expression of only the VWA domain was sufficient to restore degradation of HA-FAT10 in cells lacking Rpn10 (Figure 2.5c, f). Likewise, hRpn10 with mutated UIMs also restored HA-FAT10 degradation to wt-like levels. To confirm the earlier finding that the degradation of FAT10 is independent of ubiquitin (Hipp et al., 2005; Schmidtke et al., 2009), we analyzed the degradation of lysine deficient FAT10 in the similar experimental setup and observed no significant effect in its degradation (Supplementary Figure 2.3). Taken together, these results show that the VWA domain alone is sufficient to sustain the degradation of FAT10 and that Rpn10 was sufficient to, at least partially, sustain the degradation of FAT10 even in the absence of NUB1L.

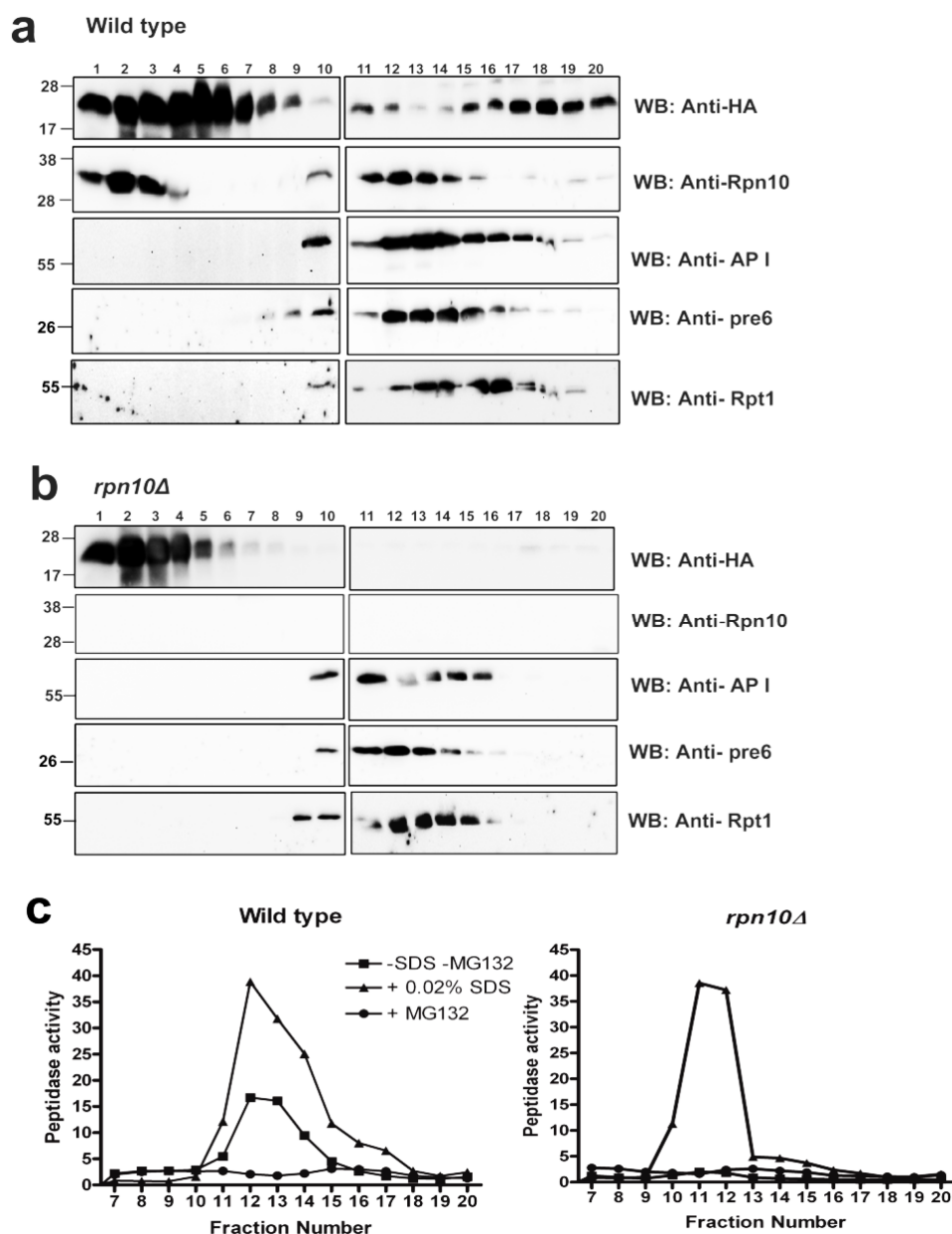


Figure 2.4 Glycerol gradient fraction analysis showing the interaction of FAT10 with the yeast proteasome. (a) Whole cell lysate from the wild type yeast transformed with a low-copy plasmid expressing HA-FAT10 was loaded onto a 10%-40% glycerol gradient and subjected to ultra-centrifugation (see Experimental Procedures). The fractions were analyzed by western blotting with anti-HA, anti-scRpn10, anti-aminopeptidase I (AP I), anti-pre6 and anti-Rpt1 antibodies. HA-FAT10 migrated to higher fractions of about 600 kDa (fraction 11-12 and 15-20). AP I, which is a decamer of 600 kDa, served as size marker. (b) Glycerol gradient fraction analysis of a whole cell lysate from *rpn10Δ* cells transformed with a low copy plasmid expressing HA-FAT10. Analysis was performed as in (a). (c) Peptidase activity analysis. The fractions of glycerol gradients were analyzed for the proteolytic activity by incubating them with the fluorogenic substrate Suc-LLVY-AMC (■). The treatment of these fractions with MG132 reduced the peptidase activity to basal level (●) which confirmed that this activity relies on the proteasome. After addition of 0.02% SDS, the peptidase activity of 20S proteasome containing fractions was increased (▲). The unspecific background activity by the substrate was subtracted from all the readings. The wild type yeast contains 20S proteasome in fractions 10-15 which corresponds to the western blot analysis. The *rpn10Δ* yeast show less peptidase activity corresponding to fractions 10-13.

An unexpected observation was that FLAG-Rpn10 or FLAG-VWA was also degraded over time (Figure 2.5b, c). We investigated whether this would be dependent on FAT10 and found that this degradation occurred independently of HA-FAT10 (Supplementary Figure 2.4).

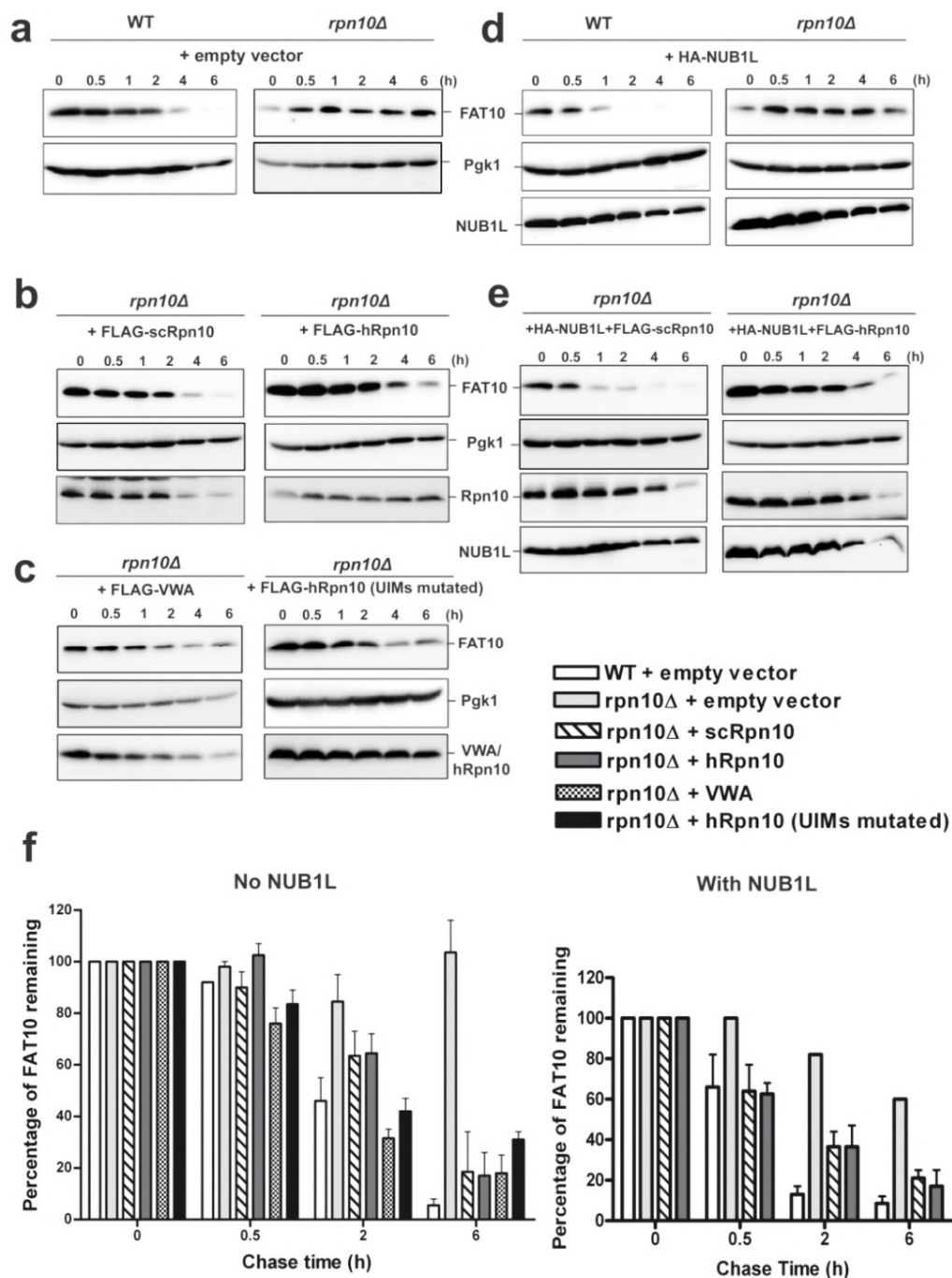


Figure 2.5 Reconstitution of FAT10 degradation in Rpn10-deficient yeast by the VWA domain of hRpn10. Cycloheximide chase analysis of FAT10 stability in WT and *rpn10Δ* cells reconstituted with hRpn10 or variants and/or NUB1L. HA-tagged FAT10 and NUB1L were visualized by anti-HA western blotting whereas FLAG-tagged proteins, scRpn10, hRpn10, VWA and hRpn10-mutated UIMs were detected via FLAG-specific antibodies. Pgk1 served as a loading control. (a) HA-FAT10 degradation in WT

and *rpn10Δ* cells. The experiment was repeated at least three times with similar outcome (quantitation is shown in (f)). (b) Degradation of FAT10 in *rpn10Δ* yeast reconstituted with FLAG-scRpn10 (left) or FLAG-hRpn10 (right). (c) Degradation of FAT10 in *rpn10Δ* yeast reconstituted with FLAG-VWA (left) or FLAG-hRpn10-UIs mutated (right). (d) Accelerated degradation of FAT10 in the presence of NUB1L in wild type but not in *rpn10Δ* yeast. (e) Accelerated degradation of FAT10 in the presence of NUB1L in *rpn10Δ* yeast reconstituted with FLAG-scRpn10 (left) or FLAG-hRpn10 (right). (f) Quantitation of HA-FAT10 cycloheximide chase experiments in the absence of NUB1L (left graph) and in the presence of NUB1L (right graph). Quantitation in each case is based on at least two independent experiments.

2.2.8 NUB1L relies on Rpn10 for promoting the degradation of FAT10

Since the degradation of FAT10 is accelerated by NUB1L (Hipp et al., 2004), and since FAT10 and NUB1L both interact with hRpn10, we investigated whether the stimulating effect of NUB1L on the degradation of FAT10 would depend on hRpn10 *in vivo*. Cycloheximide chase experiments were performed as described above, except for that HA-NUB1L was also expressed in yeast cells. The HA-FAT10 protein remained stable in *rpn10Δ* yeast cells even in the presence of HA-NUB1L but its degradation was accelerated in the wild type cells (Figure 2.5d). Following reconstitution of HA-NUB1L expressing *rpn10Δ* cells with either FLAG-scRpn10 or FLAG-hRpn10, degradation rates similar to that of the wild type strain expressing HA-NUB1L were observed (Figure 2.5e and Supplementary Figure 2.5). The quantitative analysis revealed that the half-life of HA-FAT10 was reduced to less than 1 h (Figure 2.5f, right panel). Collectively, these results show that the accelerating effect of NUB1L on FAT10 degradation is strictly dependent on hRpn10.

2.2.9 Asp11 residue in the VWA domain is crucial for the degradation of FAT10

Previous study presented the importance of Asp11 residue in the DXS motif of the VWA domain in the degradation of ubiquitin-fusion degradation (UFD) pathway proteins (Fu et al., 2001). The substitution of Asp11 with glutamate but not arginine, alanine or lysine had no effect on the degradation of UFD pathway substrates which could be due to the possibility that the salt bridge formed by Asp11 helps to maintain the structural integrity of the VWA domain and thereby, important for the association of the base with the lid (Fu et al., 2001). Considering this effect, we analyzed the degradation of FAT10 in *rpn10Δ* yeast strain reconstituted with FLAG-Rpn10ΔD11 or FLAG-hRpn10ΔD11. As expected, we observed a stabilization of FAT10 under these conditions as compared to the *rpn10Δ* reconstituted with FLAG-Rpn10 or FLAG-hRpn10 (Figure 2.6a, b). We also

analyzed if FAT10 interacts with Asp11 residue of VWA domain by performing GST-pull down assays but His-VWA Δ D11 was capable of interacting with FAT10 (Chapter 3, Figure 3.5). These results suggests that Asp11 residue is not required for the interaction with FAT10 but this residue attenuates the proteolytic activity of the proteasome probably due to loss of association of base with the lid and this led to the accumulation of FAT10. It also supported the above results presenting the degradation of FAT10 dependent on the VWA domain of hRpn10, and further argued for the proper incorporation of the VWA domain into the proteasome in the *rpn10 Δ* yeast.

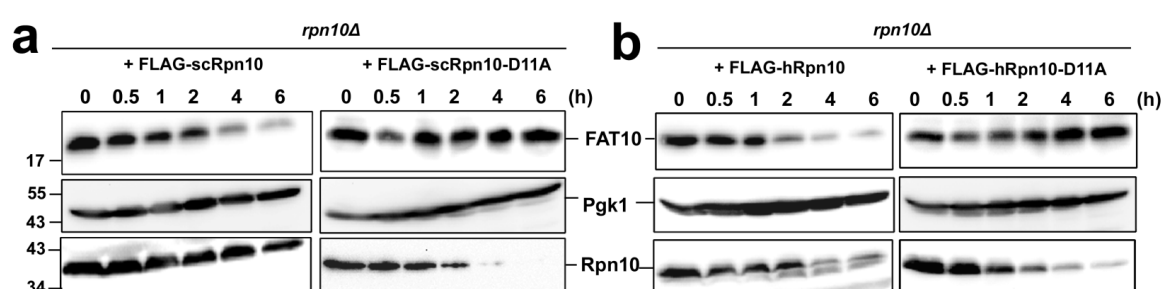


Figure 2.6 Asp11 residue in the VWA domain is important for the degradation of FAT10. Cycloheximide chase analysis of FAT10 stability in *rpn10 Δ* cells reconstituted with Rpn10 or Rpn10D11A (yeast and human). HA-tagged FAT10 was visualized by anti-HA western blotting whereas FLAG-tagged proteins, scRpn10, hRpn10, scRpn10D11A and hRpn10D11A were detected via FLAG-specific antibody. Pgk1 served as a loading control. (a) Stabilization of FAT10 in *rpn10 Δ* yeast strain reconstituted with scRpn10D11A. (b) Stabilization of FAT10 in *rpn10 Δ* yeast strain reconstituted with hRpn10D11A. The experiments were repeated at least two times with similar outcome.

2.2.10 hRpn10 VWA domain mediated degradation of FAT10 is proteasome dependent

The strict dependence of FAT10 degradation on Rpn10 suggested that FAT10 would be degraded by the proteasome in yeast. However, the majority of Rpn10 in yeast is present as free protein in the cytosol and only a fraction of it is found associated with the proteasome (Fu et al., 1998) (and Figure 2.4a). To determine whether FAT10 would indeed be degraded by the proteasome, we investigated if FAT10 could be stabilized by the proteasome inhibitor MG132 (Lee and Goldberg, 1996). We disrupted the *PDR5* gene in WT and *rpn10 Δ* cells, as wild-type yeast cells readily export MG132 via the multi-drug transporter Pdr5 rendering them resistant to MG132. Cycloheximide chase experiments in the presence of MG132 were performed in *pdr5 Δ* (NRY51) and *pdr5 Δ rpn10 Δ* (NRY53)

cells transformed with an expression plasmid for HA-FAT10 alone or in combination with an expression plasmid for FLAG-hRpn10 or FLAG-VWA. Strikingly, addition of MG132 led to a strong stabilization of FAT10 in all the transformants tested, even in the *pdr5Δrpn10Δ* strain reconstituted with only the VWA domain (Figure 2.7a-c).

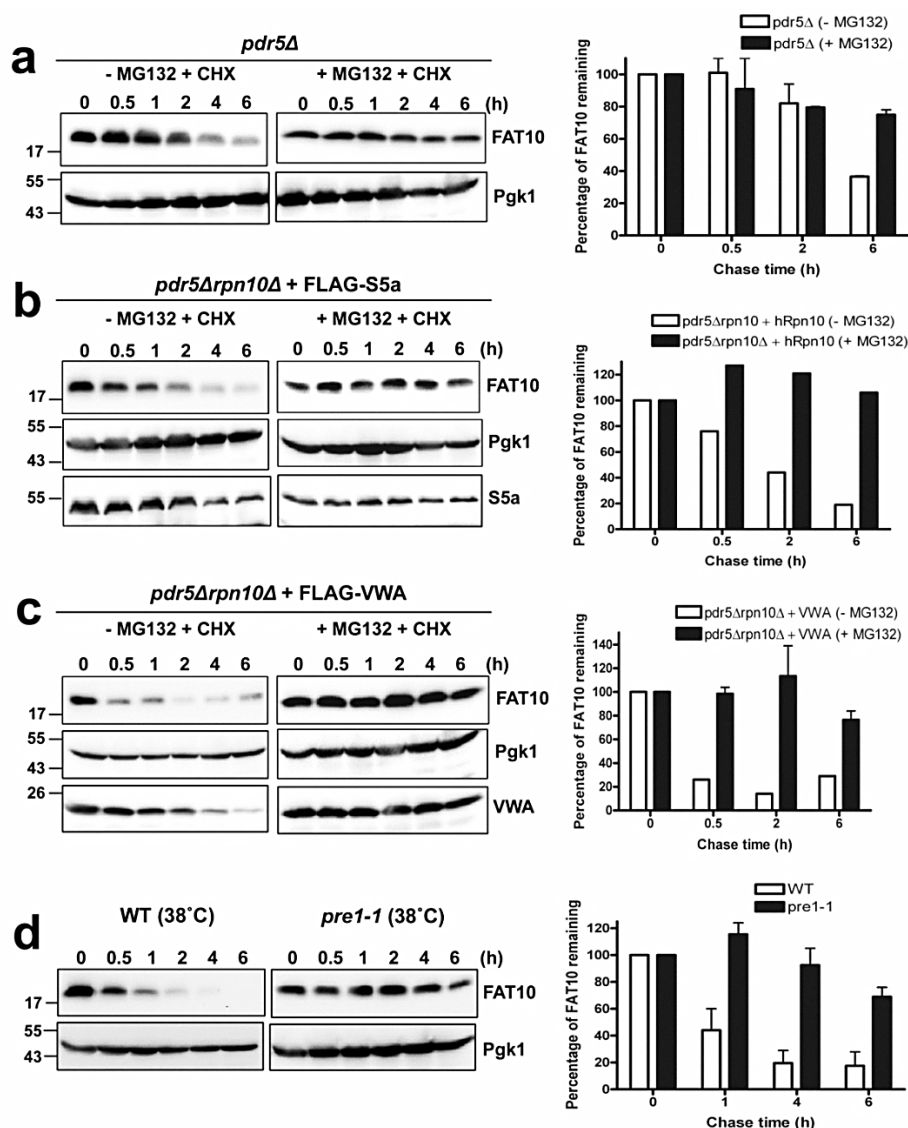


Figure 2.7 The degradation of FAT10 *in vivo* is dependent on the proteasome activity. (a) Left panel: HA-FAT10 cycloheximide chase analysis in *pdr5Δ* yeast cells in the presence or absence of the proteasome inhibitor MG132. Right panel: Quantitation of HA-FAT10 band in the western blot. (b) Left panel: Cycloheximide chase analysis as in (a) but with *pdr5Δrpn10Δ* cells ectopically expressing FLAG-hRpn10. Right panel: Quantitative assessment of HA-FAT10. (c) Left panel: Cycloheximide chase analysis as in (a) with *pdr5Δrpn10Δ* cells ectopically expressing FLAG-VWA. Right panel: Quantitative assessment of HA-FAT10. The experiments have been repeated two times yielding similar results. (d) Left panel: Degradation of HA-FAT10 in wild type (WCG4a) and *pre1-1* yeast strain. Right panel: Quantitation of HA-FAT10 degradation.

Additionally, the degradation of FAT10 was analyzed in *prel-1* yeast strain that exhibit reduced protein degradation and accumulation of ubiquitin-protein conjugates under stress conditions (Heinemeyer et al., 1991). Cycloheximide chase experiments were performed by transforming HA-FAT10 in *prel-1* and its parental wild-type yeast strain. We observed the stabilization of FAT10 in *prel-1* strain under heat stress conditions (grown at 38°C) as compared to the wild-type strain (Figure 2.7d). These data strongly suggests that the degradation of FAT10 is proteasome dependent. Furthermore, the reconstitution experiments demonstrate that the VWA domain of hRpn10 is involved in this process.

2.3 DISCUSSION

Degradation of proteins by the 26S proteasome is regulated at several levels. One of them is the docking step to the RP of the 26S proteasome which, in the case of ubiquitin system, is mediated by direct binding of poly-ubiquitin chains to the canonical proteasome subunits Rpn10 or Rpn13 or by binding to soluble ubiquitin receptors of the UBL-UBA family which in turn bind to the Rpn1 or Rpn2 subunits of the RP (Finley, 2009). Interestingly, these two binding modes seem to mutually affect each other as the degradation of poly-ubiquitylated Sic1 at the isolated 26S proteasome via Rad23 was shown to be facilitated by binding of the VWA domain of Rpn10 to the proteasome (Verma et al., 2004). Similar evidence regulating the pace of proteasomal degradation has also been observed for the FAT10 system where NUB1L was shown to accelerate FAT10 degradation about eightfold in the cells (Hipp et al., 2004). This stimulating effect relied on the N-terminal UBL domain of NUB1L required for the proteasome binding and was independent of the three UBA domains of NUB1L which bind FAT10 (Schmidtke et al., 2006). FAT10 therefore resembles ubiquitin in that it can bind directly to the proteasome as well as become tethered to the proteasome via a UBL-UBA protein, i.e. NUB1L. To better understand how NUB1L influences the rate of FAT10 degradation, we set out to identify the subunit(s) of the RP which bind FAT10. From all RP subunits, Rpn10 was the only one that interacted with FAT10 in our yeast two hybrid analysis. Interestingly, none of the subunits, known to interact with ubiquitin, namely Rpn13, or Rpt5, showed an interaction with FAT10 in this analysis. We cannot rule out from these data that other RP subunits may also bind FAT10, as false negatives cannot be excluded during yeast-two

hybrid screens, but at least Rpn13 or Rpt5 subunits did not significantly bind to FAT10 in pull down assays also (Figure 2.2b and data not shown).

The finding that FAT10 and NUB1L bind the same subunit raised the important question how NUB1L may nevertheless act as a facilitator of FAT10 mediated degradation. One possibility was that FAT10 and NUB1L bind to different domains of Rpn10. It came, therefore, as a major surprise to find both of them binding to the same domain. Even more surprising was the finding that these two ubiquitin-like proteins do not bind to the UIM domains of Rpn10, like ubiquitin, but instead bind the VWA domain of Rpn10, which has never been proposed to bind ubiquitin-like domains, thus, making the VWA domain a new UBL binding domain (Figure 2.2e). It will be interesting to investigate whether VWA domain binding is also an intrinsic property of other UBL domain protein(s). The VWA domain of various hRpn10 orthologs shares about 25% identity (Supplementary Figure 2.2a). VWA domains are characterized by a β -sheet sandwiched by multiple α -helices and they usually bind metal ions via a metal ion-dependent adhesion site (MIDAS). It was hypothesized that the VWA domain in intracellular proteins mediate protein-protein interactions which might be involved in the assembly or function of multi-protein complexes (Whittaker and Hynes, 2002). We have extensively tried to identify the residues within the VWA domain that are directly involved in FAT10- and NUB1L-binding by mutagenesis of the VWA domain. However, so far all attempts including several combined point mutations were unsuccessful in abolishing the binding whereas the deletion variants, on the other hand, could not be stably expressed (N. Rani, unpublished data). It was, therefore, quite comforting that functional data in the yeast supported the *in vitro* binding studies and the central role of the VWA domain in FAT10 degradation.

Here, we showed that the human Rpn10 subunit was able to reconstitute growth of *rpn10 Δ S. cerevisiae* cells in canavanine sensitivity assay (Figure 2.3) which, at least to our knowledge, has never been shown before. Considering that there is merely 50% identity between yeast and human Rpn10 and the fact that human Rpn10 has two UIMs while yeast protein contains only one, a functional transcomplementation of the yeast protein by the human counterpart was not necessarily expected. Rpn10 is required to keep the base and the lid of the RP firmly associated (Fu et al., 1998) and our

transcomplementation data with human Rpn10 suggest that the human ortholog can serve this structural function also in the context of the yeast 26S proteasome. Moreover, human Rpn10 co-migrated, at least partially, with the 26S proteasome in yeast transfectants indicating that it was readily incorporated into the yeast proteasome (Figure 2.4).

The reconstitution of Rpn10 deficient budding yeast cells with human Rpn10 encouraged us to test further if FAT10 degradation could be reconstituted in yeast. This again is not self-evident as FAT10 is only found in mammals and not in lower eukaryotes (Groettrup et al., 2008). The reconstitution of FAT10 degradation in yeast seemed justified as Rpn10 has been shown to be essential in mouse cells (Hamazaki et al., 2007) and is required for normal degradation of ubiquitin conjugates in *Drosophila* (Lundgren et al., 2003). We could show that FAT10 is degraded in yeast in a Rpn10 dependent manner (Figure 2.5). The fact that FAT10 degradation was supported by both, the yeast and human Rpn10, is consistent with the finding that not the UIMs (which differ in number between human and yeast) but the VWA domain of Rpn10 is required for FAT10 degradation. This system further allowed us to show that the VWA domain sufficed to robustly reconstitute FAT10 degradation (Figure 2.5e) which is in accordance with our binding studies (Figure 2.2e). Interestingly, the VWA domain could largely but not completely restore wild type-like growth of Rpn10 deficient yeast on canavanine containing plates (Figure 2.3). Consistently, mice bearing only a VWA domain survive longer than mice which completely lack mRpn10 (Hamazaki et al., 2007). These results could be interpreted in a way that the facilitator function of VWA (Verma et al., 2004) promotes the degradation of misfolded poly-ubiquitylated proteins via Rad23. The finding that NUB1L and FAT10 bind to the same domain of the same RP subunit encouraged us to re-investigate by pull-down experiments whether NUB1L might bind to either Rpn1 or Rpn13 in addition to Rpn10. Indeed, we observed that NUB1L but not FAT10 binds hRpn1 (Figure 2.2b, c) reminiscent of the UBL-UBA protein Rad23 in the ubiquitin pathway.

Earlier, the only subunit known to interact with NUB1L was hRpn10 and it was shown that the C-terminus of NUB1L containing the three UBA domains binds to hRpn10 (Tanji et al., 2005). Another study reported that the association of NUB1L with the 26S proteasome would occur via the N-terminal UBL domain (Schmidtke et al., 2006). We could show that UBL domain of NUB1L binds the hRpn1 subunit as well as hRpn10 of the 26S proteasome in accordance with the latter report (Figure 2.2c). Interestingly, we

could also show that the C-terminal UBL domain of FAT10 binds hRpn10 (Figure 2.2d) which seems to be reasonably valid as the N-terminal UBL domain of FAT10 docks to NUB1L (Schmidtke et.al., 2006).

Considering that Rpn1 binds to the Rpn10 subunit and the ATPases Rpt1/S7 and Rpt2/S4 within the base of the 19S regulator (Fu et al., 1998), our findings may explain why NUB1L serves as a potent accelerator of FAT10 degradation. Since Rpn1 knock out yeast are not viable (Hampton et al., 1996), deletion studies on the function of Rpn1 could not be performed. However, the key findings of this study can be exploited to design models how NUB1L may accelerate the degradation of FAT10 (Figure 2.8). FAT10 might directly bind to the VWA domain of hRpn10 and become degraded at a slow rate (Figure 2.8a) whereas NUB1L could bind weakly to the VWA domain of hRpn10 as well as strongly to the hRpn1 subunit. The second possibility could be that FAT10 gets transferred to hRpn10 subsequent to its interaction with the UBA domains of NUB1L which remains bound to the hRpn1 subunit (Figure 2.8b). Alternatively, conformational change(s) could be induced in hRpn10 when NUB1L binds either hRpn1 (Figure 2.8c) or Rpn10 (Figure 2.8d), thus functioning as a facilitator for the degradation of FAT10 and its conjugates. Testing the validity of the different models will help to further characterize FAT10 mediated degradation at the 26S proteasome.

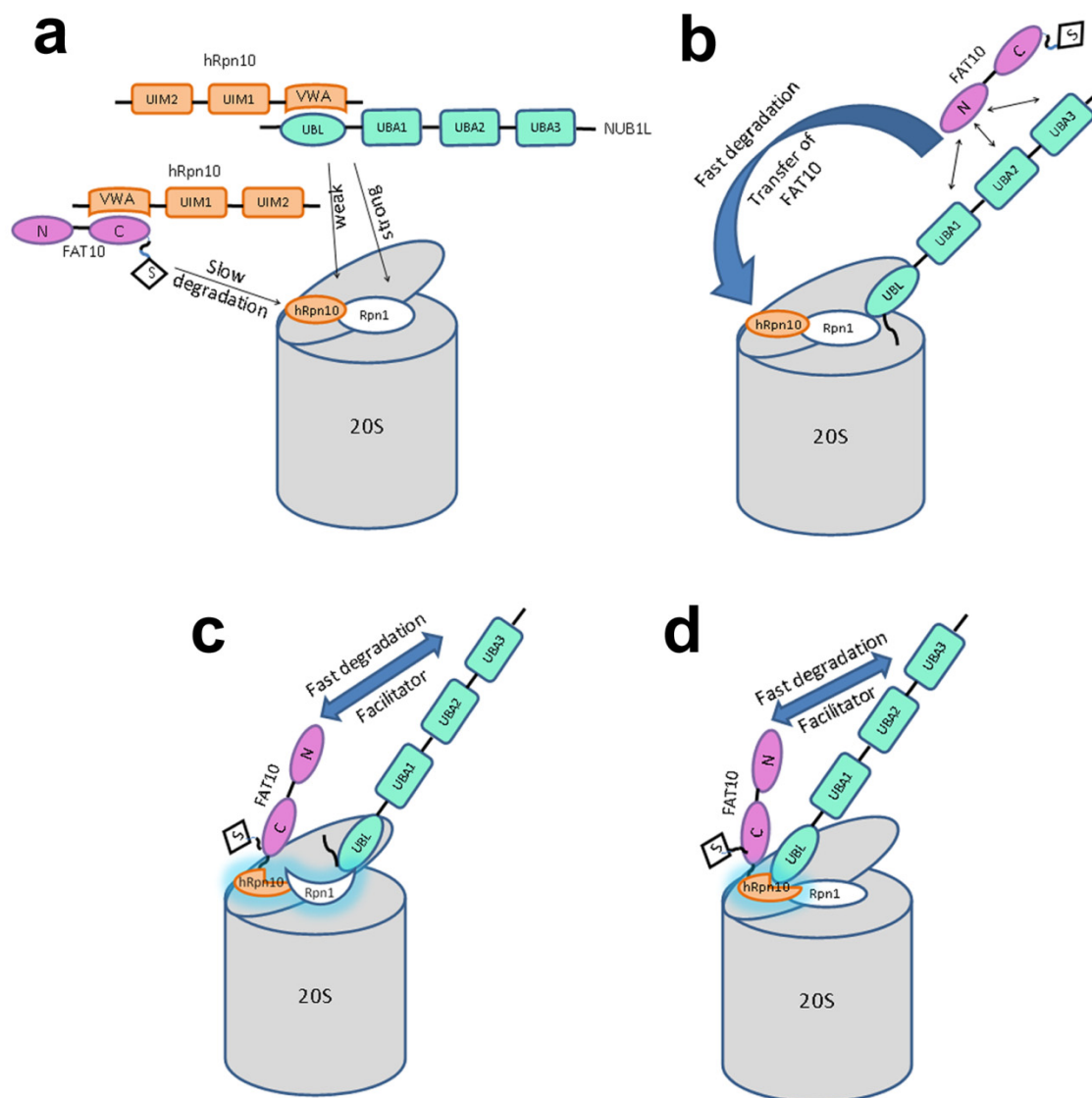


Figure 2.8 Models for the degradation of FAT10. (a) Slow degradation of FAT10 and its conjugates (represented by ‘S’) by 26S proteasome when its C-terminal UBL domain interacts with the VWA domain of hRpn10 in the absence of NUB1L. The UBL domain of NUB1L docks onto the VWA domain of hRpn10 weakly as compared to its binding to hRpn1. (b) Model showing the accelerated degradation of FAT10 when NUB1L transfers FAT10 to hRpn10. The UBL and UBA domain(s) of NUB1L interact with the hRpn1 subunit and the N-terminal UBL domain of FAT10, respectively. (c) Model showing the facilitator function of NUB1L for the degradation of FAT10 and its conjugates by the 26S proteasome. FAT10 docks onto hRpn10 while its degradation is facilitated in the presence of NUB1L, bound to hRpn1, in its vicinity by inducing conformational changes in both of the subunits. (d) Facilitator function of NUB1L when both FAT10 and NUB1L bind to hRpn10. The model suggests a conformational change in hRpn10 when NUB1L binds to it leading to an enhanced degradation of FAT10.

2.4 METHODS

2.4.1 Strains and Plasmids

Plasmid cloning and yeast strains are described in Supplementary Information Supplementary Table 2.1 and Supplementary Table 2.2).

2.4.2 Yeast Two Hybrid Analysis

See supplemental information.

2.4.3 GST-Pull Down Assay

GST, GST-FAT10, GST-NUB1L, GST-NUB1L Δ UBA, GST-NUB1L Δ UBL (see supplementary information) and His-hRpn10 (Beal et al., 1998) (kindly provided by Patrick Young, Stockholm University) were expressed in *E.coli* strain B834(DE3)pLysS as described (Hipp et al., 2004). Glutathione sepharose 4B beads (GE Healthcare) were incubated with GST, GST-FAT10 or GST-NUB1L along with purified His-hRpn10 in incubation buffer (IB) (20 mM Tris-HCl, pH 7.4, 0.1% TritonX-100) overnight at 4°C. Beads were washed 5 times with IB. For another set of experiments, the full-length His-hRpn10, His-UIM1+UIM2 domains (in pET26b, a kind gift from Patrick Young) and His-VWA domain (in pET26b) were *in vitro* transcribed and translated using TNT T7 coupled reticulocyte lysate system (Promega) and purified GST, GST-FAT10 or GST-NUB1L bound on the GSH beads was added. Proteins bound to the beads were eluted by boiling at 95°C for 5 min with SDS buffer, resolved by SDS-PAGE and subjected to western blot analysis using anti-His antibody (Sigma) or autoradiography. Experiments with GST-NUB1L Δ UBA or GST-NUB1L Δ UBL were performed similarly.

2.4.4 Transfection of HEK 293T Cells and Co-immunoprecipitation

The already described (Schmidtke et al., 2006) cDNA's of HA-FAT10-GFP, HA-Ubiquitin-GFP, GFP as well as the N- and C-terminal UBL domains of FAT10 (HA-N-FAT10-GFP and HA-C-FAT10-GFP) were transfected into HEK 293T cells and harvested the next day. Cells were washed and lysed in 250 μ l of 50 mM Tris-HCl (pH 7.8) and 0.1% TritonX-100. After centrifugation and removal of the supernatant, the expression of the proteins was determined by fluorescence measurement at 485 nm excitation wavelength and 535nm emission wavelength. 100 μ l Ni-Beads (Sigma) and 5

µg recombinant purified His-hRpn10 were incubated overnight at 4°C in PBS containing 650 mM NaCl, 0.2 % Triton X-100 and 1 mg/ml methylated BSA. The precipitate was washed and distributed into five aliquots. Equal amounts of fluorescence were loaded onto the Ni-precipitated His-hRpn10 as well as on Ni-beads only. After overnight incubation the beads were washed 5 times with PBS containing 650 mM NaCl, 0.2 % TritonX-100 and once with 5 mM Tris (pH 7.5). Detection of the load and the precipitated proteins was done by western blotting with an anti GFP-antibody (Hiss clone MMS-118P) and the bound His-hRpn10 was detected with an anti-Rpn10 antibody (Abcam) as described earlier (Schmidtke et al., 2006).

2.4.5 Yeast Strain Construction

Gene knock-outs were generated by transforming yeast with the LiOAc method with a PCR product that encoded either G418 resistance or hygromycin resistance and contained 40 bp flanking regions homologous to the gene to be knocked out. For the construction of *rpn10Δ::KanMX6* allele, pFA6a-KanMX6 (Longtine et al., 1998) plasmid was used as template for amplification of the KanMX6 cassette and plasmid pAG32 (Goldstein and McCusker, 1999) was used for construction of the *pdr5Δ::hphMX4* allele. Following transformation, cells were plated on YPD medium, incubated for ~12 hours and were then replica plated onto YPD supplemented with 300µg/ml G418 or 250µg/ml hygromycin B. The presence of the desired null allele was verified by PCR.

2.4.6 Canavanine Sensitivity Assay

Wild type yeast (MHY500) was transformed with empty vector (YEplac181) and the *rpn10Δ* yeast strain (NRY5) was transformed with empty plasmid (YEplac181) or YEplac181 containing FLAG-scRpn10, FLAG-hRpn10, FLAG-VWA or FLAG-hRpn10 (UIMs mutated) and plated on selective plates lacking leucine. After three days of growth, single transformants were selected and inoculated in synthetic media lacking leucine. Cells were grown to log phase (OD₆₀₀ ~1.0), serially diluted (5 fold steps) and spotted on selective plates lacking either leucine (control) or arginine and leucine (with 3µg/ml canavanine). Plates were incubated at 30°C for 5 days.

2.4.7 Glycerol Gradient Centrifugation

Wild type and *rpn10Δ* yeast cells transformed with plasmid p414Met25-FAT10 were grown in selective medium to an OD₆₀₀ of 3.0. After harvesting, cell pellets were resuspended in lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 2 mM ATP, 0.5 mM DTT, 100 mM NaCl and protease inhibitors) and lysed using French press. The supernatant from the lysed cells was loaded onto a 10%-40% glycerol gradient prepared in lysis buffer. The tubes were ultracentrifuged at 40,000 rpm in a Beckmann SW41 rotor for 16 h at 4°C. 600 µl fractions were collected from the top of the gradient and proteins were precipitated by the methanol-chloroform method. Proteins were resolved on SDS-PAGE and blotted with anti-HA-7, anti-AP I (kindly provided by Michael Thumm, Göttingen University) or anti Rpn10 (Abcam) antibodies. For the peptidase activity assay, 20 µl of each fraction was added to 100 µl of fluorogenic substrate Suc-LLVY-AMC (Bachem) with a final concentration of 100 µM in a black 96 well plate and incubated at 37°C for 30 minutes. The peptidase activity was recorded as the measure of fluorescence by Tecan SpectraFluor Plus (excitation λ = 360 nm and emission λ = 465 nm).

2.4.8 Cycloheximide Chase

Cycloheximide (0.25 mg/ml) was added to logarithmically growing yeast cultures (30°C), and aliquots were removed at the indicated times. The experiment with *pre1-1* strain (kindly provided by Wolfgang Heinemeyer, TU Munich, Germany) was performed as described earlier (Heinemeyer et al., 1991). Lysates were prepared immediately using the NaOH lysis method (Kushnirov, 2000) and boiled for 5 min. HA-FAT10 (cloned in p416Met25 vector) and HA-NUB1L (cloned in p414GPD vector) were visualized by western blotting with an anti-HA-7 (Sigma) antibody, hRpn10 and variants (cloned in YEplac181 vector) were detected using an anti-FLAG-HRP antibody (Sigma). Protein degradation after immunoblotting was quantified using Quantity One software (Bio-Rad) and normalized using an anti-Pgk1 (Invitrogen) loading control.

2.4.9 Site directed mutagenesis

Oligonucleotide primers were designed complementary to opposite strands of the vector with the desired mutation. The extension of primers by *Phusion* polymerase generated a plasmid with desired mutation containing staggered nicks. The cycling reaction included denaturation cycle at 95°C for 30 sec; and 12-18 (depending on the number of mutations)

cycles at 95°C for 30sec, 55°C for 1 min and 68°C for 5 min. Following PCR cycles, the product was treated with *Dpn I* enzyme which specifically cleaved methylated and hemimethylated DNA and hence digested the non-mutated parental DNA template. The vector containing desired mutation was further transformed in competent cells and the isolated plasmids from the bacterial colonies were sequenced to identify the plasmid with the desired mutation.

2.4.10 Proteasome Inhibition

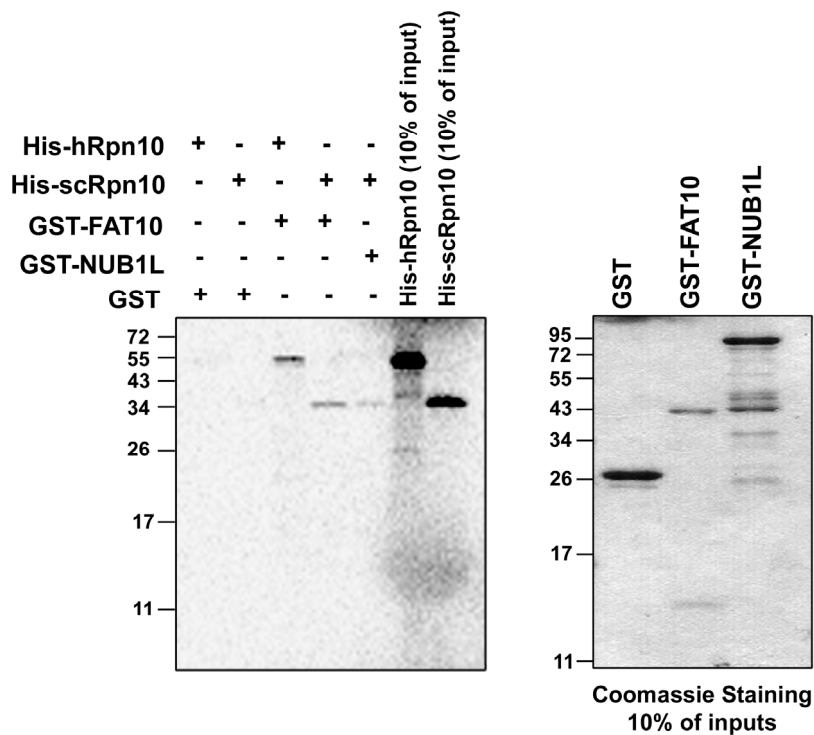
A yeast strain lacking the *PDR5* gene was used for experiments involving inhibition of the proteasome. The proteasome inhibitor MG132 (Sigma) was added to a final concentration of 70 μ M to the cultures prior to the start of the cycloheximide chase (Ji et al., 2009). To ensure proper proteasome inhibition, fresh MG132 was added every 90 minutes during the course of the experiment. FAT10 stability was visualized via immunoblotting with an anti-HA antibody.

2.5 ACKNOWLEDGEMENTS

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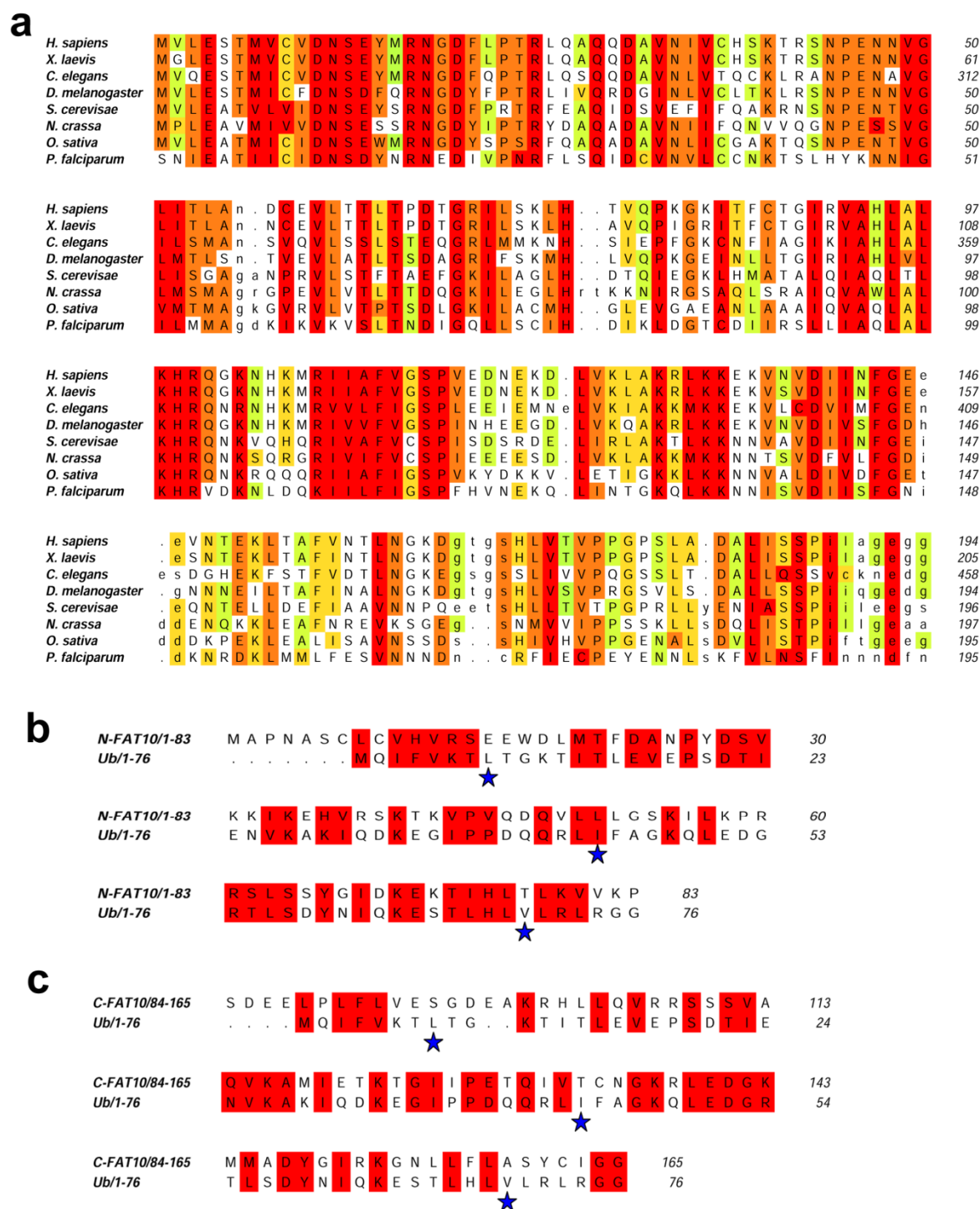
2.6 SUPPLEMENTARY INFORMATION

2.6.1 Supplementary Figures

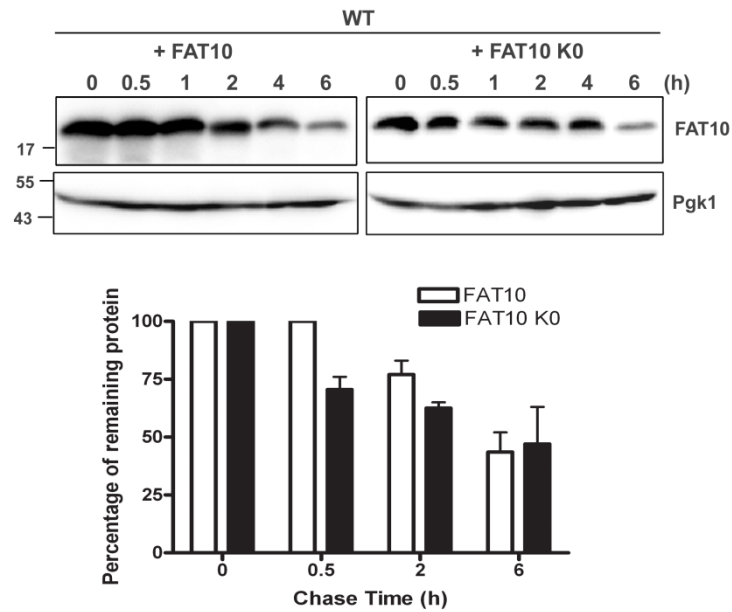


Supplementary Figure 2.1 Interaction of scRpn10 with FAT10 and NUB1L. Recombinant GST, GST-FAT10 or GST-NUB1L was incubated with *in vitro* transcribed and translated His-hRpn10 or His-scRpn10 along with GSH beads. The pull down showed that His-scRpn10 interacts with GST-FAT10 as well as GST-NUB1L. The interaction of hRpn10 with GST-FAT10 served as positive control.

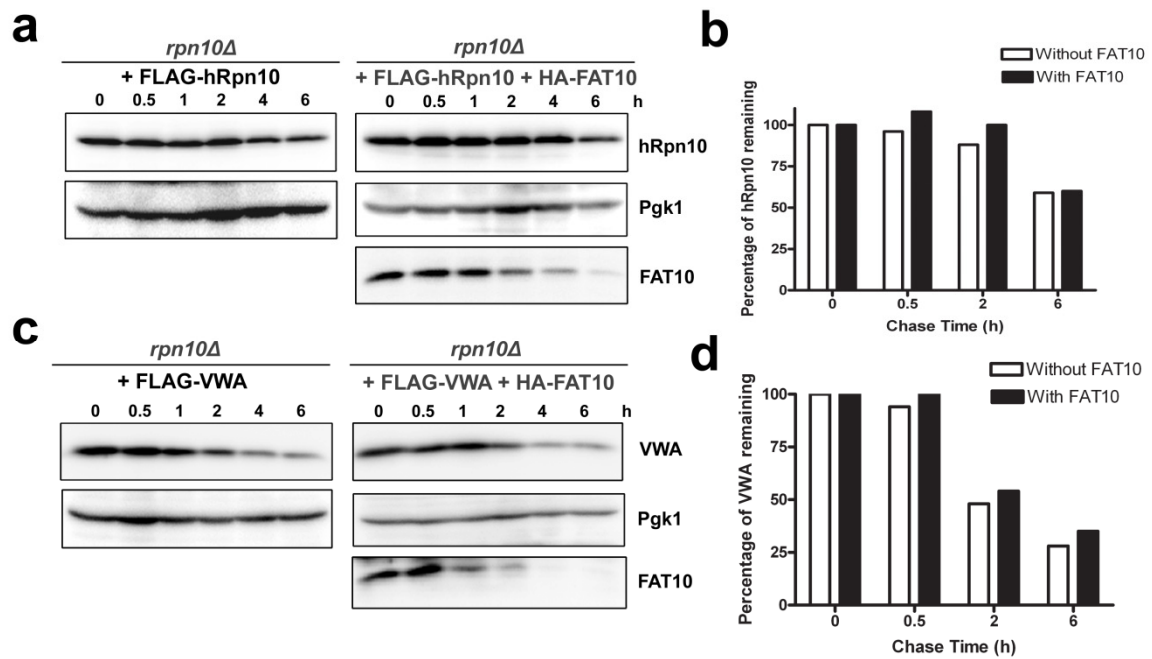
2. Proteasomal targeting of FAT10 by the VWA domain of hRpn10 and NUB1L



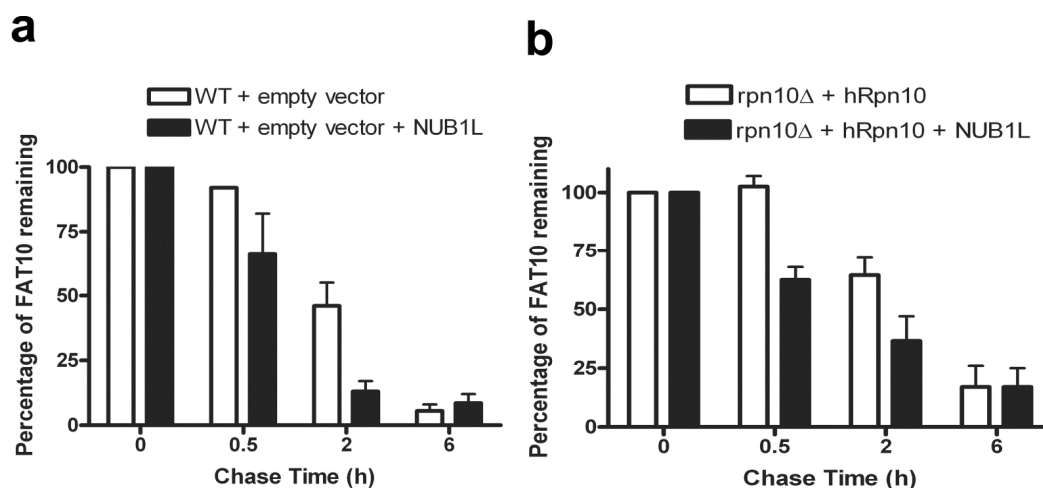
Supplementary Figure 2.2 Amino acid sequence alignments. (a) Multiple alignment of amino acid sequences of the VWA domain of various orthologs of Rpn10 of the 26S proteasomes. The degree of sequence similarity is highlighted using the cyan-to-red color code using ALINE software. The percent identity of the sequences is approximately 25% as calculated in Jalview. (b) Amino acid sequence alignment of the N-terminal UBL domain of FAT10 and ubiquitin showing the non-alignment of Leu8 and Val70 present in ubiquitin (annotated with stars). (c) The alignment of the C-terminal UBL domain of FAT10 and ubiquitin amino acid sequence showing the non-alignment of Leu8, Ile44 and Val70 of ubiquitin (annotated with stars).



Supplementary Figure 2.3 Degradation of FAT10 is independent of ubiquitin. Upper panel: Cycloheximide chase for 6 hours was performed after transformation of wild type yeast with either FAT10 or lysine deficient FAT10 (FAT10 K0). Pgk1 blots served as loading control. Lower panel: Quantitative assessment of the degradation of FAT10 or FAT10 K0 showing the degradation of FAT10 K0 comparable to the degradation of FAT10.



Supplementary Figure 2.4 No effect of FAT10 on the degradation of hRpn10 or VWA domain. (a) Cycloheximide chase for 6 hours was performed, as explained earlier, in *rpn10Δ* yeast reconstituted with only FLAG-hRpn10 or together with HA-FAT10. Pgk1 western blots served as loading controls. The anti-FLAG-HRP blot showed that the degradation of hRpn10 is independent of FAT10 expression. (b) Quantitative assessment of the western blot in panel (a) showing no effect on the rate of degradation of hRpn10 in the presence and absence of FAT10. (c) A similar experiment was performed for the analysis of the degradation of the VWA domain of hRpn10 in the presence and absence of FAT10. The western blot with anti-FLAG-HRP antibody showed that there is no effect of FAT10 on the degradation of the VWA domain. (d) Graph representing the quantitative analysis of the VWA protein bands in (c).



Supplementary Figure 2.5 NUB1L accelerated the degradation of FAT10 in the presence of hRpn10.

(a) Graph representing the quantitative assessment of the FAT10 protein bands in the western blot shown in Fig. 5a and b (data from at least two set of experiments). More than 75% of FAT10 was degraded in about 2 h in the presence of NUB1L (black bars). (b) Cycloheximide chase of FAT10 showing that hRpn10 can reconstitute the degradation of FAT10 (white bars) which gets accelerated in the presence of NUB1L (black bars). The graph represents the mean value from two sets of experiments.

2.6.2 Supplementary Methods

2.6.2.1 Yeast two hybrid analysis

The cDNAs encoding human FAT10 and NUB1L were cloned into the plasmid pGADT7 (Clontech) containing the GAL4 activation domain and were used as bait to screen all subunits of the human 19S regulatory particle. The cDNA of each individual human 19S RP subunits cloned into the pGBD vector (Clontech) (Yao et al., 2006) was generously provided by Robert E. Cohen (Colorado State University); Rpn13 (Husnjak et al., 2008) was also included in this test (a kind gift from Koraljka Husnjak, Frankfurt University). The bait construct was transformed into the yeast strain AH109 by standard LiCl transformation (Gietz and Schiestl, 2007) and tested for self-activation by X-gal filter assay on selection plates lacking either leucine (for FAT10 and NUB1L) or tryptophan (for RP subunits). For the interaction analysis, FAT10 or NUB1L was co-transformed with the prey constructs encoding the different RP subunits. The co-transformants were grown on selection plates in the presence of 1 mM 3-AT at 30°C. After incubation for 4 days, the colonies were replicated on Whatman filter paper, frozen in liquid nitrogen, soaked in PBS containing X-gal (100 µg/ml) and incubated at room temperature. The blue color development indicated a positive interaction. For the positive control, pLexA-

FAT10 (Aichele et al., 2010) was co-transformed with pGADT7-NUB1L in the NMY51 yeast strain.

2.6.2.2 Multiple protein sequence alignment

The amino acid sequence of the VWA domain of hRpn10 of 26S proteasome from different organisms was obtained from Conserved Domains Database (Marchler-Bauer et al., 2011) and were aligned and formatted using Jalview (Waterhouse et al., 2009) and ALINE (Bond and Schüttelkopf, 2009). The percent identity was calculated using Jalview. The alignments for N- and C-terminal FAT10 with ubiquitin were performed using CLUSTAL W (Thompson et al., 1994) and formatted using ALINE.

Supplementary Table 2.1 Yeast strains. The table representing the yeast strains used in this study along with the designation and genotype.

Strains	Genotype	References
AH109	<i>MATa, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ, MEL1</i>	Clontech
NMY51	<i>MATa his3Δ200 trp1-901 leu2-3,112 LYS2::(lexAop)4-HIS3 ura3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4</i>	Dualsystems Biotech
MHY500	<i>MATa, his3-Δ200, leu2-3,112 ura3-52, lys2-801 trp1-1</i>	(Chen et al., 1993)
NR5	MHY500 with <i>rpn10Δ::kanMX6</i>	This study
NR51	MHY500 with <i>pdr5Δ::hphMX4</i>	This study
NR53	MHY500 with <i>rpn10Δ::kanMX6, pdr5Δ::hphMX4</i>	This study
WCG4a	<i>MATa, his3-11,15 leu2-3,112 ura3 Can^S GAL2</i>	(Heinemeyer et al., 1997)
YHI29/1	<i>pre1-1</i>	(Palanimurugan et al., 2004)

Supplementary Table 2.2 Gene cloning. The table showing the genes cloned into different vectors along with the restriction sites used.

Insert	Vector	Restriction site	Reference for vectors
FAT10	pGADT7	BamHI and XhoI	Clontech Laboratories
NUB1L	pGADT7	NdeI and ClaI	Clontech Laboratories
hRpn10	YEplac181	EcoRI and KpnI	(Gietz and Sugino, 1988)
VWA	pET26b	NdeI and HindIII	Novagen
VWA	YEplac181	EcoRI and KpnI	-----
UIM1UIM2	YEplac181	EcoRI and KpnI	-----
hRpn10 (UIMs mutated)	YEplac181	EcoRI and KpnI	-----
FAT10	p416Met25	SpeI and Sall	(Mumberg et al., 1994)
NUB1L	p414GPD	SpeI and Sall	(Mumberg et al., 1994)
scRpn10	pET45b(+)	KpnI and HindIII	Novagen
NUB1LAUBA	pGEX2TKS	NotI and XbaI	GE Healthcare (pGEX2TK modified)
NUB1LAUBL	pGEX2TKS	NotI and XbaI	-----
FAT10 K0	p416Met25	SpeI and Sall	-----
Rpn10ΔD11	YEplac181	EcoRI and KpnI	-----
hRpn10ΔD11	YEplac181	EcoRI and KpnI	-----

3. Studies on the Structure of the hRpn10 Subunit of the 26S Proteasome and the UBL-UBA Domain Protein NUB1L

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3.1 INTRODUCTION

The major ubiquitin receptor in the 26S proteasome was recognized as Rpn10 which initiates the degradation of proteins conjugated to polyubiquitin chains (Deveraux et al., 1995a; Ferrell et al., 1996; Voges et al., 1999). The Rpn10 subunit also interacts with proteins containing UBL/UBA domains like Rad23/hHR23a (yeast/human) and Dsk2/PLIC1 (yeast/human) via the UBL domain (Funakoshi et al., 2002; Hiyama et al., 1999; Schaubert et al., 1998; Walters et al., 2002), whereas another UBA domain containing protein p62 (sequestosome-1) interacts via the PB1 domain (which is similar to the UBL domain as it forms a ubiquitin-like β -grasp fold) (Bjorkqvist et al., 2008). The hRpn10 subunit is characterized by three domains, namely, the N-terminal von Willebrand A (VWA) domain and two C-terminal ubiquitin-interacting motifs (UIM1 and UIM2) (Hofmann and Falquet, 2001; Whittaker and Hynes, 2002; Zhang et al., 2009). The UIM domain is composed of ~20 amino acids with α -helical structure. It is usually found in proteins involved in ubiquitylation and ubiquitin metabolism, or known to interact with ubiquitin-like modifiers (Hofmann and Falquet, 2001). The VWA domain is characterized by Rossmann folds consisting of β -sheet sandwiched by multiple α -helices. The domain is found in extracellular matrix proteins and in integrin receptors, and usually involved in cell adhesion (Whittaker and Hynes, 2002). One of the most important noncontiguous sequence motif in this domain is a metal-ion dependent adhesion site (MIDAS) (D-x-S-x-S....T....D) which is involved in its binding to metal ions (Whittaker and Hynes, 2002). The amino acid sequence alignment of this domain in hRpn10 subunit in various orthologs is shown in the Supplementary Figure 2.2 (Chapter 2).

Polyubiquitin interacts with hRpn10 through UIMs (Beal et al., 1998) but in our previous studies we observed that the VWA domain of hRpn10 interacts with FAT10 (Figure 2.2e, Chapter 2). NUB1L, a non-covalent interaction partner of FAT10, also binds to the VWA domain of the hRpn10 subunit (Figure 2.2e, Chapter 2). NUB1L is composed of a N-terminal UBL domain and three C-terminal UBA domains (Tanaka et al., 2003). UBL domains mimic ubiquitin in function and structure. They also form a β -grasp fold with an α -helix similar to ubiquitin. They play an essential role in the interaction with the 26S proteasome (Hartmann-Petersen and Gordon, 2004; Hochstrasser, 2009; Jentsch and Pyrowolakis, 2000; Su and Lau, 2009). The UBA domain consists of approximately 45 amino acids and is found in diverse proteins involved in the ubiquitin-proteasome

pathway. It is characterized by relatively poor sequence conservation (Hofmann and Bucher, 1996). It comprises of a compact bundle of three α -helices and a small conserved hydrophobic surface formed by the C-terminus of α -helix 1, loop 1, and α -helix 3 (Madura, 2002). There are functional differences between the UBA domain proteins (Withers-Ward et al., 2000) suggesting that the hydrophobic surface may be a more general protein–protein interaction module. Amino acid residues, which are conserved, contribute to the structural similarity of UBA domains whereas diverse residues allow the interaction with different proteins. The alignment of representative UBA domains in the protein database as obtained by SMART analysis shows several consensus sequences (Figure 3.1 and Figure 3.2).

Various bioinformatics prediction, analysis and modeling tools can be utilized to generate hypotheses and theoretical models. The most extensively employed application is to derive functional information about proteins. Another important application includes designing site-directed mutagenesis experiments and thereby predicting ligand binding site(s). The protein data bank (PDB) is widely used as a source of information about the structure of proteins, both experimentally and theoretically. The protein structure prediction methods are basically classified into two categories: (a) comparative modeling methods predict the structure based on the known protein structures (Bowie et al., 1991; Sali and Blundell, 1993), which could be either a sequence-sequence comparison based approach (Karplus et al., 1998) or a sequence-structure comparison based approach (threading) (Bowie et al., 1991; Jones et al., 1992); (b) *ab initio* methods predict structures directly from the protein sequence (Li and Scheraga, 1987; Skolnick and Kolinski, 1991). One of the most reliable software for predicting the three-dimensional structure of proteins is HHpred (Soding et al., 2005), which is based on the identification of homologous sequences with the known structures by searching the local or global alignment using alignment database like Pfam (Sonnhammer et al., 1998) or SMART (Ponting et al., 1999; Schultz et al., 1998). This method works quite well because the structural changes are slow during evolution as compared to sequences and therefore, homologous proteins may have a similar structure even if their sequences have diverged. This is the first server which utilizes the pairwise comparison of hidden Markov models (HMMs), which improves the sensitivity. The program calculates the three-dimensional structure using MODELLER software (Sali and Blundell, 1993).

MMU27462+1_3	SQESINQ	LVY	M	--	GFDTV--	VAEA	LRVFG	GN-VOL	RAQT	LAH
ATHAKIN10A_1_1	DEEILQEVIN	M	--	GFDRN--	HLIESLRNRT	QN-DGT	VITYY	LIL		
HS467511_1	LIESLSQMLS	M	--	GFSDGG	WLTRLQTKN	YD-IGA	LDT	IQY		
MMU27462-1_2	RLENVNTIRG	M	--	GYSTQ--	AAKQ	LRHQAR	GN-LDD	ILKV	OLS	
hsu75362_1	PEEIVAIITS	M	--	GFQRN--	QAIQ	LRATN	NN-LER	LDW	IFS	
osu63530_1	LEATIQSILE	MGG		IUDR-D	IVLH	LSAAF	NN-PER	VEY	LYS	
CBL_MOUSE_1	LSSEIERIMS	Q	--	GYSYQ--	DIQK	LVIAH	NN-TEM	KNI	ORE	
hs2671010_1	VDAKIAKIMG	E	--	GFAFE--	EVKR	LEIAQ	NN-VEV	RSI	ORE	
YBE7_YEAST_1	KSLAVEELSG	M	--	GFTEE--	EAHNL	EKCN	WD-LEA	TNE	ULD	
UBPC-YEAST_2	DEVSLTSMLS	M	--	GLNPN--	LCKK	LILNN	GD-VNR	SVEW	VFN	
HUMORF_1_1	ELTALESIE	M	--	GFPRG--	RAEK	LALTG	NQG	EAM	MDW	OME
HUMHHR23A_1_1	YETMLTEIMS	M	--	GFERE--	RVVA	LRASY	NN-PHR	VEY	ILT	
hsu75362-1	DESSVMQAE	M	--	GFPLE--	ACRK	LVYFTG	NMG	EV	FNW	IIV
CELC06E2_2_1	QKKKVDSIE	M	--	GFSL--	ESIL	LGSD	WN-LAD	REQ	OLE	
osu63530-1	EDEAILRTEP	M	--	GFDR--	LVL	DVFFACN	KD-EOL	ANY	OLD	
stsnflpkn_1	DEEILQQVSR	M	--	GLDRD--	QLLD	SLQKRI	QD-DAT	VAYY	OLY	
T24B8.7	YQDNQDMTIT	M	--	GFKSE--	EIDT	LRLSN	ND-VEK	IQY	LTR	
HUMHHR23A-1_2	EKEATEROKA	L	--	GFPE--	LVIO	YFACE	KN-ENL	AME	OLS	
CEUT27A3_6	VRFVAGEML	M	--	GFCE--	ACYR	DAYYSN	GN-VEI	SNW	OME	
yd28_schpo	LNSKIACQVS	M	--	GFDP--	EAAQ	LDARN	GD-LDV	ASF	LL-	
cez20_5	QEENVLAITG	M	--	GFYRE--	QTIA	LRAAF	WN-PDR	VEF	OLN	
DMUBCD4_1_1	CDSKIQRORD	M	--	GFIDEH--	EARAV	LSKEN	WN-LEK	TEG	IFS	
gts1_yeast	YSRQLAEIKD	M	--	GFSDT-N	KNLD	LSAH	GN-INR	IDY	CEK	
upba_dicdi	NSQDVNIIG	M	--	GFIDS--	QAKL	LNKNTK	GN-LER	ADW	IFS	
hsdkg24_1	FEKKVKQIID	IT	--	GFKNQD--	ECVI	LHDCN	GD-VNR	INV	OLE	
hsnadglct_1	PATDIDRILR	A	--	GFTLQ--	EALG	LRHVG	GN-ADL	LLV	OLA	
CEF15C11_2	YASOLEEQQS	M	--	GFSDR-A	RNV	ALTATF	GD-LNA	VER	OLN	
hsstpkemk_1	DPRRTLMVS	M	--	GFYTRE--	EIQD	SLVGQR	YN-EVM	TYL	OLG	
RA23_YEAST_1	RNETIERIME	M	--	GFYQRE--	EVER	LRAAF	NN-PDR	VEY	OLM	
ATTS1169_1_1	LEEKVKRIVE	M	--	GFGDA--	QVRS	LISSG	GD-ENL	LEK	ICS	
ceu22183_1	RIEKLIIQIFQ	L	--	GFNKA--	AILES	VEKEK	FE-DIH	TYL	OLG	
RF2P_DROME	INKSIHAMMA	M	--	GFSEGA	WLTQ	LESVQ	GN-ISA	LDV	MNV	
yang_schpo	YAEQLSQINE	M	--	GFVDF-E	RNVQ	LRRSG	GN-VOG	IES	OLS	
upba-dicdi	NQEVLDITLS	M	--	GFPLV--	RCKK	LLATG	GKD	REL	MNV	IFE
KP78_HUMAN_1	DQKRIDIMVG	M	--	GFYSQE--	EIQES	LSKMK	YD--EIT	ATY	OLL	
MMU27462_1_1	DPSKVHNLQ	L	--	GFETAQ--	EARLG	RACD	GN-VDH	ATH	ISN	
SPAC3F10_13_1	LDTKIKTKN	M	--	GFVSES--	DAKD	SLECG	YD-VES	REF	IFS	
cef49c12_8	YEQAATIRE	F	--	GFEND-E	LIQL	LEQAN	GD-VQA	MEF	IID	
UBC1_BOVIN_1	YTKKIDENCA	M	--	GFDRN--	AVIV	LSKSKS	WD-VET	TEL	OLS	
YSCL9931_9_1	TRMIVERITE	I	--	GFVSSD--	EALL	LLQND	MN-ENE	AGE	LTR	
SC130KBXV_53_1	DNQAVKSOLE	M	--	GFIPHE--	VAVD	LQRTG	GN-LEA	VNE	IFS	
CET12G3_4_1	TEKTFDRULE	M	--	GFSSYN--	VTVA	LIRANG	SN-LEM	CLOA	ULH	
YEX3_YEAST_1	PEQTIKQMD	L	--	GFPRD--	AVVK	LKQTN	GN-AEF	ASL	OFQ	
UBPC_YEAST_1	NQCSISQIE	M	--	GFTON--	ASVR	LFTNG	NQDAES	MNV	OFQ	
RNMARK1_1	DAKRIDIMVT	M	--	GFARD--	EIND	LVSQK	YD-EVM	TYI	OLG	
Consensus/60%	.ppplppLbs	M..		GFscp..	psbpALp.ss	ss.hpbAspb	Lbp			

Figure 3.1 Multiple sequence alignment of UBA domains representing the consensus amino acid residues. Multiple sequence alignment of UBA domains in 45 homologous proteins, as available on the SMART server. The high degree consensus sequences are highlighted. Proteins are represented on the left side by their PDB IDs.



Figure 3.2 Hidden Markov Model Logo of UBA domains representing the consensus amino acid residues. The logo was obtained using SMART software based on an alignment of 45 homologous protein sequences bearing UBA domains. The total width of the red-shaped (dark and light) stack visualizes the expected number of inserted letters in alignment. The total stack height is computed as the information content of the column. The relative height of each letter within the stack is proportional to its frequency at that position. The highly conserved residues shown are MGF...AL..A...L.

In this study we employed comparative modeling methods to predict structures of the VWA domain of the hRpn10 subunit and NUB1L. The prediction of amino acid residues, involved in the interaction with FAT10 and NUB1L, was based on the modeled structure and the conservation pattern of VWA domains. These insights were validated by performing site-directed mutagenesis experiments. Unfortunately, all mutations analyzed theoretically as well as experimentally were not important for the interaction. Therefore, another approach was employed to answer this question, which involved obtaining the structure of the VWA domain by X-ray crystallography. To achieve this, we expressed the VWA protein in *E.coli* strain using SUMO fusion technology (Panavas et al., 2009). The purification was accomplished by using affinity and gel filtration chromatography. Similarly, we also purified full-length hRpn10 protein by utilizing affinity and ion-exchange chromatography. Currently, we are scaling up the purification of protein to obtain high yield of protein. This study will give an insight into the new interaction site on the VWA domain of the hRpn10 subunit which has never been reported.

On the other hand, we also modeled the structure of NUB1L and strikingly, observed that NUB1L is composed of four UBA domains instead of three. The alignment of the newly identified domain with the previously known UBA domains confirmed our structural

analysis. We predict that this domain might also be important for the interaction with FAT10 and therefore, in the future studies, it should be taken into consideration.

3.2 RESULTS

3.2.1 Predicted secondary and tertiary structure of the hRpn10 subunit

The hRpn10 subunit is composed of a VWA domain at the N-terminus and two UIM domains at the C-terminus (Hofmann and Falquet, 2001; Whittaker and Hynes, 2002). The major region of interest in the Rpn10 subunit has been the UIM domains, which interact with several proteins including polyubiquitin (Alves-Rodrigues et al., 1998; Beal et al., 1998; Riedinger et al., 2010; Zhang et al., 2009). The structure of the VWA domain of Rpn10 was unknown for several years. Recently, the structure of full-length Rpn10 subunit of *Schizosaccharomyces pombe* was determined by means of X-ray crystallography (VWA domain structure) and NMR spectroscopy (UIM domain structure) (Riedinger et al., 2010). Due to our intriguing finding that FAT10 and NUB1L interact with the VWA domain of hRpn10 subunit and not with UIM domains (Figure 2.2e, Chapter 2), we became interested in determining and defining motifs in the VWA domain important for this interaction. This could be achieved by solving its structure either by bioinformatics approach or X-ray crystallography. According to the secondary structure prediction performed by using PSIPRED software (Jones, 1999), the VWA domain of hRpn10 comprised of long stretches of α -helical structures and short stretches of β -sheets (Figure 3.3A). This predicted structure had a p-value of $3e-11$ (using threading) and a percentage identity of 46.2 with the VWA domain of *S. pombe*.

The three-dimensional structure of the VWA domain of hRpn10 was determined by using HHpred software, which utilizes homologous sequences and hidden Markov models (HMMs) for structure prediction. The best homologous sequences (PDB ID: 3ibs, 1yx4 and 2dba) were chosen manually (Figure 3.3B) to model the three-dimensional structure of the full-length hRpn10. The N-terminal structure was accomplished by using the known structure of conserved hypothetical protein BatB from *Bacteroides thetaiotaomicron* (PDB ID: 3ibs) having the VWA domain with an identity of 19% to the human VWA domain of Rpn10. The structure of central regions representing UIM-1 and

UIM-2 was obtained from the already known structure of UIM domains (PDB ID: 1yx4). The C-terminal region was structured using the structure of tetratrico peptide repeat of human smooth muscle cell associated protein-1, isoform 2 (PDB ID: 2dba). The N-terminal VWA domain represented a compact structure with β -sheets surrounded by α -helices. UIM domains formed a less complex α -helical structure. The C-terminal also formed a compact structure with α -helical structures.

3.2.2 Site-directed mutagenesis in the VWA domain of hRpn10 to study its interaction with FAT10 and NUB1L

After analyzing the predicted structure of the VWA domain in hRpn10, alanine scanning of the putative interacting amino acids was performed. The interaction of the mutated VWA domain with FAT10 was studied by performing a GST-pull down assay as mentioned before (Chapter 2). The selection of amino acid residues was based on the charged conserved residues and the hydrophobic patches in the VWA domain (Figure 3.4).

Initially, we mutated the charged and highly conserved residues like D11/E14, R17, D20, K98, R100 and K103 (Figure 3.5A, B). The mutations performed after detailed analysis of the three-dimensional modeled structure included RNGD (17-20), HSK (37-39), NPE (44-46), SP (114-115), KK (131-132) and SH (167-168) (Figure 3.5C, D). These residues were conserved and exposed on the surface, as examined by the predicted 3-D structure. Unfortunately, these mutations were not enough to prevent the interaction between FAT10 and the VWA domain of hRpn10. Probably, either a group of these mutated amino acids or may be another set of amino acids are required for the interaction. We attempted to identify the region of interaction by using the N- and C-terminal parts of the VWA domain, but as expected from the structure, the protein could not be expressed recombinantly (data not shown).

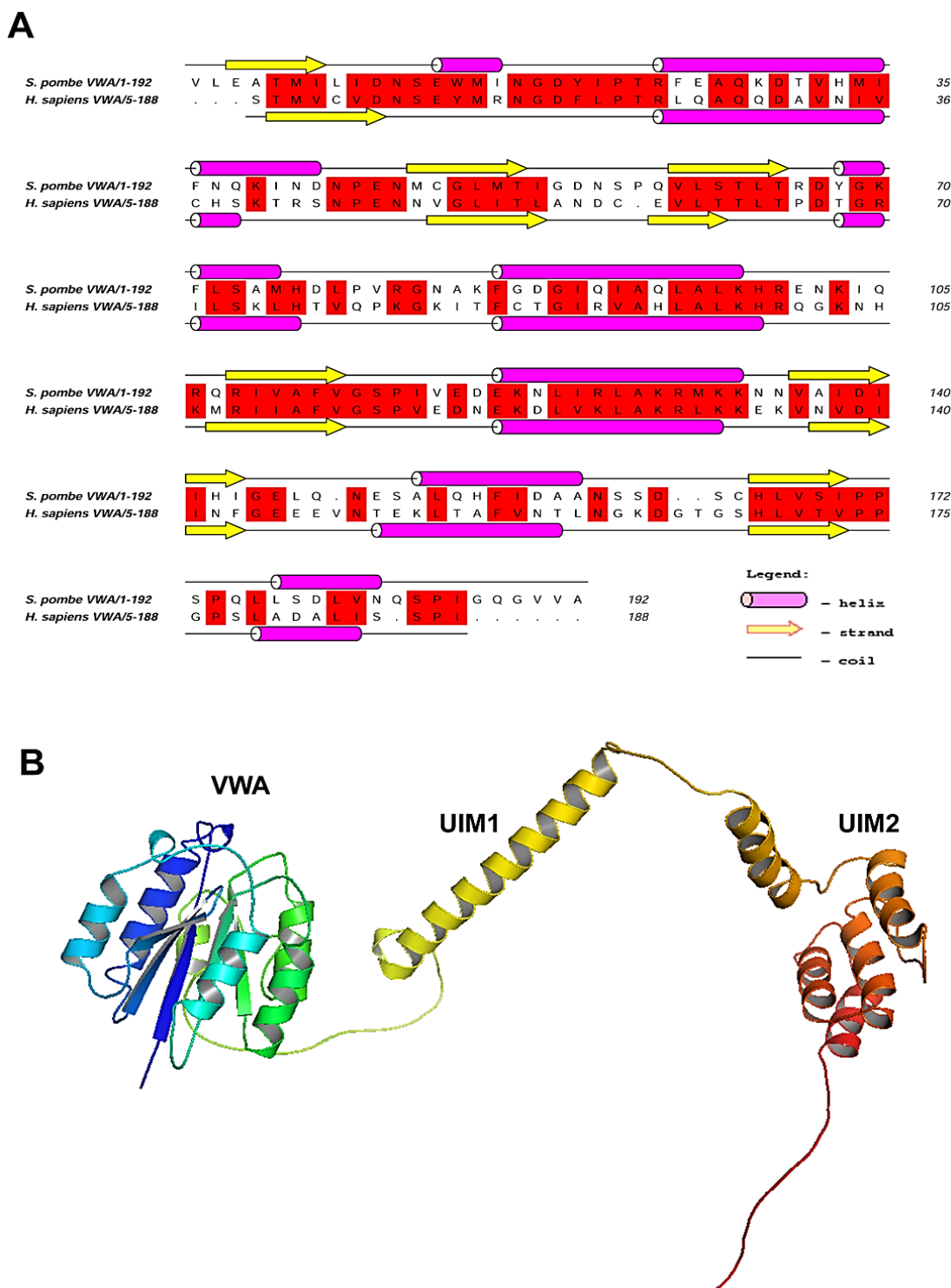


Figure 3.3 Predicted secondary and tertiary structure of hRpn10. (A) Amino acid sequence alignment of the VWA domain of Rpn10 in *H. sapiens* and *S. pombe*. The highlighted red color represents conserved residues. Alignment was performed using MUSCLE software and formatted using Jalview and ALINER software. The secondary structure was obtained from PSIPRED server. Yellow arrowheads and pink cylinders represent β -sheets and α -helices, respectively. The percentage identity between the two homologous sequences is 46.2. (B) The tertiary structure of hRpn10 was obtained by using HHpred server. The structure of the N- and C-terminal region was obtained separately and then combined together.

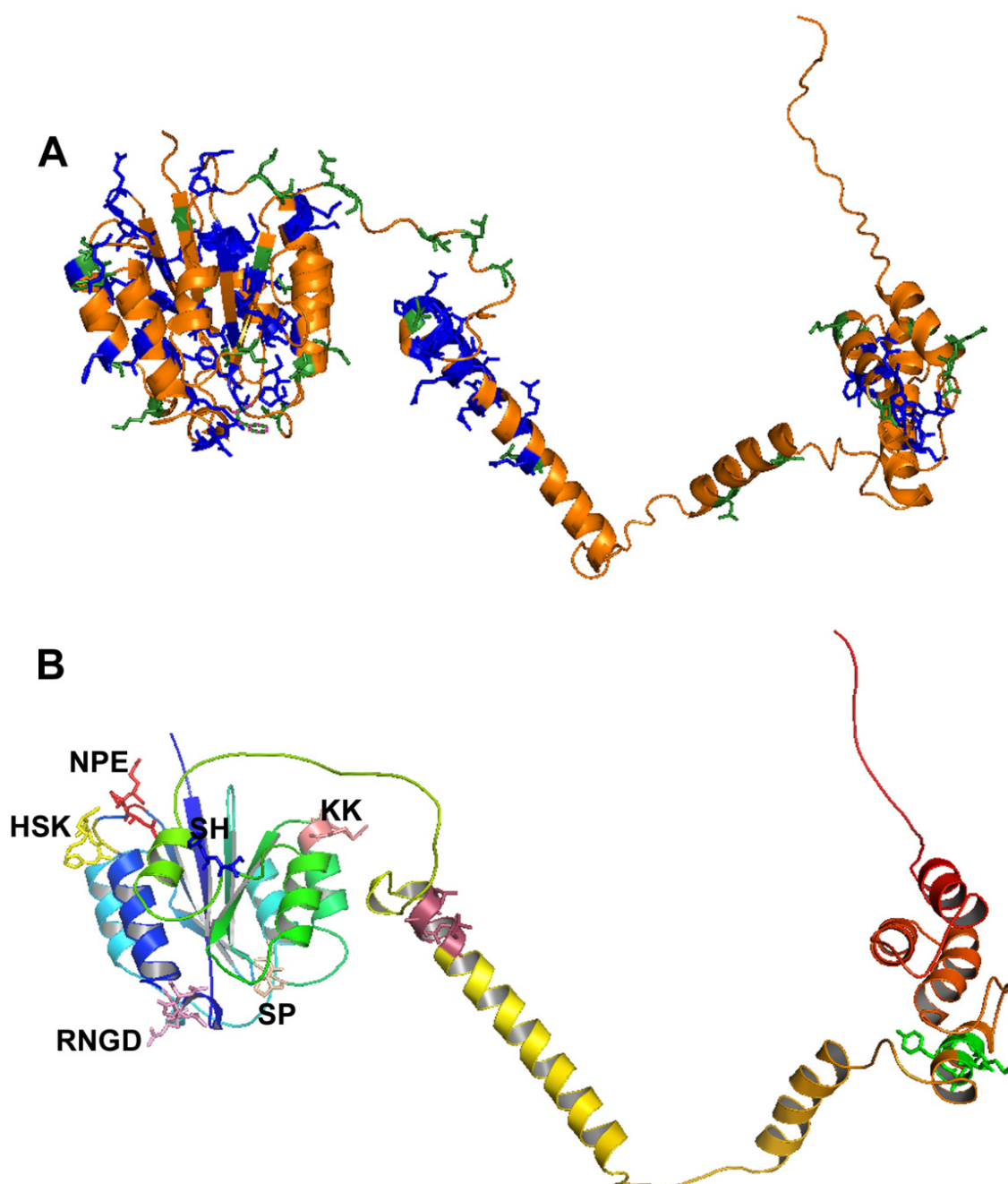


Figure 3.4 Representation of the conserved and hydrophobic residues in the tertiary structure of hRpn10. (A) CLANS software was used to predict the conservation of residues in hRpn10. Different groups include insect/drosophila, vertebrates, fungi and plants. The tertiary structure was formatted using Pymol software. Amino acid residues conserved across groups (>70%) are marked in blue and residues conserved within groups (>70%) but variable between groups (<40%) are marked in green. (B) Representation of some of the conserved and exposed residues in different colors over the predicted structure model of hRpn10 which were further analyzed in the binding assay.

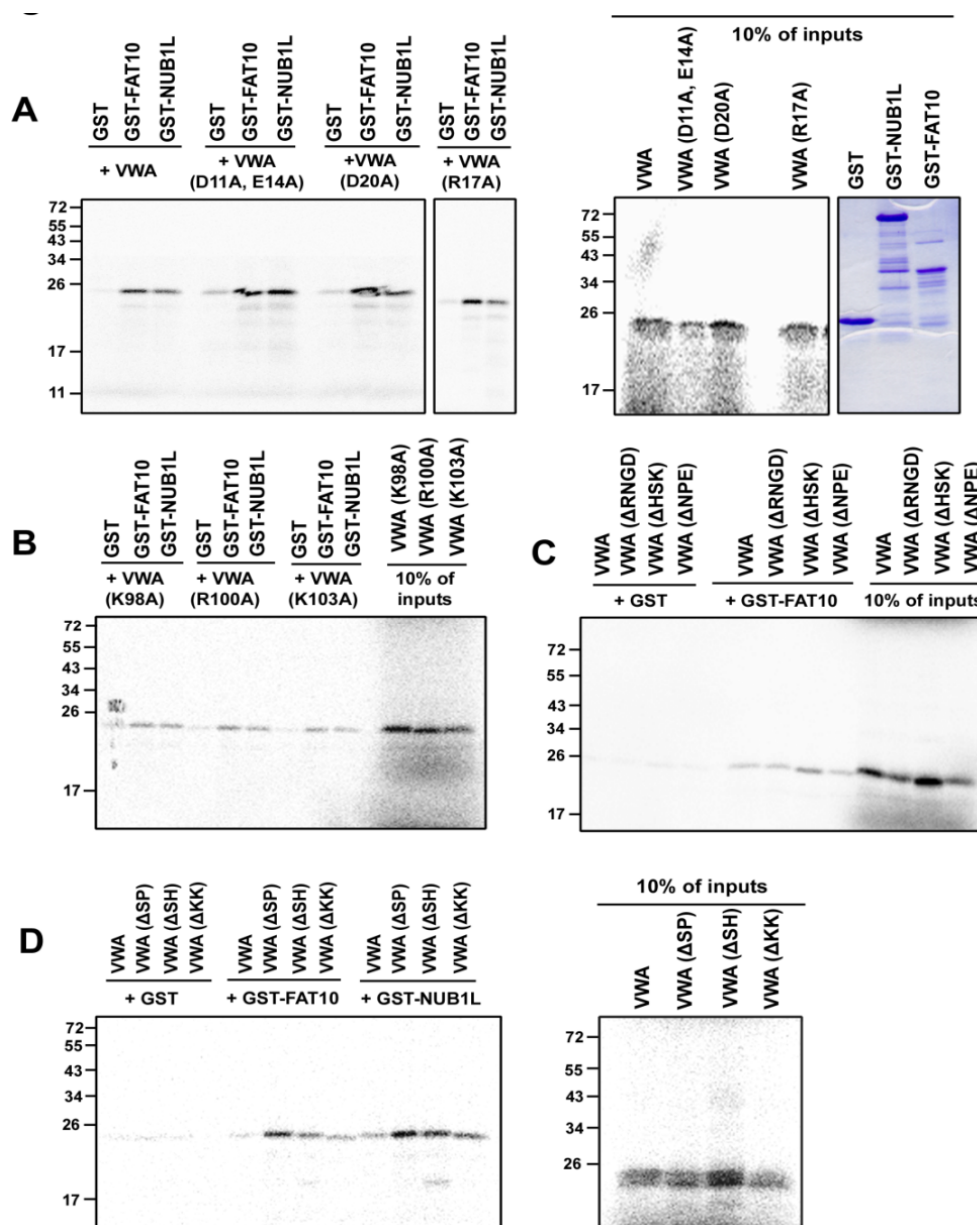


Figure 3.5 Amino acid residues of the VWA domain of hRpn10, predicted for its interaction with FAT10 and NUB1L, were not essential. (A) Left panels: GST pull-down assay representing the interaction of mutated (D11A/E14A, D20A and R17A) VWA domain of hRpn10. Recombinant GST, GST-FAT10 or GST-NUB1L was incubated together with *in vitro* transcribed and translated ^{35} S-labelled His-VWA or mutated VWA along with GSH beads and the possible interaction was observed by autoradiography. The binding of mutated VWA protein to the GST protein served as a negative control and the binding of FAT10 and NUB1L to the radioactive VWA domain served as a positive control. This selection of residues was based on the high level of conservation. Right panels: 10% of inputs of the radioactive mutated VWA domains and GST-tagged recombinant proteins. (B) GST-pull down assay as mentioned above. K98, R100 and K103 residues were mutated to alanine. This selection was also based on the conservation pattern. (C) GST-pull down assay as mentioned above. Group mutations included RNGD, HSK and NPE, which was based on the conservation as well as exposed residues as represented in Figure 3.4B. (D) Left panel: GST- VWA domain (performed similarly as above). Right panel: Autoradiograph pull down assay with SP, SH and KK mutations in showing the 10% of inputs of VWA and the mutated VWA proteins. All experiments were performed at least two times with similar results.

3.2.3 Characterization of the VWA protein

Another approach, to identify amino acids in the VWA domain responsible for its interaction with FAT10, was to co-crystallize the two proteins and then identify the interacting residues. Initially, the His-SUMO-VWA protein was purified by employing affinity chromatography. The purity of the protein was analyzed by coomassie staining and the concentration was determined by Bradford assay (Figure 3.6A, left panel). After the cleavage of the His-SUMO tag from the His-SUMO-VWA protein, the coomassie staining showed a prominent band of the VWA protein of ~23 kDa but a high molecular weight protein of ~70 kDa was also detected (Figure 3.6A, right panel). This impurity might be the heat shock protein Hsp70 which binds unspecifically to several proteins to assist in the proper folding of the protein. Mass spectrometry analysis also confirmed the 23 kDa protein to be the VWA domain. The purified protein was unstable and therefore, an attempt to remove Hsp70 led to the precipitation of the protein. Further purification steps were attempted to remove the impurity detected in the protein preparation. Gel filtration chromatography using the Superdex 75 10/300 GL column (GE Healthcare) was employed. Fractions B7-B11 were collected (Figure 3.6B) and passed through the Superdex 100 10/300 GL column (GE Healthcare) for further processing. Fractions C4-C9 showed a high purity of the protein (Figure 3.6C) but with a very low concentration. Further scaling up of the process is required to proceed with the crystallization of the protein.

3.2.4 CD spectra of the purified VWA protein

Circular dichroism relies on the principle of differential absorption of circular polarized light by chiral substances (Kelly et al., 2005). The difference in absorbance between the left- and right-handed polarized light is measured in terms of ellipticity in degrees. The secondary structure of the protein can be studied by using this technique but it does not provide detailed structure information. We used this spectrum to determine the proper folding of the protein in solution. The graph obtained represented proper folding of the VWA protein having α -helical structure (Figure 3.7A).

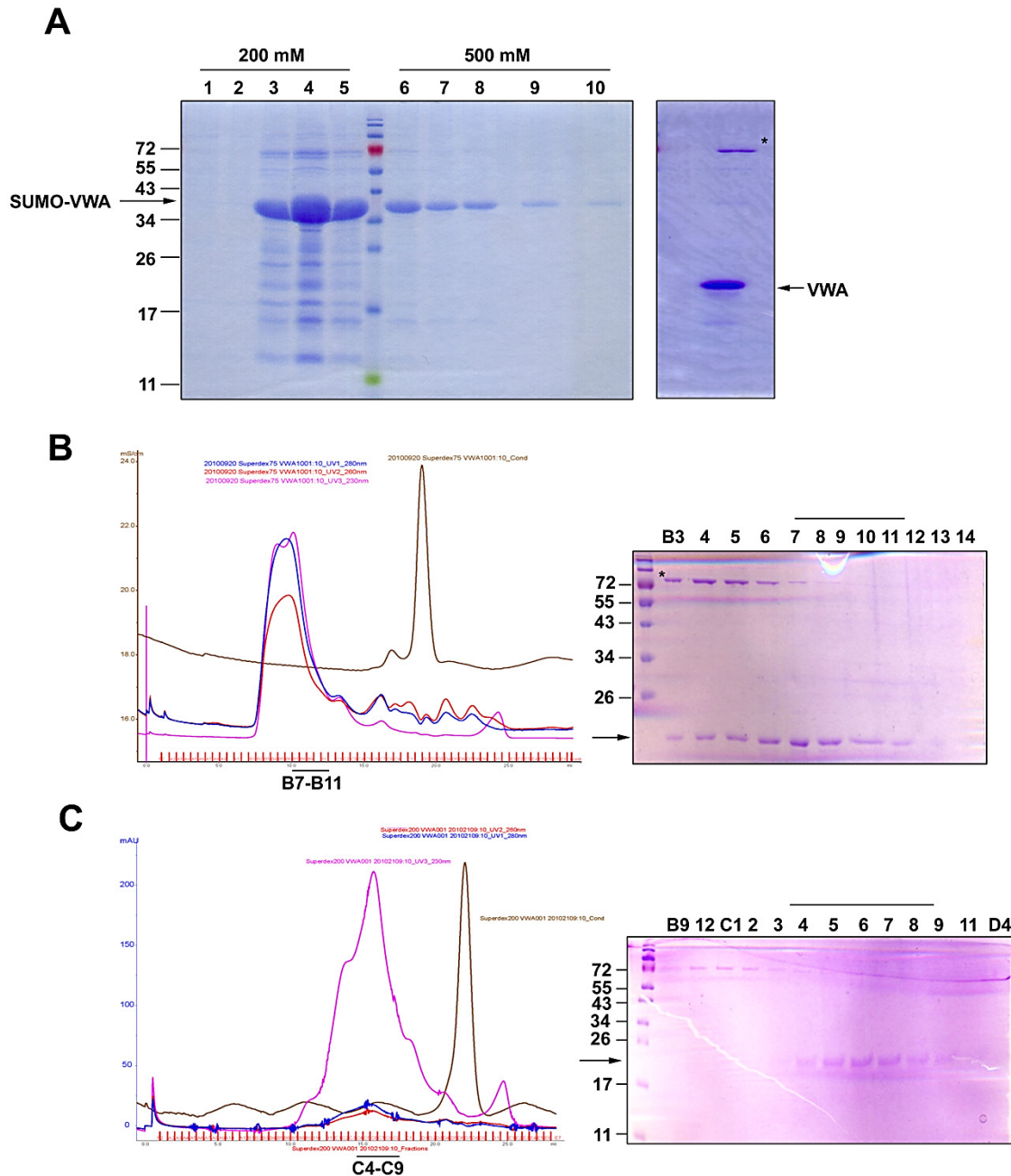


Figure 3.6 Chromatogram and SDS-PAGE of the purified VWA protein. (A) Left panel: The lysate from *E. coli* cells expressing VWA protein was loaded onto His-column and the His-SUMO-VWA protein was eluted in either 200 mM (lanes 1-5) or 500 mM (lanes 6-10) of imidazole. The purity of the protein was determined by coomassie staining. Right panel: Coomassie staining of the cleaved VWA protein is shown with an impurity of ~70 kDa (marked with asterisk). (B) Left panel: Chromatogram representing the elution of purified VWA protein after loading the fractions from (A) on Superdex 75 10/300 GL column. The protein eluted as single peak shown in blue, red and pink colors at different UV wavelengths. Right panel: Fractions obtained within this peak were analyzed by SDS-PAGE. Fractions B7-B11 showed high purity marked with an arrow head. (C) Fraction B7-B11 from (B) were collected and loaded onto a Superdex 200 10/300 GL column for further purification. Right panel: The purity of the protein was determined by coomassie staining. Fractions C4-C9 (marked by an arrow head) contains VWA protein separated from impurities.

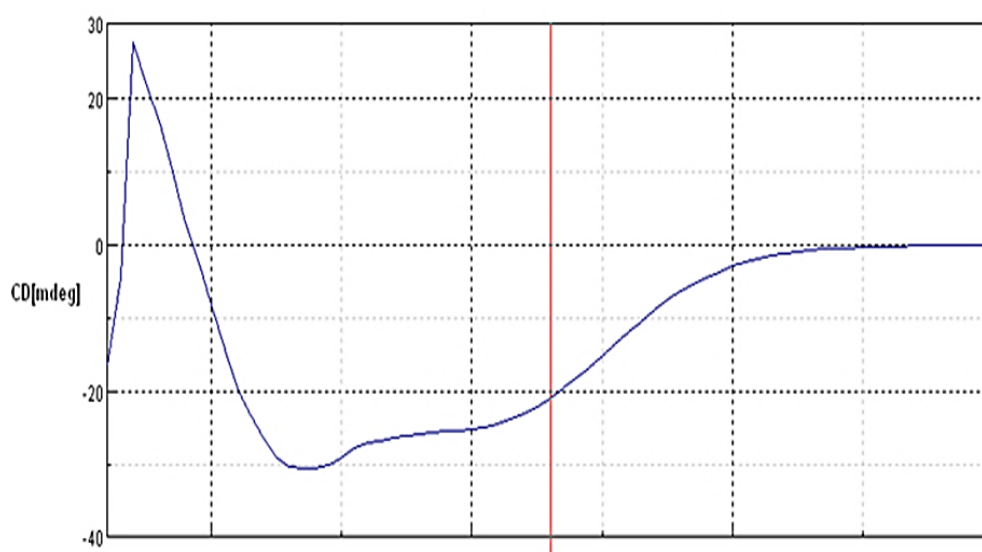
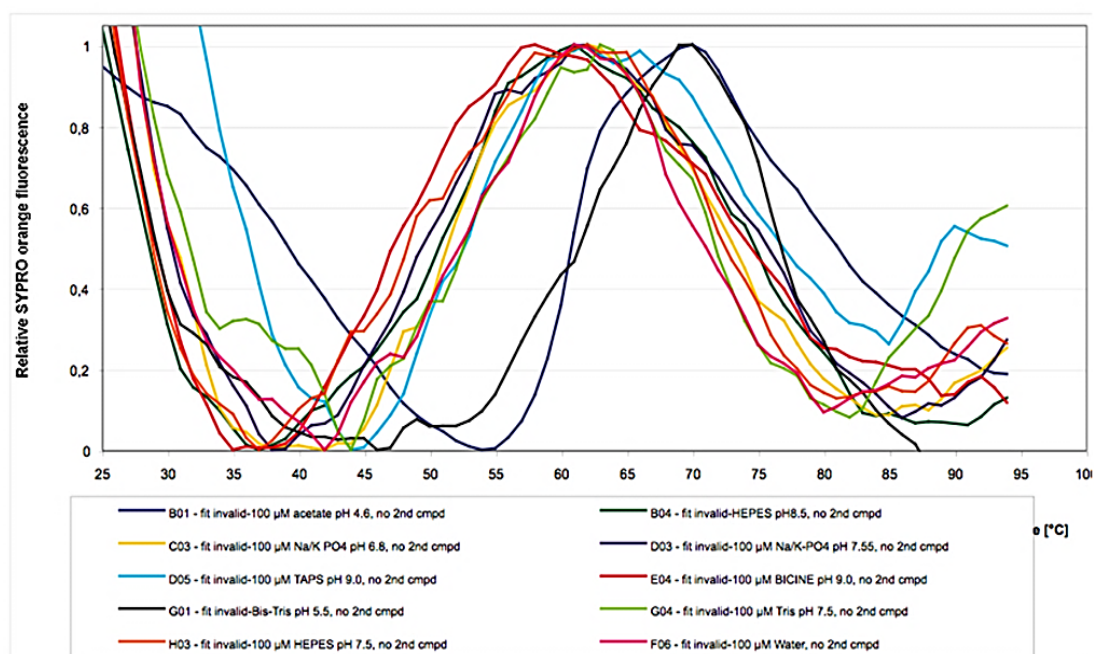
A**B**

Figure 3.7 CD spectra of the purified protein and thermofluor analysis for X-ray crystallography. (A) The CD spectrum of the purified VWA protein representing α -helical structure. **(B)** Thermofluor graph showing the stability of protein in two buffers: 100 μ M acetate pH 4.6 and Bis-Tris pH 5.5.

3.2.5 Thermofluor

This technique was employed to optimize the buffer conditions suitable for the stability of protein (Ericsson et al., 2006). It utilizes a fluorescent dye Sypro Orange, which has low quantum yield in aqueous solution but is highly fluorescent in nonpolar environments with low dielectric constants such as hydrophobic sites in proteins. The dye binds to the hydrophobic region of the protein when it starts to unfold, which results in a significant increase in fluorescence emission. After reaching a maximum value, the fluorescence intensity reduces, probably due to the precipitation of denatured protein and fluorescent probe. The application of different buffers demonstrated the stability of the protein in two buffers: 100 μ M acetate buffer (pH 4.6) and Bis-Tris (pH 5.5) (Figure 3.7B). Since the theoretical pI of VWA protein is $\sim$ 8.3 (as determined using the ExPASy server) (Wilkins et al., 1999), the protein solubility should be minimal around this pH. These two buffers had a pH value far below the pI of the protein and hence, the protein was apparently stable.

3.2.6 Purification of the full-length hRpn10

Alternatively, we attempted to purify the full-length hRpn10 protein due to difficulties in obtaining pure, non-aggregated VWA protein. The first steps of purification were performed as described earlier (Beal et al., 1998) (Figure 3.8). The purity of the protein was quite high but further downstream steps of purification were employed using Resource Q column (GE Healthcare), which was based on ion exchange chromatography. For the initial experiments the protein was eluted using a gradient of buffer with a maximum of 1M NaCl concentration but it was not sufficient to elute a considerable amount of pure hRpn10 protein. Unfortunately, the yield was very low due to the loss of protein during these purification steps (data not shown). Scaling up of the purification steps and establishment of the suitable buffer conditions, may be high salt concentrations, are required to achieve a high purity.

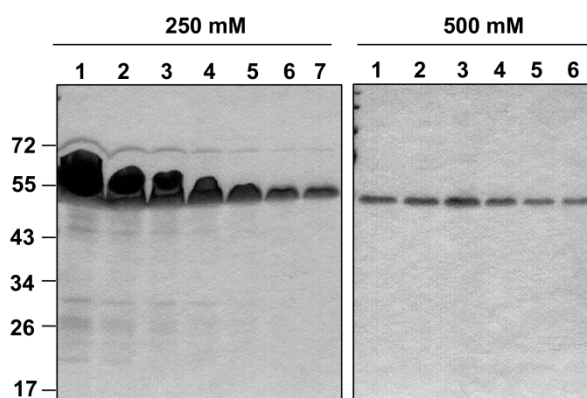


Figure 3.8 Purification of full-length hRpn10 protein. Coomassie staining of purified recombinant full-length His-hRpn10. The protein was eluted with 250 mM (left panel) and 500 mM (right panel).

3.2.7 Predicted secondary and tertiary structure of NUB1L

NUB1L consists of a N-terminal ubiquitin-like domain (UBL) and three C-terminally located ubiquitin-associated domains (UBA) (Tanaka et al., 2003). In our previous studies, we observed that the UBL domain of NUB1L interacts with the Rpn1 subunit of the proteasome with a high affinity (Figure 2.2b, Chapter 2). Yet, another interaction of NUB1L with the proteasome is through the VWA domain of the hRpn10 subunit (Figure 2.2e, Chapter 2). NUB1L interacts with FAT10 through its UBA domains and has been reported to accelerate the degradation of FAT10 (Hipp et al., 2004; Schmidtke et al., 2006) but, interestingly, it was reported that the interaction of NUB1L with FAT10 is not required for this function of NUB1L (Schmidtke et al., 2006). To better understand the mechanism of accelerated degradation of FAT10 by NUB1L and also to determine the docking sites of the three interacting proteins, i.e., FAT10, NUB1L and hRpn10, we pursued to obtain the three-dimensional structure of NUB1L.

For the initial studies, we obtained the structure with the help of the HHpred software (Figure 3.9A), which may later be confirmed by X-ray crystallography of the protein. Surprisingly, we observed four similar helical bundles with three loops (shown in blue color) in the predicted tertiary structure of NUB1L (Figure 3.9B).

The UBL domain formed β -grasp folds with an α -helix (shown in orange color). Further, the multiple alignment of the amino acid sequences of these helical structures depicted

that some hydrophobic residues were conserved when aligned with the known UBA domains of hHR23A (ubiquitin receptor) (Bertolaet et al., 2001; Chen et al., 2001; Rao and Sastry, 2002), which suggested that they represent the UBA domains (Figure 3.10). This confirmed that NUB1L is comprised of four UBA domains and not three.

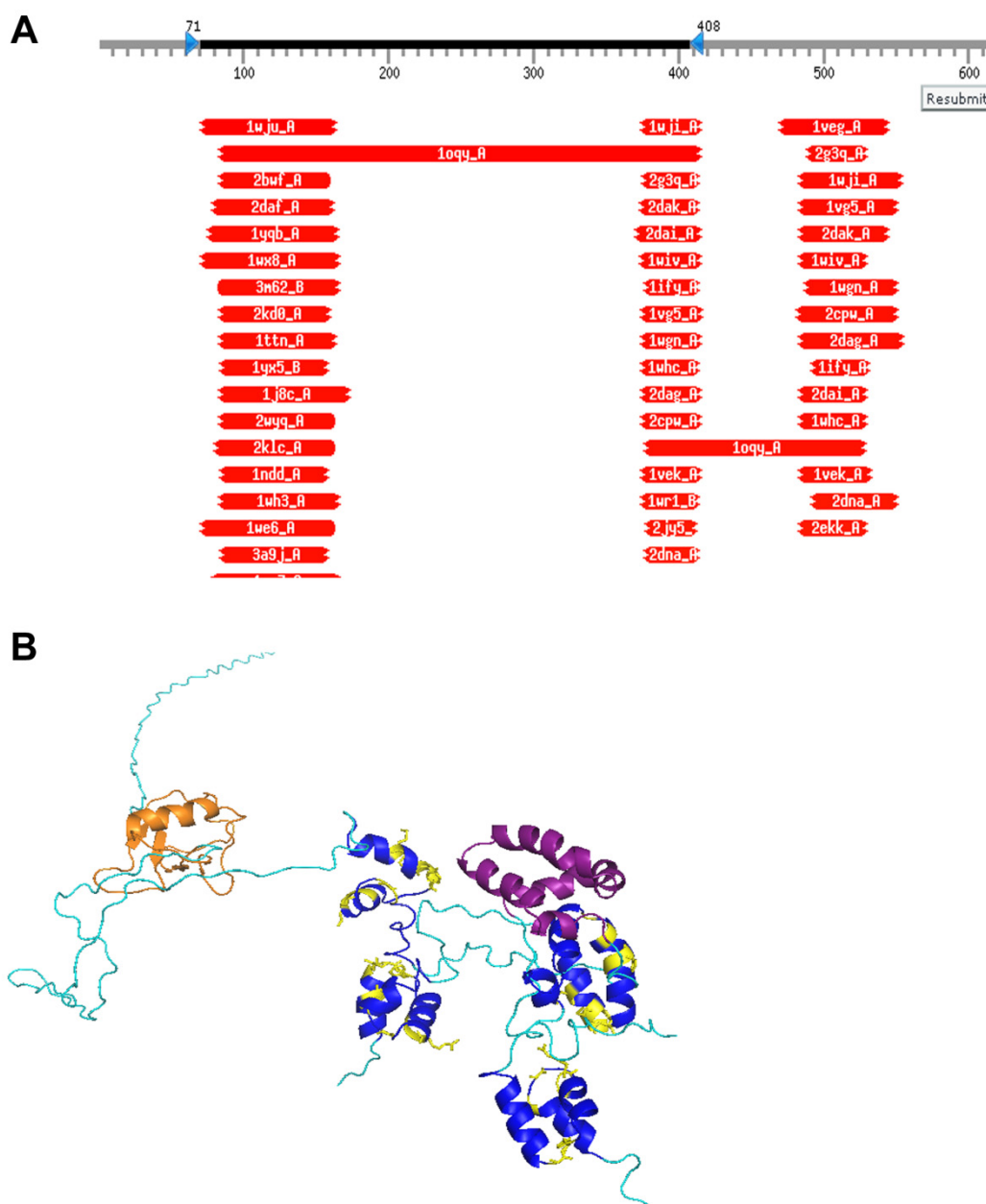


Figure 3.9 Tertiary structure prediction of NUB1L. (A) Results obtained from HHpred server representing sequences homologous to NUB1L with the known tertiary structures. The red bars show the PDB ID of the protein. The sequence of 1oqy_A was selected manually for determining the structure of N- and C-terminal NUB1L separately and then combining them together. (B) Predicted tertiary structure of NUB1L. The UBL domain is shown in orange color and the four UBA domains are shown in blue color. The highly conserved regions in UBA domain are marked in yellow. The extreme N- and C-terminal regions are shown as unstructured regions due to no homology to other proteins with known structure.

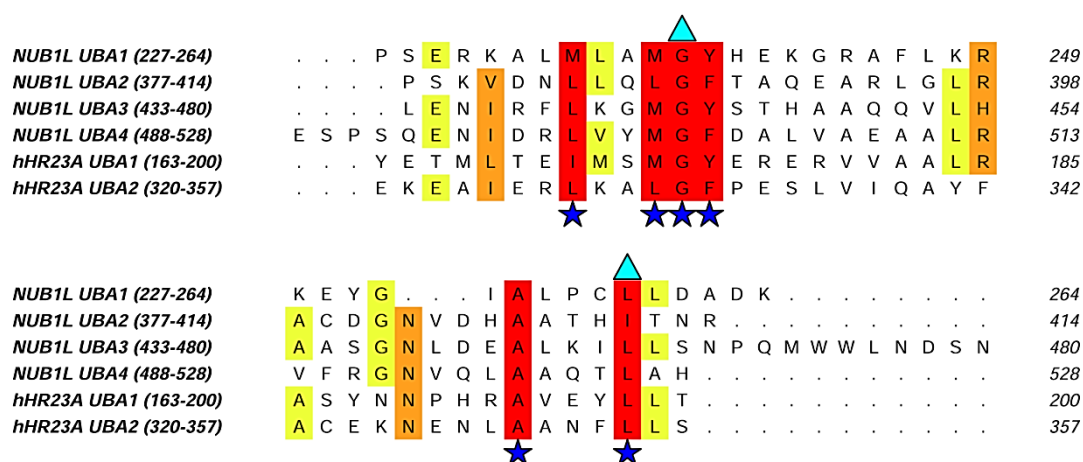


Figure 3.10 Multiple amino acid sequence alignment of the UBA domains of NUB1L and hHR23A. Amino acid sequence alignment was obtained by using MUSCLE software. Blue stars represent the highly conserved residues whereas cyan triangles represent the conserved residues important for maintaining the structural integrity of UBA domains. The intensity of the color is modulated by the degree of the conservation, with intense color representing the most conserved residues.

3.3 DISCUSSION

The predicted tertiary structure of hRpn10 shows the compact VWA domain and loosely packed α -helical UIM domains. Looking closely at the structure, one could postulate that the proteins interacting with the hRpn10 subunit would bind the UIM domain but surprisingly, the biochemical assays showed that FAT10 and NUB1L bind the VWA domain and indeed the degradation of FAT10 is dependent on the VWA domain (Chapter 2). The predicted structure of the VWA domain depicts the presence of central β -sheets with one anti-parallel strand surrounded by α -helices (Figure 3.3). This anti-parallel strand differentiates the VWA domain from closely related Rossmann or nucleotide binding domains (Springer, 2006). Site-directed mutagenesis was performed, taking into consideration that the stability of the structure was not disturbed. Even some mutations (D11 and E14) were performed in the MIDAS motif, which is recognized as an essential binding motif for the metal ions (Whittaker and Hynes, 2002) but these residues were not important for the interaction with FAT10 and NUB1L (Figure 3.5). Unfortunately, it was difficult to determine the interacting amino acids for a 194 amino acid long domain by this approach. The X-ray crystal structure of these three proteins is not yet determined. We, therefore, adopted the method of X-ray crystallography to obtain the three-

dimensional structure of hRpn10 and NUB1L. Since the structure of UIM domains in complex with two monoubiquitin molecules is already known (Narasimhan et al., 2005), we pursued to determine the structure of only the VWA domain of hRpn10. The stability of the purified VWA protein was insufficient, as could be predicted by the presence of Hsp70 co-purified with the VWA protein (Figure 3.6). The CD spectrum suggested proper folding of the purified protein in solution (Figure 3.7). In an attempt to remove this impurity, we included ATP in the lysis buffer for the release of Hsp70 but this led to the precipitation of protein after some time. Other attempts included removing the 70 kDa protein from the 22 kDa protein by size-exclusion chromatography (Figure 3.6b, c). This approach was also not very successful although we obtained some fractions of pure protein. Probably Hsp70 is in complex with the VWA domain and not easily separable by size-exclusion chromatography. As observed from the structure of UIM domains, there are large hydrophobic patches on its surface and probably these domains are essential to maintain the stability of the protein. This is also supported by the fact that the expression of *in vitro* transcribed and translated VWA protein was moderate. Therefore, we attempted to purify full-length hRpn10 protein in good yield and scaling up of this process is still required (Figure 3.8).

On the other hand, we also focused to predict the tertiary structure of NUB1L to better understand its binding to hRpn10. Interestingly, the structure revealed four compact three-helix bundles, which is the characteristic of the UBA domain (Figure 3.9B). Alignment of the amino acid sequence of these structures displayed high conservation of unusually large hydrophobic residues usually observed in UBA domains. Met171 in helix 1, Met173 to Tyr175 in loop 1, Val195, Leu198 and Leu199 in helix 3 of the UBA-1 domain of hHR23A were determined as constituting a large hydrophobic surface patch whose side chains are partially exposed on the surface (Mueller and Feigon, 2002). Some of these residues were highly conserved in all the four identified UBA domains of NUB1L (Figure 3.10, marked with blue stars). It is assumed that these hydrophobic residues are required either for the structural integrity or as a binding site for other proteins (Mueller and Feigon, 2002). The highly conserved Gly174 and Asn190 in hHR23A were postulated to be necessary for the conformation and integrity of the structure (Mueller and Feigon, 2002) and these correspond to the Gly (238, 387, 443, 502) and Asn (249, 398 and 513) in the four UBA domains of NUB1L (Figure 3.10).

It has been reported that NUB1L Δ UBA1-3 does not interact with FAT10 in the GST-pull down assay, but surprisingly its interaction with the proteasome by the UBL domain can accelerate the degradation of FAT10, as demonstrated by the pulse chase experiments (Schmidtke et al., 2006). Our finding of a new putative UBA domain in NUB1L could explain this discrepancy. Probably the fourth UBA domain, which was not taken into consideration in the pulse chase experiments, may interact weakly with FAT10 *in vivo* and the accelerated degradation of FAT10 was attained because of the interaction of NUB1L with the proteasome.

UBA domains are present in many unrelated proteins and were postulated to bind ubiquitin (Hofmann and Bucher, 1996). It would be interesting to determine if UBA domains of NUB1L could also interact with mono-ubiquitin or multi-ubiquitin. Leu198 in UBA-1 and Leu355 in UBA-2 of hHR23A were postulated to be essential for the binding to ubiquitin (Bertolaet et al., 2001). These residues were predicted to be important for maintaining the structural integrity of the protein and therefore may not provide any information on the binding interface (Bertolaet et al., 2001; Mueller and Feigon, 2002). But it was shown that certain other proteins could still interact with the UBA domains in hHR23A bearing these mutations, which suggested that the structure was maintained (Bertolaet et al., 2001). These residues correspond to Leu259, Ile411, Leu467 and Leu526 in UBA domains (1-4) in NUB1L (Figure 3.10, marked with cyan triangle). A conserved glycine residue in UBA-1 domain in Rhp23 (fission yeast homologue of hHR23A) was also described to be essential residue for binding to ubiquitin (Wilkinson et al., 2001). Interestingly, this residue corresponds to Gly238, Gly387, Gly443 and Gly502 in the UBA domains (1-4) in NUB1L (Figure 3.10, marked with cyan triangle). This indicates a high possibility that NUB1L could also interact with mono- or multi-ubiquitin. Probably, the expression of NUB1L is regulated by ubiquitin. Certain Ub-isopeptidases (isopeptidase T, PUSP13, UBP5) also have UBA domains with conserved residues (Chen et al., 2001). Probably, these isopeptidases may also interact with FAT10 via the UBA domain.

The N-terminal UBL domain is characterized by three β -sheets and an α -helical structure (Figure 3.9B). It was reported earlier that the C-terminal region of NUB1L is required for its interaction with hRpn10 but in our studies we observed a reduction in the binding of NUB1L to hRpn10 in the absence of UBL domain (Figure 2.2c, Chapter 2) which

suggested a role of UBL domain in this binding. hHR23A interacts with the UIM-2 domain of hRpn10 subunit via Leu10, Ile49 and Met75 which corresponds to the Leu8, Ile44 and Val70 residues of ubiquitin binding to the UIM domains (Mueller and Feigon, 2003) (Figure 3.11A, marked with blue triangle). Interestingly, the alignment of the UBL domain of hHR23A and NUB1L demonstrates that these residues are not aligned (Figure 3.11B, marked with blue triangle). These residues in NUB1L are not aligned with ubiquitin as well (Figure 3.11C, marked with blue triangle), which argues for its different binding site in the hRpn10 subunit (i.e., VWA domain) (Chapter 2).

It will be important to determine these aspects to get a better insight into the field of FAT10. Co-crystallization of hRpn10 with FAT10 or NUB1L may provide evidence of the interaction sites, which would give an insight into a new protein-protein interaction site in the VWA domain of hRpn10. This can also further help in determining the mechanism of accelerated degradation of FAT10 in the presence of NUB1L by performing mutational studies and competition assays. The high percentage of identity in the conserved region in the UBA domains of hHR23A and NUB1L argues for the similarity in their function(s). The two proteins share a similar function of acting like an adaptor for targeting proteins for proteasomal degradation (Elsasser et al., 2004; Hipp et al., 2004). Determination of binding sites in NUB1L may also provide its functional relevance, e.g., UBA-2 domain of hHR23A binds Vpr of HIV-1 (Dieckmann et al., 1998; Withers-Ward et al., 1997) and this could also be possible for NUB1L.

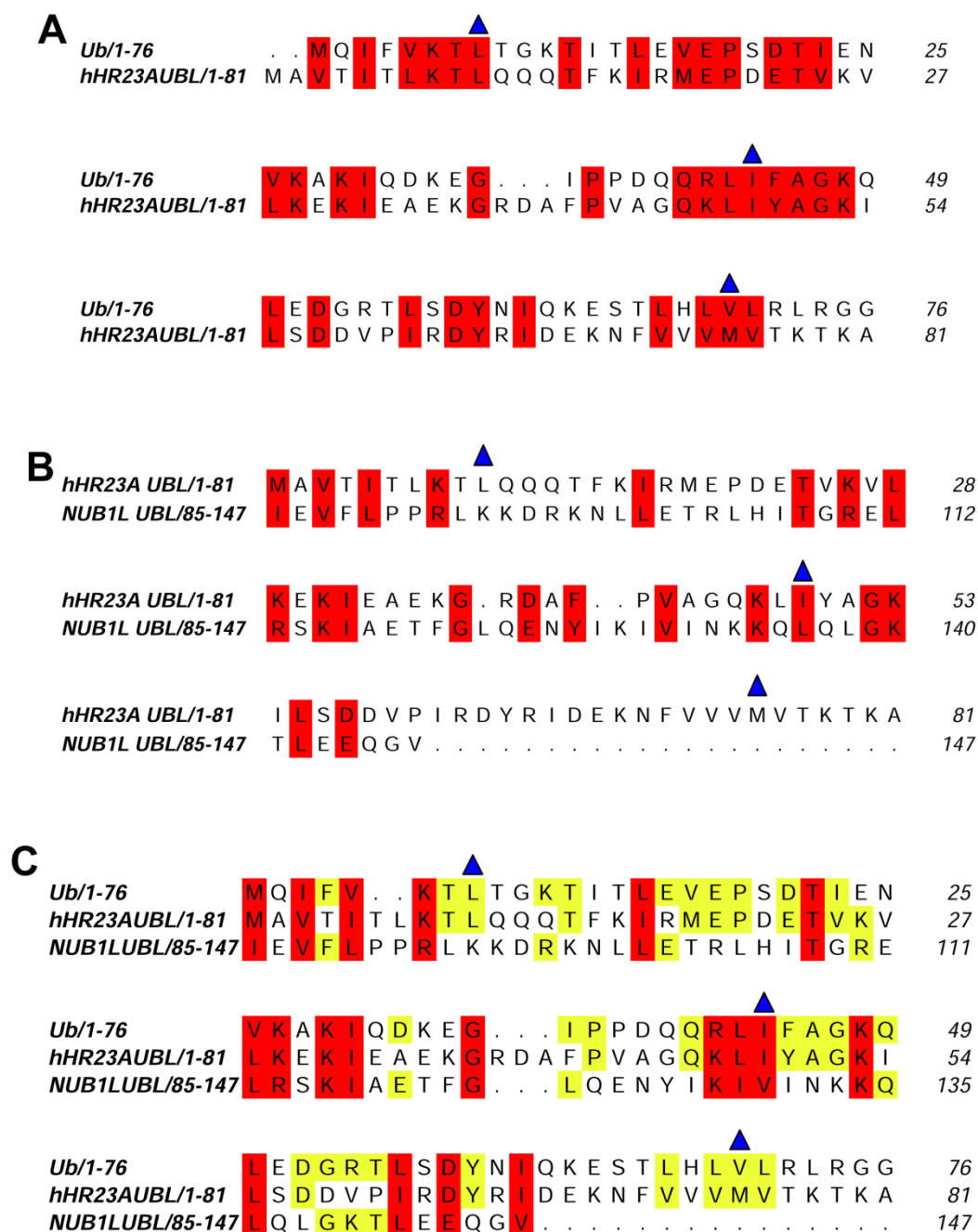


Figure 3.11 Amino acid sequence alignment of the UBL domains of hHR23A and NUB1L. (A) Amino acid sequence alignment of Ub and the UBL domain of hHR23A representing that Leu8, Ile44 and Val70 residues of ubiquitin corresponds to the Leu10, Ile49 and Met75 residues of hHR23A (marked with blue triangles). (B) Amino acid residues essential for the binding of the UBL domain of hHR23A to the UIM-2 domain of hRpn10 (marked with blue triangles) does not align with residues in the UBL domain of NUB1L. The alignment was performed using MUSCLE software and further formatted using Jalview and ALINE softwares. Similarities in these domains are marked in red. (C) Leu8, Ile44 and Val70 amino acids in Ub does not align with the UBL domain of NUB1L (marked with blue triangles). The intensity of the color is modulated by the degree of the conservation, with intense color representing the most conserved residues.

3.4 METHODS

3.4.1 Site-directed mutagenesis

Oligonucleotide primers were designed complementary to opposite strands of the vector with the desired mutation(s). The extension of primers by *Phusion* polymerase generated a plasmid with desired mutation containing staggered nicks. The cycling reaction included denaturation cycle at 95°C for 30 sec; and 12-18 cycles (depending on the number of mutations) at 95°C for 30 sec, 55°C for 1 min and 68°C for 5 min. Following PCR cycles, the product was treated with *Dpn I* enzyme, which specifically cleaved methylated and hemimethylated DNA and hence, digested the non-mutated parental DNA template. The vector containing desired mutation was further transformed in competent cells and the isolated plasmids from the bacterial colonies were sequenced to identify the plasmid with the desired mutation.

3.4.2 GST-pull down assay

The assays were performed as mentioned before (Chapter 2).

3.4.3 Purification of VWA protein

The cDNA encoding the *vwa* domain was cloned into a SUMO vector (Panavas et al., 2009) in frame with SUMO via Bsa I and Xho I restriction sites. His-SUMO-VWA protein was expressed in *E. coli* strain *B834(DE3)pLysS*, induced with 0.1 mM IPTG and incubated at 30°C for 5 hrs. The protein was purified using His column (GE Healthcare) according to the manufacturer's protocol. The His-SUMO tag was cleaved by incubating the protein overnight at 4°C with 4 µg of purified Ulp1 (kindly provided by Nicola Catone, Biotechnology Institute Thurgau, Switzerland) per mg of the purified protein. The VWA protein was obtained by affinity chromatography using a His column. The desired protein was collected in the flow through and concentrated. Further purification steps were performed by using Superdex 75 and Superdex 200 10/300 GL column (GE Healthcare) using 20 mM PBS (pH 7.4), 150 mM NaCl, 3 mM KCl buffer.

3.4.4 CD spectra analysis

The protein was diluted in sodium phosphate buffer, pH 8.0, to a final concentration of 0.1 mg/ml and all the measurements were performed at 20°C in a 0.1 cm quartz cuvette using a J-810 CD-spectropolarimeter (Jasco). Five spectra were analyzed to optimize the signal to noise ratio in the range from 260 nm to 190 nm. The bandwidth was adjusted to 2.0 nm and a sensitivity of 100 mdeg was used to obtain the spectra. The data pitch was 0.1 nm, the time response 1 sec and the scanning rate of 20 nm min⁻¹.

3.4.5 Thermofluor procedure

2 µg of protein was heated in the indicated buffers along with 0.1% SYPRO Orange dye. The melting curve was calculated by the real-time RT-PCR cycler MX3005P (Agilent Stratagene) and analyzed with MAXPro-QPCR software 4.10 (Agilent Stratagene).

3.4.6 Purification of hRpn10 protein

hRpn10 cloned into the pET26b vector was a kind gift of Patrick Young (Stockholm University). The protein was expressed as described before (Chapter 2, (Beal et al., 1998). Further purification steps were performed using Resource Q column (GE Healthcare). The protein was eluted with 20 mM Na₃PO₄ buffer with an increasing gradient of NaCl (1M).

3.4.7 Sequence retrieval

The amino acid sequences were retrieved from the protein database from NCBI (<http://www.ncbi.nlm.nih.gov/protein/>).

3.4.8 Primary amino acid sequence alignment

MUSCLE (MUltiple Sequence Comparison by Log- Expectation) software (Edgar, 2004) was adapted to obtain the multiple sequence alignments. Further the sequences were aligned and formatted using Jalview (Waterhouse et al., 2009) and ALINE softwares (Bond and Schuttelkopf, 2009). The percent identity was obtained from the PSIPRED server.

3.4.9 Secondary structure prediction

The secondary structure was predicted by using the PSIPRED server (<http://bioinf.cs.ucl.ac.uk/psipred/>) (Jones, 1999).

3.4.10 Three-dimensional structure prediction

The three-dimensional structure was predicted using HHpred server (<http://toolkit.tuebingen.mpg.de/hhpred>) (Soding et al., 2005). The best homologous sequence (retrieved from Protein Data Bank) was selected manually to model the structure. The output file in the pdb format was visualized and formatted using the PyMOL software (<http://www.pymol.org/>).

4. Investigating the Role of FAT10 in Neurodegenerative Diseases

Neha Rani, Katrin S. Lindenberg, G. Bernhard Landwehrmeyer and Marcus
Groettrup

4.1 INTRODUCTION

Neurodegenerative diseases like Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD) and fronto temporal dementia have a similar characteristic of aggregation of abnormal proteins (Taylor et al., 2002). These aggregates consist of ubiquitylated protein conjugates, which are the hallmark of neurodegeneration (Ciechanover and Brundin, 2003). The mechanism for the formation of these aggregates remains unclear but it is well known that ubiquitin immunoreactivity is associated with such diseases. Ubiquitin covalently modifies the misfolded or unwanted toxic proteins for degradation by the 26S proteasome. This pathway, known as the ubiquitin-proteasome system (UPS), involves several enzymatic steps which include activating enzymes (E1), conjugating enzymes (E2) and the ligases (E3). The C-terminal diglycine motif of ubiquitin forms an isopeptide bond with the lysine residue of the substrate, and thus, marks the protein for degradation by the 26S proteasome (Ciechanover and Brundin, 2003). A failure within this pathway results in the accumulation of misfolded proteins which may lead to the pathogenesis of neurodegenerative disease (Alves-Rodrigues et al., 1998; Ciechanover and Brundin, 2003; Rubinshtein, 2006; Tansey et al., 2007).

Another important pathway for the removal of such proteins is the autophagy-lysosome pathway. This involves the formation of double-membrane-bounded structures called autophagosomes, which fuse with lysosomes to form autophagolysosomes, and then their contents are degraded by acidic hydrolases of the lysosome. The ubiquitin-proteasome pathway is more efficient than the basal levels of autophagy, so if proteins have access to both pathways, proteasomes are the preferred and dominating route. When a cytosolic protein is aggregate-prone and a poor substrate for the proteasome, then autophagy becomes the major route of clearance by default. As of now, specific signals are not known which target such proteins to autophagosomes (Rubinshtein, 2006). The demand for basal autophagy differs among tissues, it is particularly important in the liver and other tissues where the cells, such as neurons and myocytes, do not divide after differentiation. Autophagy has a beneficial effect of protecting against neurodegeneration, but how this is attained is not completely understood. One factor involved in this pathway is p62/sequestosome-1 (SQSTM1), which recognizes polyubiquitylated proteins in aggregates via a ubiquitin-binding domain and subsequently binds to LC3/Atg8, via LC3

Interacting Region (LIR), on the autophagosome membrane to target proteins for degradation (Mizushima et al., 2008).

PD and HD are both late-onset diseases and associated with the accumulation of intracellular toxic proteins. PD is associated with the loss of dopaminergic (DA) neurons, formation of cytoplasmic inclusions of aggregated proteins (Lewy bodies (LBs)), and neuroinflammation. Mutations in Parkin, DJ-1 and PINK1 have been linked to recessively inherited parkinsonism whereas mutations in α -synuclein and LRRK2 (also known as Dardarin) have been linked to dominantly inherited parkinsonism (Dauer and Przedborski, 2003; Tansey et al., 2007). HD is characterized by the repetition (> 35 repeats) of glutamine (CAG) residues in the gene encoding huntingtin (htt). This mutation results in neuronal dysfunction associated with movement disorders and psychiatric symptoms (Bjorkqvist et al., 2008). The N-terminal fragment comprising the first 100-150 residues with the expanded polyglutamine tract is believed to be the cause of toxicity and aggregates of these N-terminal fragments are found in Huntingtin bodies (Sarkar and Rubinsztein, 2008; Zoghbi and Orr, 2000). It has been shown that histone deacetylase 6 (HDAC6), a microtubule-associated deacetylase, which is a component of aggresomes, could protect cells by facilitating removal of harmful misfolded protein aggregates from the cytoplasm and concentrating them into aggresomes for efficient processing and degradation (Kawaguchi et al., 2003). However, it still remains a controversy whether these aggregates are cytoprotective or destructive. Neuroinflammation is an established, though incompletely understood feature of neurodegeneration. It is characterized by the presence of activated microglia and reactive astrocytes in the parenchyma of the central nervous system (CNS) and increased production of cytokines, chemokines, prostaglandins, complement cascade proteins, and reactive oxygen and nitrogen species (ROS/RNS). This can result in the vulneration of the blood brain barrier and direct participation of the adaptive immune system. Microglia secrete several cytokines, basically tumor necrosis factor (TNF), IL-1, and IL-6 families, interferon gamma (IFN- γ), and transforming growth factor-beta (TGF- β) and these modulate inflammatory processes and the permeability of blood-brain barrier (Ciesielska et al., 2007; Reale et al., 2009; Tansey et al., 2007).

In addition to ubiquitin, FAT10, which belongs to the family of ubiquitin-like modifiers, serves as an independent signal for the degradation of its conjugated proteins (Hipp et al.,

2005; Schmidtke et al., 2009). It is inducible by the pro-inflammatory cytokines, IFN- γ and TNF- α , and also highly up-regulated in cancers of liver and colon (Lukasiak et al., 2008; Raasi et al., 1999). Earlier it has been reported that FAT10 interacts non-covalently with HDAC6, which could function as a linker between the dynein motor complex and FAT10ylated protein cargo during its transport to the aggresome (Kalveram et al., 2008). Moreover, NUB1L, a non-covalent interaction partner of FAT10, which accelerates the degradation of FAT10 by the proteasome (Hipp et al., 2004), accumulates in Lewy bodies of PD and dementia with Lewy bodies (DLB) (Tanji et al., 2007; Tanji et al., 2006).

The present work seeks to elucidate further the physiological role of FAT10 in neurodegenerative diseases, in support of the earlier work where FAT10 was shown to be localized to aggresomes after proteasome inhibition, in cells (Kalveram et al., 2008). To investigate the possible link between FAT10 and such diseases, we examined FAT10 mRNA level in the brain sections of both HD and α -synuclein transgenic mice. Using quantitative real-time RT-PCR analyses, we observed that the expression of FAT10 mRNA correlates with the degree of inflammation in the brain of diseased mice, as determined by the induction of the pro-inflammatory cytokines, IFN- γ and TNF- α mRNA. Interestingly, we recognized FAT10 protein in aggregates from the brains of HD transgenic mice. Moreover, immunohistochemistry study showed the presence of FAT10 in the brain of different neurodegenerative disease cases. Taken together, the induction of FAT10 at the transcription level and its presence in aggregates (probably sequestered to aggregates via HDAC6) in PD and HD mouse models suggest that it could play a role in such diseases, perhaps by targeting misfolded proteins for their degradation either by the proteasome pathway (during early stages of the disease) or by autophagy (during proteasome inhibition).

4.2 RESULTS

4.2.1 Expression of FAT10 mRNA in different organs of C57BL/6 mouse after LCMV infection

Previously it was reported that thymus and lymph nodes express the highest amounts of FAT10 whereas in the brain FAT10 expression was the lowest, as determined by the

FAT10 mRNA levels in a set of mouse organs (Lukasiak et al., 2008). The synergistic induction of FAT10 in the presence of pro-inflammatory cytokines, IFN- γ and TNF- α (Raasi et al., 1999), provided the rational to test the induction of FAT10 after seven days of intravenous (i.v.) infection with lymphocytic choriomeningitis virus (LCMV). LCMV is a single-stranded, non-cytopathic RNA virus, which does not cause disease by itself but rather elicits a T-cell response that results in immuno-pathologic disease. LCMV-induced disease is mediated by CD8⁺ T cells in immuno-competent mice and by CD4⁺ T cells in mice that are deficient in CD8⁺ T cells (Hildeman and Muller 2000). Total mRNA was isolated from different mouse organs and quantitative real-time RT-PCR analysis of FAT10 expression was performed. FAT10 mRNA expression in the kidney was significantly induced approx. 40-fold as compared to the uninfected organ (Figure 4.1). The mRNA expression was up-regulated in uterus, liver and ovaries but no significant induction was detected in spleen, lymph node, lung, thymus and brain (Figure 4.1). FAT10 was not induced in the brain after i.v. infection, probably due to the lack of cytokines in the brain because of the presence of the blood-brain barrier (immune-privileged organ).

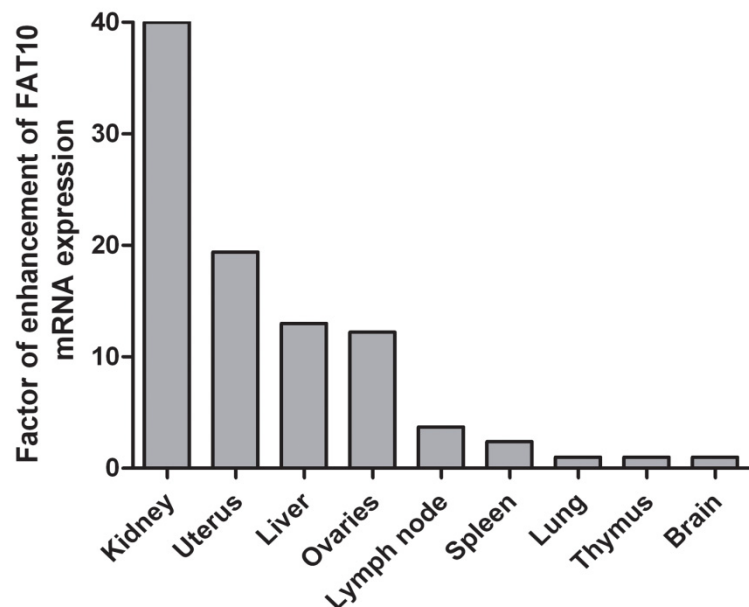


Figure 4.1 Quantification of FAT10 mRNA levels in different mouse organs after intravenous LCMV infection. Total RNA was isolated from the indicated organs of C57BL/6 mouse and real-time RT-PCR analysis was performed using 1 μ g of RNA. The values were normalized to HPRT gene expression and the overexpression was calculated relative to the respective uninfected organs, which was set to unity. PCR efficiencies were obtained from four serial dilutions and the amplification of the desired product was confirmed by a melting point curve analysis followed by agarose gel electrophoresis.

4.2.2 Induction of mRNA expression level of FAT10 in the brain of C57BL/6 mouse after intracranial infection with LCMV

The above results instigated us to investigate the mRNA expression of FAT10 in the mouse brain after intracranial (i.c.) infection with LCMV. Intracranial infection of mice with LCMV is a well characterized experimental model that exemplifies the inflammatory responses in the central nervous system (Asensio et al., 1999; Buchmeier et al., 1980; Doherty et al., 1990). Immuno-competent adult mice infected with LCMV develop an acute monophasic disease characterized by infiltrating mononuclear cells in the meninges, choroid plexus and ependymal membranes leading to convulsive seizures and death of animals after 6-8 days (Buchmeier et al., 1980). Previous report showed a marked increase in cerebral chemokines and pro-inflammatory cytokines including TNF- α , IL-1 α , IFN- γ and IL-1 β , at day 6 following LCMV infection (Asensio et al., 1999). According to our real-time RT-PCR analysis, we observed that FAT10 mRNA was highly induced by about 560-fold in the infected brain as compared to the uninfected brain (Figure 4.2A). We analyzed the mRNA expression of IFN- γ and TNF- α in the brain after mice were injected with LCMV in two different ways, i.e., intravenously and intracranially. As expected from the up-regulation of FAT10, we witnessed induction of IFN- γ by about 45-fold (Figure 4.2B) and TNF- α by about 60-fold (Figure 4.2C) after i.c. infection, which suggested that FAT10 up-regulation is strictly dependent on proinflammatory immune response elicited in the brain.

4.2.3 Induction of FAT10 mRNA in the brain of transgenic Huntington's disease mouse model

The brain has been considered as immune-privileged organ due to the presence of the blood-brain barrier and the lack of a lymphatic system but it is now well established that the brain can mount an inflammatory response (Tansey et al., 2007). The elevation of FAT10 mRNA in the brain, after an immune response is elicited, gave us an incentive to study if FAT10 could be detected in neurodegenerative diseases like Huntington's disease, Parkinson's disease or Alzheimer's disease. Due to inadequacy of resources in obtaining diseased human tissues, we performed experiments on transgenic (Tg) Huntington's disease mice (R6/2) (Mangiarini et al., 1996). The inter-connection between the peripheral inflammation and CNS pathology in HD is not very well established

although it has been studied. Several cytokines of the innate immune system like IL-6, IL-4, IL-10 and TNF- α , are up-regulated early in the infection and further increased with the progression of the disease both centrally and peripherally in the brain whereas the level of IFN- γ mRNA increases late in infection (Bjorkqvist et al., 2008; Campbell et al., 1994).

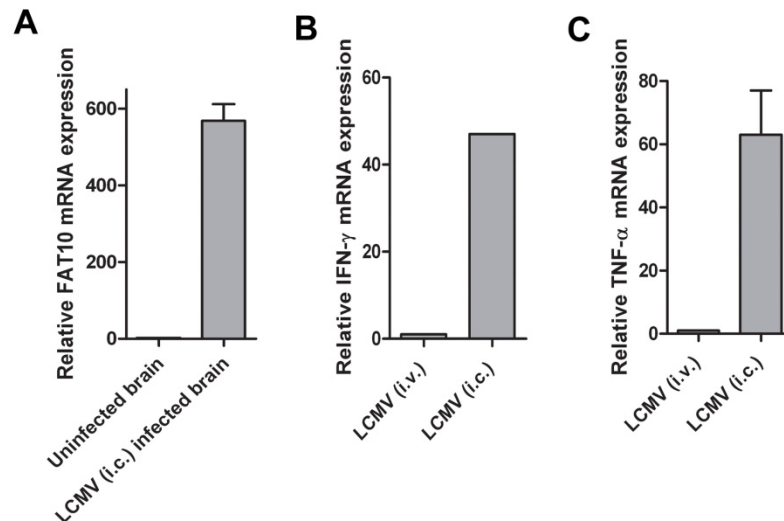


Figure 4.2 Induction of mRNA level of FAT10, IFN- γ and TNF- α in the brain of mice after intracranial (i.c.) LCMV infection. (A) mRNA expression in i.c. infected (LCMV) brain was determined by real-time RT-PCR and the induction was calculated relative to the uninfected brain sample and normalized to HPRT expression. (B) IFN- γ and (C) TNF- α mRNA expression after i.c. infection was calculated similarly as above, relative to the mRNA expression after intravenous (i.v.) LCMV infection. Error bars represent standard error calculated from two independent sets of experiments.

These studies supported the hypothesis that FAT10 could be up-regulated in the brain of HD transgenic mice (R6/2). R6/2 mice are phenotypically normal at an age of 2 weeks, motor impairment can be detected from 5-6 weeks of age, and the disease progresses at 12 weeks (Mangiarini et al., 1996). In accordance with the above findings, we observed an induction in the FAT10 mRNA levels in most of the brain samples (R6/2 20, 66, 160, 300 and 361) as compared to the wild-type brain samples (Figure 4.3A, B). The quantitation of two different experiments is shown in different graphs. The induction level varied from about 75-fold to 8-fold. Some samples (R6/2 18 and 376) did not show a significant induction in FAT10 expression, which co-related very well with the low expression of IFN- γ (Figure 4.3C).

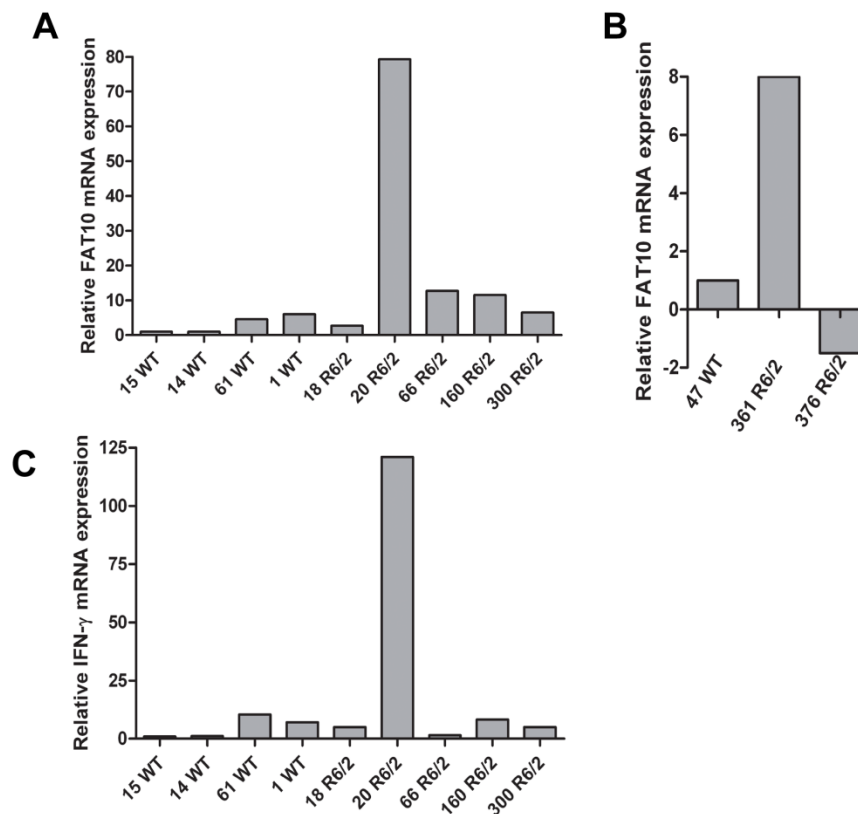


Figure 4.3 Co-expression of FAT10 and IFN- γ mRNA in brain samples of Huntington's disease transgenic mice. (A, B) Quantification of FAT10 and (C) IFN- γ mRNA levels in the brain of the indicated transgenic mouse (R6/2) as determined by the real-time RT-PCR, relative to the lowest mRNA level observed in the wild-type (WT) mouse. Normalization was performed with HPRT gene expression.

4.2.4 Induction of FAT10 mRNA in the brain of alpha-synuclein transgenic mouse

Parkinson's disease (PD) is associated with the loss of dopaminergic neurons in the ventral midbrain with cell bodies in the substantia nigra pars compacta (SNpc) (Tansey et al., 2007). The macrophages in the brain, known as microglia, get activated upon the invasion of pathogens, trauma, stroke and infection. Other immune factors include the production of reactive oxygen and nitrogen species (ROS/RNS), cytokines and chemokines (Tansey et al., 2007). We investigated if FAT10 expression could be induced in this disease as well and if it is a characteristic of aggregate-forming neurodegenerative diseases. Total mRNA was isolated from a section of several α -synuclein Tg mice brains (Freichel et al., 2007; Kahle et al., 2001). FAT10 mRNA was induced in most of the analyzed α -synuclein Tg (old age) mice brain within a range of 2- to approx. 32-fold as

compared to the young Tg mice (sample 176 and 177; Fig. 4A) or wild-type mice (sample 1 and 3; Figure 4.4B). This variation in the induction of FAT10 coincides with the variation in the level of immune response generated which was evident by the induction of IFN- γ in the brain of diseased mice (Figure 4.4C, D). FAT10 mRNA induction was more obvious in the hind brain (HB) rather than the forebrain (FB). Immunohistochemical staining of α -synuclein in the brain of Tg mice showed widespread expression but after proteinase K (PK) treatment, which removes all but aggregated α -synuclein, a signal was detected in the hind brain only (data not shown). These PK-resistant α -synuclein-immunoreactive structures are supposed to be abnormal inclusions (Lewy bodies, Lewy neuritis and Lewy dots) or diffuse neuropil staining (Tanji et al., 2010). This suggests the formation of aggregates and the induction of FAT10 in the hind brain region of Tg mice but this does not imply that FAT10 is substantially located in the aggregates.

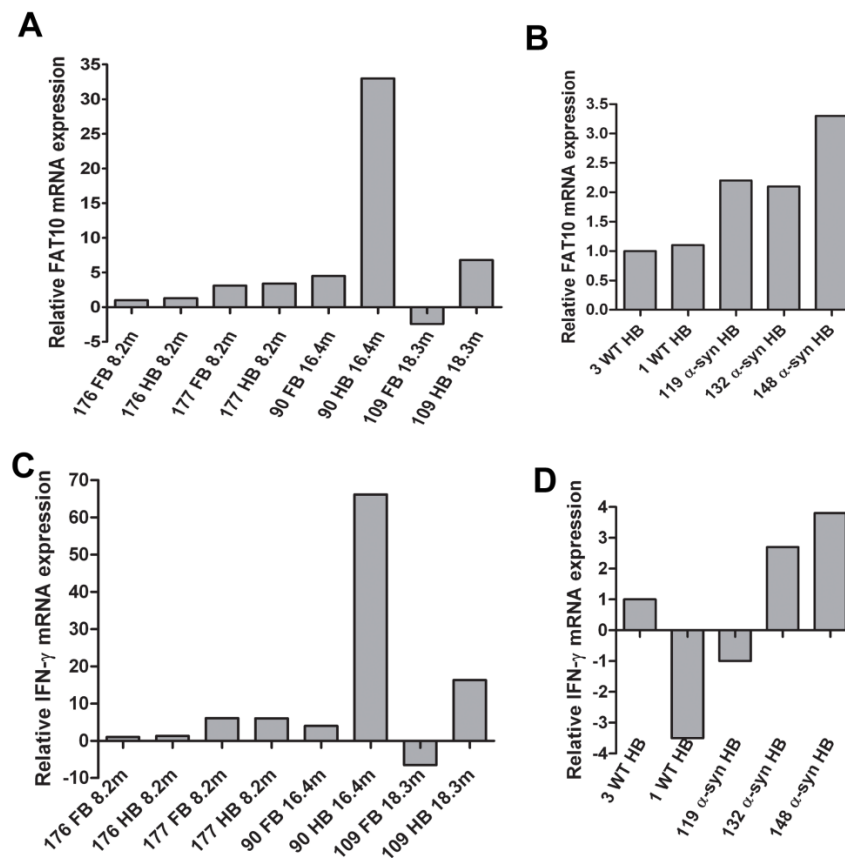


Figure 4.4 Co-expression of FAT10 and IFN- γ mRNA in the brain samples of α -synuclein transgenic mice. (A, B) FAT10 and (C, D) IFN- γ mRNA expression in the indicated samples of the fore brain (FB) or hind brain (HB), relative to the lowest mRNA level observed in the young mice (8.2 months old) or wild-type mouse. Normalization was performed with HPRT gene expression.

4.2.5 FAT10 protein is the component of aggregates in the brain of transgenic Huntington's disease mouse

HD transgenic mouse has deposition of aggregates in the brain due the expression of polyglutamine repeats (Davies et al., 1997; DiFiglia et al., 1997; Zoghbi and Orr, 2000) and this is the basis of pathogenesis (Lunkes et al., 2002). As reported earlier, FAT10 localizes to aggresomes under proteasome inhibition conditions, in cells (Kalveram et al., 2008); and moreover, NUB1L, a non-covalent interaction partner of FAT10, localizes to Lewy bodies (Tanji et al., 2007), we set out to determine the presence of FAT10 in such aggregates. The aggregates cannot be solubilized in SDS buffer by heat treatment and, therefore, aggregates were dissolved by following a formic acid protocol (Lunkes et al., 2002). Considering that the mRNA expression does not always correlate with the expression of protein, we analyzed the expression of FAT10 protein in such brain samples. Subsequent to the solubilization of aggregates by formic acid treatment (see experimental procedures), FAT10 protein was analyzed by western blotting. Surprisingly, FAT10 was detectable in the lysate from the Tg mice as compared to the lysate from wild-type mice, which do not form aggregates (Figure 4.5A). The polyclonal antibody against FAT10 formed a smear in Tg mice samples, which represent some high molecular weight FAT10 conjugates (Figure 4.5A). Endogenous FAT10 induced by IFN- γ and TNF- α served as a positive control. Full-length endogenous huntingtin protein (~ 350 kDa) was not detected, probably because of low sensitivity of the antibody or due to less transfer of high molecular weight proteins on the nylon membrane. The R6/2 Tg mouse expresses exon-1 with polyGln repeats of ~ 150 kDa (Mangiarini et al., 1996). This band was also not detectable with the antibody since the protein has high tendency to aggregate, and hence, does not migrate in the SDS-gel efficiently but we observed another small fragment of ~ 55 kDa in the Tg mice samples but not in wild-type (Figure 4.5B), which probably represent the N-terminal htt fragment prone to form nuclear aggregates (Lunkes et al., 2002). Anti-GAPDH blotting served as a loading control (Figure 4.5C, left panel) and this band was not visible in the wild-type samples after addition of formic acid (Figure 4.5C, right panel), which strongly supported the absence of aggregates in wild-type brain samples, and hence, the validity of the experiment. This implicates that FAT10 expression is up-regulated, and is the component of aggregates, in the brain of HD transgenic mouse model.

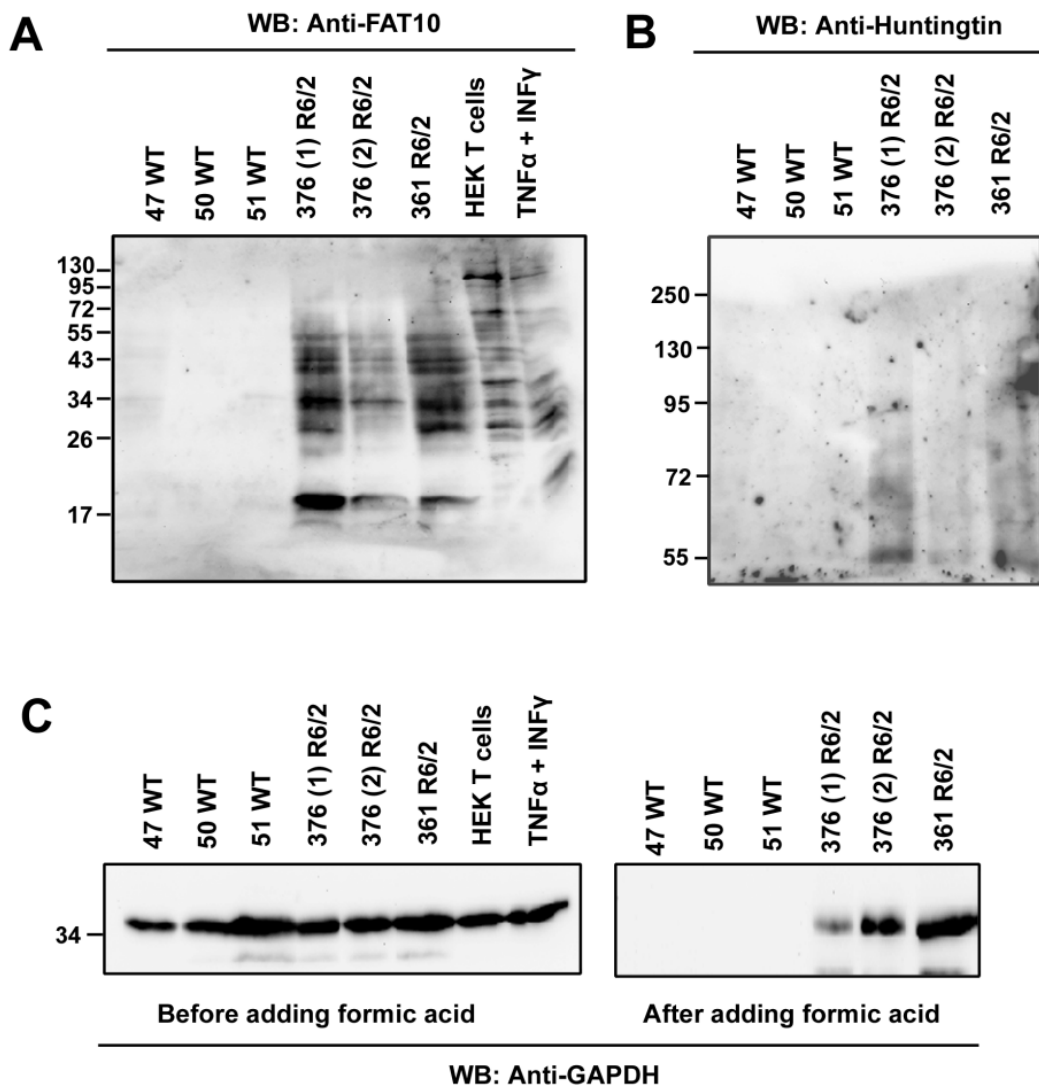


Figure 4.5 FAT10 protein expression in the brain samples of R6/2 mice. Total protein was extracted from the brain samples using the formic acid protocol (see experimental procedures). (A) Protein lysate blotted with anti-FAT10 antibody showing abundant levels of FAT10 (~ 18 kDa) in the aggregates of R6/2 mice brain samples. HEK293T cells served as negative control whereas cells induced with TNF- α and IFN- γ served as positive control. (B) Protein lysates blotted with anti-huntingtin antibody showing the presence of 55 kDa htt fragment (see results for detail) in the samples from R6/2 mice. (C) Left panel: Western blot showing GAPDH as a loading control. Right panel: Western blot showing the absence of aggregates, and hence, GAPDH in the wild-type brain samples.

4.2.6 FAT10-positive aggregates detected in the human HD and PD transgenic mouse brain

Formation of aggregates is the characteristic feature of many neurodegenerative diseases. PD is associated with the presence of Lewy bodies (LB), which are round or spherical in shape, generally 5 to 30 μm in diameter and characterized by a central hyaline core.

These are acidophilic and surrounded by a pale halo. In order to confirm the above finding, we established immunohistochemical stainings in both human and mouse models of neurodegenerative disease. The brain section of PD transgenic mouse was stained with FAT10 specific antibody which depicted Lewy body-like structures positive for FAT10 (Figure 4.6A, B). Pre-immune serum staining and blocking the antibody with FAT10 peptide served as negative controls, showing the specificity of the antibody (Figure 4.6B). The human PD section showed no obvious FAT10-positive aggregates, which were α -synuclein positive, possibly because of sparsely populated aggregates/Lewy bodies, and therefore, the antibody was not able to detect FAT10. We also investigated this feature in other neurodegenerative diseases. The brain section of human Alzheimer's disease demonstrated positive staining for FAT10 although it was not evident if FAT10 was localized in the β -amyloid positive aggregates (Figure 4.6C). FAT10-positive aggregates, positive for huntingtin (htt, 2B4), could also be observed in the human HD brain section (Figure 4.6D). Together, this finding supports the physiological existence of FAT10 in aggregates in neurodegenerative diseases, to some extent. Although our findings and the past findings supports the localization of FAT10 in aggregates but, in future, it has to be carefully determined which cell types express FAT10.

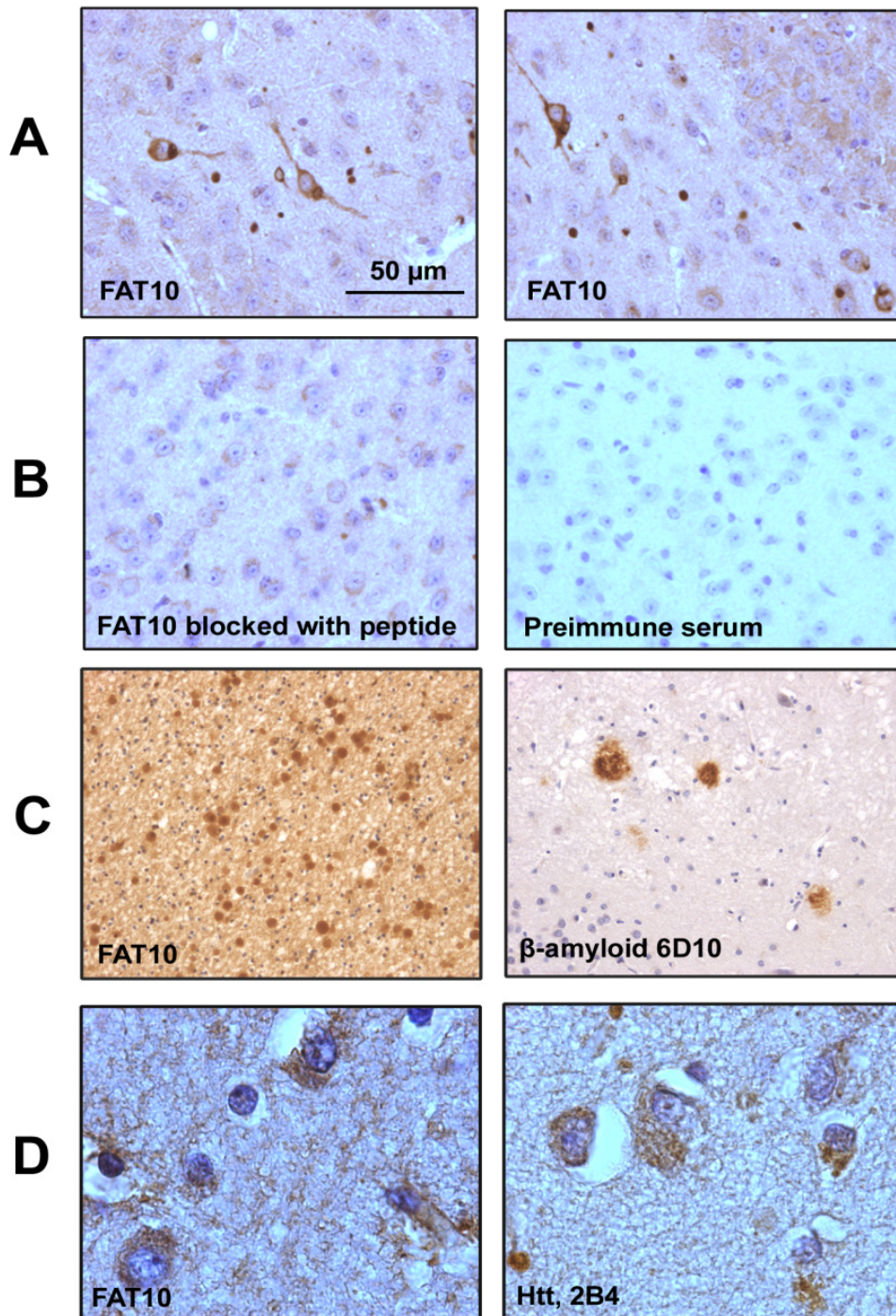


Figure 4.6 Presence of FAT10-positive aggregates in the brain section of neurodegenerative disease mouse models or human tissues. Immunohistochemical staining in the brain section of α -synuclein transgenic mouse (A) with FAT10-specific antibody showing the presence of FAT10 (brown staining) in the Lewy body-like structures, (B) left panel: with the FAT10-specific antibody blocked with FAT10 peptide showing the specificity of antibody, right panel: with the pre-immune serum showing no signal for FAT10. (C) The brain section from Alzheimer's disease patient stained with either FAT10-antibody (left panel) showing positive FAT10 "bodies", or with an antibody specific for β -amyloid (right panel). (D) Human HD brain tissue section showing aggregates positive for FAT10 (left panel), and huntingtin (htt, 2B4) (right panel).

4.2.7 FAT10 induction is not dependent on misfolded protein stress conditions

As noticed above, FAT10 expression was highly induced in the brain of HD and PD Tg mice, therefore, we questioned if this induction was due to misfolded protein stress condition in such cases. Misfolded proteins resulting from genetic mutations, inappropriate protein assembly, aberrant modifications and environmental stress are the inevitable byproducts of biogenesis (Kawaguchi et al., 2003). Aggresome formation augments in response to increasing levels of misfolded protein in cells (Alves-Rodrigues et al., 1998; Johnston et al., 1998). In this context, we analyzed the mRNA expression of FAT10 in HEK293T cells after inhibiting the proteasome using MG132 at 10 μ M concentration for 6h but there was no induction of FAT10 mRNA (Figure 4.7A). Misfolded protein stress was also induced with puromycin (protein synthesis inhibitor) in Hela cells, as well as canavanine (structural analogue of arginine which gets incorporated in proteins causing toxicity), and thapsigargin (inhibitor of sarco/endoplasmic reticulum Ca⁺² ATPase) (Kawaguchi et al., 2003) in HEK293T cells (Figure 4.7B, C). Different concentrations and time duration for the induction of stress had no significant effect on the expression of FAT10 mRNA as determined by real-time RT-PCR. This supported the above findings that FAT10 expression is strictly dependent on the immune response generated. Although we investigated this question by employing several different stress conditions, stress as a FAT10 inducer cannot be excluded completely because other stress conditions like oxidative stress or heat stress still remain to be investigated.

4.3 DISCUSSION

Several evidences suggest the role of FAT10 in the regulation of immune system: first, it is encoded in the major histocompatibility complex (MHC) class I region close to the HLA-F gene (Liu et al., 1999). Second, its expression is highly up-regulated in the presence of pro-inflammatory cytokines, IFN- γ and TNF- α (Raasi et al., 1999). Third, it is over-expressed in hepatocellular carcinoma and other gastrointestinal and gynecological cancers (Lee et al., 2003; Lukasiak et al., 2008). Fourth, it is highly expressed in lymphoid organs like thymus, lymph nodes and spleen (Canaan et al., 2006;

Lee et al., 2003; Lukasiak et al., 2008), and last but not the least, FAT10-deficient mice show hypersensitivity to lipopolysaccharide (Canaan et al., 2006). Furthermore, the interaction of FAT10 with HDAC6, and hence, its localization in aggresomes under proteasome inhibition suggested a possible role of FAT10 in transporting the misfolded proteins to aggresomes for their removal (Kalveram et al., 2008). All these factors were in favor of the hypothesis that FAT10 could play a significant role in neurodegenerative diseases involving the formation of aggregates in the brain due to proteasome inhibition.

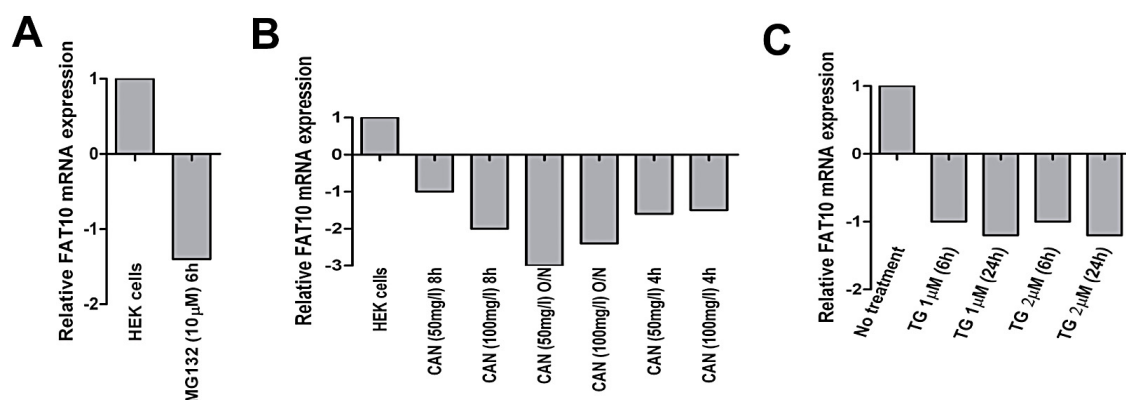


Figure 4.7 Misfolded protein stress has no significant effect on the expression of FAT10 mRNA. Total RNA was isolated and real-time RT-PCR was performed. Relative FAT10 mRNA expression in HEK293T cells is depicted in the graph when cells were treated with (A) proteasome inhibitor (MG132), (B) an analogue of arginine (canavanine, CAN), and (C) thapsigargin (TG), for the indicated time duration and concentration. *O/N: overnight.

Since, the expression of FAT10 is least in the brain, therefore, a relevant question was if FAT10 expression could be up-regulated after an immune response is elicited in the brain. This was addressed by infecting C57BL/6 mouse with LCMV either intravenously or intracranially. When LCMV was injected intravenously, FAT10 induction was not detected in the brain (Figure 4.1). The cellular basis of CNS immune privilege rests mainly on the absence of dendritic cells in the healthy CNS (Ransohoff and Cardona, 2010). But surprisingly, after 6 days of intracranial infection with LCMV, FAT10 was approx. 560-fold up-regulated in the brain of infected mouse as compared to the uninfected mouse (Figure 4.2A). Consistent with this, we observed a substantial increase in the level of IFN- γ and TNF- α (Figure 4.2B, C), which supports the earlier study

showing a marked increase in the pro-inflammatory cytokine genes in the brain, including TNF- α , IL-1 α , IFN- γ and IL-1 β after LCMV infection intra-cranially (Asensio et al., 1999). This deduced an important conclusion that *fat10* gene can be induced in the brain and its expression is controlled by the pro-inflammatory cytokines.

Next, we analyzed the expression of FAT10 in the brain of HD and α -synuclein transgenic mice. These diseases are characterized by the formation of aggregates in the brain usually at an old age. UbK48 isopeptide levels significantly increase early in pathogenesis in the cortex and striatum of R6/2 mice compared to non-transgenic littermates (Bennett et al., 2007), and therefore, we expected an increase in the expression of FAT10 as well. Real-time RT-PCR analysis for FAT10 showed an increase in the expression, which coincide with the expression pattern of IFN- γ . It was interesting to note that the induction was not the same in all the tissue samples from brain but there was a large variation. This was consistent with the difference in the level of cytokine induction in different parts of the brain. This also suggests that FAT10 is expressed in certain localized regions or in some specialized cell types in the brain. In future, it will be important to determine which cell type expresses FAT10 in the brain. The CNS consists of basically four cell types: neurons, astrocytes, oligodendrocytes and microglia. CNS trauma, infection, inflammatory and neoplastic diseases are all accompanied by a loss of blood-brain barrier integrity, and it has been considered axiomatic that exposure to plasma proteins activates microglia. Upon activation, microglia can produce numerous protein mediators, including those categorized as cytokines (both pro-inflammatory and anti-inflammatory cytokines), growth factors, chemokines and neurotrophins. In addition, they can express molecules that are associated with an ability to stimulate T cells with antigen (a feature typical of antigen presenting cells) (Ransohoff and Cardona, 2010). This information, together with the fact that FAT10 expresses abundantly in lymphoid organs, suggests the possibility that FAT10 is primarily expressed in microglia cells although this has to be carefully examined in future.

We also assessed the expression of FAT10 protein in the aggregates (insoluble fraction) from these animal models. The identification of FAT10 protein in the SDS-insoluble fraction of the whole lysate from R6/2 mice as compared to the wild type-mice (Figure 4.5A) argues for a significance of FAT10 in such diseases. The authenticity of R6/2 mouse can be judged by its wide application in HD studies (Bennett et al., 2007; Bett et

al., 2009; Bjorkqvist et al., 2008; Yamanaka et al., 2008) although one drawback of this model could be that it expresses only a fragment of mutant huntingtin, exon 1 (Mangiarini et al., 1996). It shares extensive behavioral and molecular phenotypes with Hdh^{Q150/Q150}, a knock-in mouse model of HD (Woodman et al., 2007). This result co-related with the immuno-staining which clearly indicated the presence of FAT10 in aggregates in human HD samples as well as in α -synuclein Tg mouse (Figure 4.6).

Recently, we identified a novel substrate of FAT10 conjugation. A covalent as well as non-covalent interaction of FAT10 with p62 was observed *in vivo* (Aichem A. et al., unpublished). p62 could be recruited to protein aggregates, which are ubiquitinated, because of its ability to bind polyubiquitin via the UBA domain (Donaldson et al., 2003). p62 is induced by proteasome inhibition (Ishii et al., 1997; Kuusisto et al., 2001; Thompson et al., 2003) and also as a response to the expression of mutant huntingtin (Nagaoka et al., 2004). It is implicated that p62 may be involved in linking polyubiquitinated protein aggregates to the autophagic machinery, thus, accelerating the clearance of such aggregates, and thereby, contributing to reduced toxicity of mutant huntingtin expression (Bjorkoy et al., 2005). Lewy bodies may serve a similar role like aggresomes in neurons in PD and related disorders (Johnston et al., 1998; Kopito, 2000). FAT10 is a cytosolic protein which is merely expressed in the brain. Taken together, these findings suggest that FAT10 expression is tightly controlled in the brain under normal conditions but induced when an immune response is elicited. Apparently, FAT10ylated proteins could be removed from aggregates in the case of neurodegenerative diseases by its mutual association with p62 and HDAC6. A plausible assumption would be that FAT10 is sequestered to aggresomes by HDAC6 under stress conditions, and then along with its conjugates become associated with p62, thus, enhancing the degradation of misfolded proteins via 26S proteasome or autophagy. Probably, FAT10 finds another way (apart from proteasomal degradation) to remove the misfolded proteins when proteasome function is inhibited because of the overload with such proteins. This could suggest a protective role of FAT10 in neurodegenerative diseases. Further investigation will help to understand the role of FAT10 in neurodegenerative diseases. An interesting attempt would be to cross-breed FAT10-deficient mouse with the diseased transgenic mouse and observe the change in the phenotype of the newly generated FAT10 deficient and HD or PD heterozygous mouse as compared to the corresponding wild-type mouse. As implicated from earlier studies that

FAT10 is highly up-regulated in liver and colon carcinoma (Lee et al., 2003), in future, it would be interesting to investigate the expression and role of FAT10 in the liver Mallory bodies and hyaline bodies in hepatocellular carcinoma, which contains p62 as well as polyubiquitylated proteins (Zatloukal et al., 2002).

4.4 METHODS

4.4.1 Mice

C57BL/6 mice were bred at the animal facility of University of Constance and treated in accordance with institutional guidelines. Brain tissue samples of α -synuclein transgenic mice were obtained from Boehringer Ingelheim Pharma GmbH & Co. (Biberach, Germany). Brain tissue samples of HD transgenic mice (R6/2) were obtained from Center for Clinical Research, University of Ulm.

4.4.2 Virus infection

LCMV-WE was originally obtained from F. Lehmann-Grube (Hamburg, Germany) and propagated on the fibroblast line L929. Mice were either infected with 200 pfu of LCMV-WE intravenously or 30 pfu intracranially.

4.4.3 RNA preparation and real-time RT-PCR

RNA was prepared from tissues using the Nucleospin RNA II kit (Machery Nagel) according to the manufacturer's protocol. The purity and integrity of RNA was checked on 1% agarose gel, and quantified by a spectrophotometer by measuring absorbance at 260 nm. cDNA was synthesized using the Reverse Transcription System kit (Promega) and the relative gene expression was determined with LightCycler Fast Start DNA Master SYBR Green I Kit (Roche) using LightCycler Instrument (Roche). The relative gene expression of IFN- γ was determined by using TaqMan Master Kit (Roche) using IFN- γ specific probe (Roche). Normalization was performed by using a house keeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT). The real-time RT-PCR for FAT10 and HPRT was conducted as described by Lukasiak et al. 2008. The PCR primers

for IFN- γ used were: forward: 5'-atc tgg agg aac tgg caa aa- 3'; reverse: 5'-ttc aag act tca aag agt ctg agg- 3'. The PCR primers for TNF- α used were: forward: 5' -gaa ctgg cag aag agg cac t- 3'; reverse: 5' -gg tct ggg cca tag aac tga- 3'. The specificity of the product was checked by melting curve analysis and by running the product on an agarose gel. The data obtained was analyzed by using the mathematical approach for relative gene expression quantification in real-time RT-PCR, called the Pfaffl method (Pfaffl, 2001) which calculates C_t values and the PCR efficiencies of the respective primer pair. An Excel based program REST (Pfaffl et al., 2002) calculated the relative gene expression of a target in comparison to a reference gene.

4.4.4 PolyGln aggregates dissociation from Htt transgenic mice

The frozen brain tissues were homogenized in ice-cold RIPA buffer (50mM Tris.HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and EDTA free complete protease inhibitor tablet (Roche)) using a homogenizer. After sonification (2 cycles for 20 sec each), the homogenates were filtered through cheese cloth; and 2% SDS, 5% β -mercaptoethanol and 15% glycerol was added. Samples were boiled for 5 min at 95°C followed by sonification (2 cycles for 20 sec each) and centrifugation at 100g for 5 min. Thereafter, SDS-soluble and SDS-resistant proteins were separated by microcentrifugation (Beckman, TLA-55) at 100,000g for 1h at 4°C. The pellet was washed twice with chloroform and resuspended in 100% formic acid. Samples were incubated at 37°C for 30 min and 0.1% SDS was added. Some of the extract was preserved and rest was dried using speed vac. The resulting dried material was resuspended in SDS-buffer and boiled for 5 min at 95°C before loading on the SDS-gel.

4.4.5 Proteasome inhibition, canavanine, puromycin and thapsigargin treatment

HEK293T cells were cultivated in DMEM supplemented with 10% FCS and 100 μ g/ml penicillin/streptomycin (Invitrogen) and then were treated with 10 μ M MG132 (Sigma) in DMSO for 5h and control cells were treated equally with DMSO. To study the effect of misfolded protein stress, the cells were treated with either 50 μ g/ml or 100 μ g/ml of canavanine (Sigma) for 4h, 8h and overnight. Puromycin (Sigma) treatment was performed for 2h at a concentration of 5 μ g/ml in Hela cells. ER stress was induced in

HEK293T cells by using thapsigargin (Sigma) at a concentration of 1 μ M or 2 μ M for 6h and 24h.

4.4.6 Western blot analysis

Proteins from the whole lysate were blotted with antibodies raised against FAT10 (rabbit polyclonal) (Hipp et al., 2005), huntingtin (Abcam), or GAPDH (Sigma). The secondary antibody (swine anti-rabbit or goat anti-mouse coupled with horse radish peroxidase) was detected by the Supersignal Substrate Western Blotting Kit (Pierce).

4.4.7 Immunohistochemistry

These experiments were performed at the Experimental Neurology Department at University of Ulm, Germany, using standard procedures.

4.5 ACKNOWLEDGEMENTS

We thank Dr. Thomas Ciossek (Boehringer Ingelheim Pharma GmbH & Co.) and Prof. Dr. Bastian Hengerer for providing transgenic mice material and technical comments.

5. USE1 is a Bispecific Conjugating Enzyme for Ubiquitin and FAT10 which FAT10ylates itself in *cis*

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5.1 INTRODUCTION

The covalent conjugation with modifiers of the ubiquitin family is a versatile and widely used post-translational modification that may change the function, the fate or the localization of specific target proteins. The formation of an isopeptide bond between the C-terminus of ubiquitin and a lysine residue in a target protein requires that the C-terminal glycine residue of ubiquitin becomes activated by an E1 type enzyme which first adenylates ubiquitin and then transfers it onto a cysteine residue of the E1 enzyme to form a thioester. The activated ubiquitin is subsequently passed onto a cysteine in the active site of a ubiquitin conjugating enzyme (E2). The ubiquitin-charged E2 enzyme and a specific substrate protein are then both bound by a ubiquitin ligase (E3) which catalyses the transfer of the activated ubiquitin onto the substrate protein (Hershko and Ciechanover, 1998). In humans there are dozens of E2 enzymes and hundreds of E3 enzymes, the specificity of which ensures that ubiquitylation is a highly regulated and substrate selective event. The initial activation of ubiquitin was for decades believed to be accomplished solely by a single enzyme designated UBE1 (Ciechanover et al., 1981; Haas et al., 1982), until recently when it was shown that a second E1 type enzyme, called UBA6 (UBE1L2, E1-L2, MOP-4), exists, which can also activate ubiquitin (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007). Interestingly, UBE1 and UBA6 do not cooperate with the same set of E2 enzymes and one E2 enzyme has been described which can accept ubiquitin only from UBA6 but not from UBE1. This E2 enzyme, which was originally named E2Z (Gu et al., 2007), has accordingly been renamed as UBA6-specific E2 enzyme (USE)1 (Jin et al., 2007).

The principle of ubiquitin activation and conjugation via an E1-E2-E3 cascade also applies to several well-investigated ubiquitin-like modifiers including SUMO-1/2/3, NEDD8, ISG15, and ATG8/12. Each of these subfamilies of modifiers use their private E1, E2, and E3 enzymes to ensure, that a unique modifier is specifically transferred via the respective private E2 and E3 enzymes onto the correct target substrate (Huang et al., 2005; Kerscher et al., 2006). This elaborate specificity allows separate regulation of conjugation with each of the known ubiquitin-like modifiers and it ensures that a given substrate is not derivatized by the wrong modifier. This principle of wholly specific enzymes was found to be violated when it was discovered recently, that the E1 enzyme UBA6 activates not only ubiquitin, but also the ubiquitin-like modifier FAT10 (Chiu et

al., 2007). This finding poses the interesting question how this E1 enzyme can discriminate between an E2 enzyme that needs to be charged with activated ubiquitin or FAT10 (Groettrup et al., 2008). Since the E2 enzymes Ubc5 and Ubc13 can be charged with activated ubiquitin but not with FAT10 *in vitro* (Chiu et al., 2007), it is possible that the discrimination between ubiquitin and FAT10 may occur on the level of E2 enzymes.

FAT10 is an interferon (IFN) γ - and tumour necrosis factor (TNF) α -inducible ubiquitin-like modifier, which is encoded in the major histocompatibility complex (MHC) class I locus and is constitutively expressed in mature dendritic cells and B cells (Bates et al., 1997; Liu et al., 1999; Raasi et al., 1999). FAT10 consists of two ubiquitin-like domains in tandem array and bears a free diglycine motif at its C-terminus, which is required for the covalent conjugation to so far unidentified target proteins (Chiu et al., 2007; Raasi et al., 2001). The biological function of FAT10 remains poorly understood but it has been shown that overexpression of FAT10 leads to caspase dependent apoptosis in a mouse fibroblast cell line (Raasi et al., 1999) and renal tubular epithelial cells (Ross et al., 2006). In addition, FAT10 has been shown to serve as a signal for proteasomal degradation independently of the ubiquitylation system (Hipp et al., 2005; Schmidtke et al., 2009). NEDD8 ultimate buster long (NUB1L), a cytokine inducible linker protein which non-covalently interacts with FAT10 via its three C-terminal ubiquitin-associated (UBA) domains and with the 26S proteasome via its N-terminal ubiquitin-like (UBL) domain, accelerates the degradation of FAT10 and FAT10-linked proteins (Hipp et al., 2004; Schmidtke et al., 2006).

In this study, we sought to further characterize the conjugation pathway of FAT10. Stimulated by the identification of USE1 as an interaction partner of FAT10 in a yeast two hybrid screening, we characterized USE1 as a conjugating enzyme for FAT10 *in vitro* and *in vivo*. Interestingly, USE1 turned out to be not only a major E2 enzyme for FAT10 but also the first substrate of FAT10 as USE1 was demonstrated to auto-FAT10ylate itself in *cis*.

5.2 RESULTS

5.2.1 Identification USE1 as an interaction partner of FAT10

It has recently been shown that UBA6 is an E1 enzyme not only for ubiquitin but also for FAT10 (Chiu et al., 2007). During *in vitro* activation experiments we had obtained independent evidence that UBA6 can activate FAT10; however, in some of the experiments an activation of FAT10 by UBE1L was also observed (C. Pelzer, unpublished data). To further investigate the interaction between these E1 enzymes and FAT10, a yeast two hybrid assay was performed. FAT10 could only interact with UBA6, but not with the closely related E1-type enzymes UBE1 or UBE1L, whereas ISG15, used as control, interacted specifically with UBE1L, but not with UBE1 or UBA6 (Figure 5.1). The interaction between the proteins described was clear in an X-Gal filter assay and as judged from the colour change on selective growth plates. Therefore, our data is consistent with UBA6 being the E1 enzyme for FAT10, since ISG15, which has a similar structure as the one predicted for FAT10 (Groettrup et al., 2008; Narasimhan et al., 2005), cannot interact with UBA6.

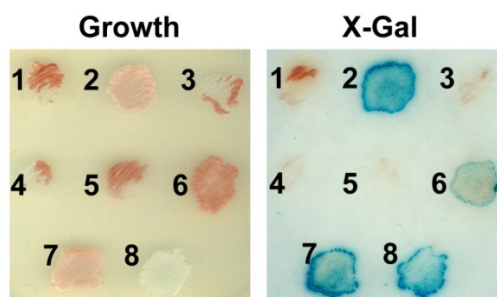


Figure 5.1 FAT10 interacts specifically with UBA6 and USE1 in yeast two hybrid assay. Yeast NMY51 were co-transformed with pLexA-N-ISG15 and pACT2-UBE1 (1), pACT2-UBE1L (2) or pACT2-UBA6 (3), respectively, or with pLexA-FAT10 and pACT2-UBE1 (4), pACT2-UBE1L (5), pACT2-UBA6 (6), pACT2-USE1short (7) or pACT2-USE1 full length (8), respectively, and grown on 3-AT containing selection plates lacking tryptophan, leucine and histidine (left panel). An X-Gal filter assay was performed to verify the interaction of the respective proteins (right panel). A representative experiment from a total of three experiments with similar outcome is shown.

In order to identify a possible E2 enzyme for FAT10, a yeast two hybrid screen was performed with human FAT10 serving as a bait for screening of a human thymus library. This approach was chosen as it has been shown previously that, at least in some cases, E2

enzymes can non-covalently interact directly with their cognate ubiquitin-like modifiers (Knipscheer et al., 2007). Sequencing of the positive clones revealed that, besides the known interaction partner NUB1L (Hipp et al., 2004), a truncated version of the E2 enzyme USE1 was among the identified putative interaction partners of FAT10. This result was further pursued because USE1 has been shown to take over activated ubiquitin from UBA6 (Jin et al., 2007), the E1 enzyme of FAT10 (Chiu et al., 2007), and because we could show by yeast two hybrid interaction assay, that USE1 specifically interacted with UBA6 but not with UBE1 or UBE1L (Figure 5.2 and note 1). The isolated cDNA of USE1 was lacking 259 nucleotides at the 5' end, designated USE1short. Hence, it was necessary to also test full-length USE1, which is extended N-terminally (Jin et al., 2007). The complete USE1 cDNA was therefore cloned into the prey vector and a yeast two hybrid assay was carried out again. The full-length USE1 also gave a blue colour change in the X-Gal filter assay demonstrating the interaction of the proteins (Figure 5.1, right panel). In addition to the growth on the selection plates already indicating the interaction of FAT10 and USE1, the co-transformants showed a colour change to faint pink for USE1short or to white for full-length USE1 (Figure 5.1, left panel). From the difference in colour it seemed that full-length USE1 interacts more strongly with FAT10. Thus, we concluded that both USE1short and the full-length USE1 interact with FAT10 under the conditions in the yeast two hybrid assay and we hypothesized that USE1 might act as an E2 enzyme for FAT10.

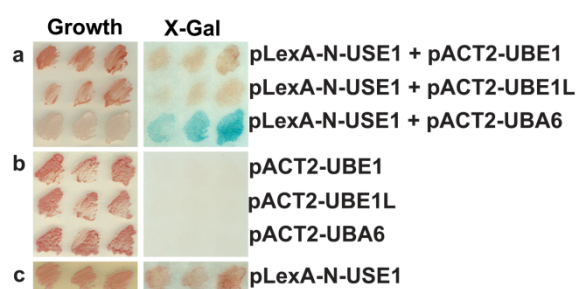


Figure 5.2 Specific interaction of USE1 with UBA6 in yeast two-hybrid assay. (a) *S. cerevisiae* strain NMY51 was co-transformed with pLexA-N-USE1 and pACT2-UBE1, pACT2-UBE1L or pACT2-UBA6 and grown on 3-AT-containing selection plates lacking tryptophan, leucine and histidine (left panel). Protein-protein interaction was visualised by an X-Gal filter assay (right panel). (b,c). Yeast NMY51, expressing the single proteins as indicated, were grown on selection plates lacking leucine in case of the pACT2 constructs or tryptophan in case of pLexA-N-USE1 (left panels) and tested for absence of self-activation by an X-Gal assay (right panels). A representative experiment is shown out of a total of three experiments performed.

5.2.2 USE1 serves as a conjugating enzyme for FAT10 *in vitro* and *in vivo*

To test *in vitro* whether the transfer of FAT10 onto USE1 can occur, *in vitro* transcribed and translated FLAG-His-FAT10 or FLAG-His-FAT10 Δ GG was incubated with recombinant His-tagged USE1 in the presence of recombinant GST-UBA6. The *in vitro* conjugation assay revealed that FAT10 was covalently linked to USE1 and the amount of the FAT10-charged GST-UBA6 decreased simultaneously (Figure 5.3a). FLAG-His-FAT10 Δ GG served as a negative control, and was not transferred onto His-USE1 protein. Similar experiments performed with ubiquitin (Figure 5.3b) showed that HA-tagged ubiquitin readily conjugated to USE1 in accordance with a previous report (Jin et al., 2007). These results indicate that USE1 is not exclusively a conjugating enzyme for ubiquitin but also serves as an E2 enzyme for FAT10 *in vitro*.

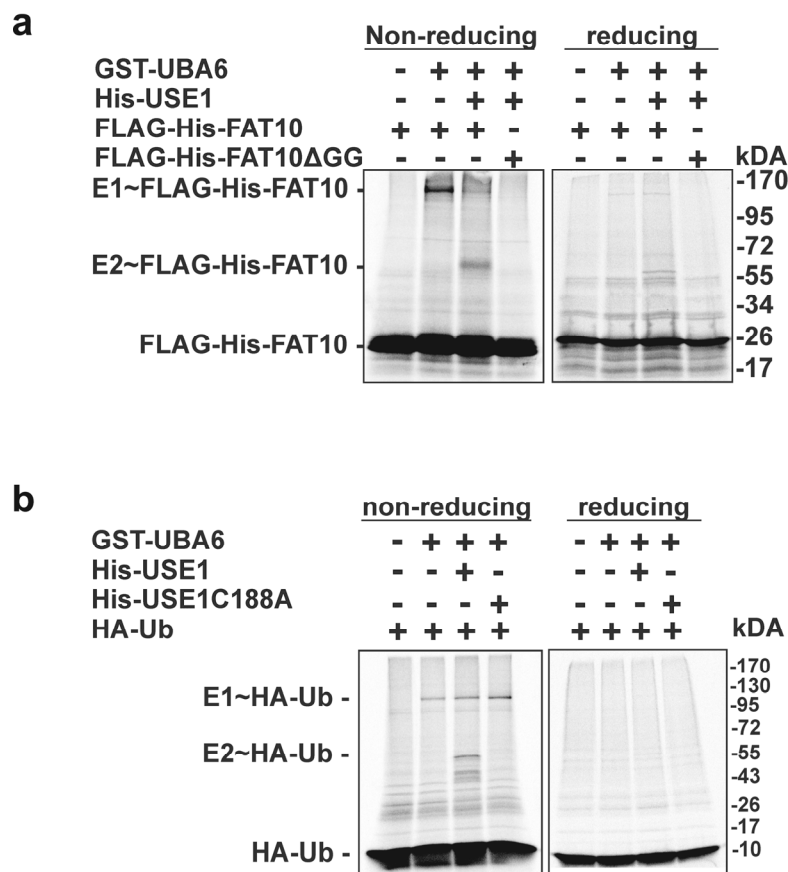


Figure 5.3 FAT10 is transferred from UBA6 onto USE1 *in vitro*. (a) Bacterially expressed affinity purified His-USE1 was incubated together with ATP, recombinant GST-UBA6 and *in vitro* transcribed and translated ^{35} S-labelled FLAG-His-FAT10 bound to anti-FLAG beads (lane 3 from the left). As negative controls FLAG-His-FAT10 alone (lane 1), FLAG-His-FAT10 and GST-UBA6 (lane 2), and FLAG-His-FAT10 Δ GG with GST-UBA6 and His-USE1 (lane 4) were used. Samples were subjected to non-reducing

(left) and reducing (right) SDS-PAGE and visualized by autoradiography. The positions of FAT10, the FAT10-USE1 conjugate (E2~Flag-His-FAT10) and the FAT10-UBA6 conjugate (E1~Flag-His-FAT10) are indicated at the left. **(b)** As a control bacterially expressed affinity purified His-USE1 was incubated together with ATP, recombinant GST-UBA6 and *in vitro* transcribed and translated ³⁵S-labelled HA-ubiquitin bound to anti-HA beads (lane 3 from the left). As negative controls HA-ubiquitin alone (lane 1), HA-ubiquitin with GST-UBA6 (lane2), or HA-ubiquitin with GST-UBA6 and His-[C¹⁸⁸A]USE1 (lane 4) were used. Samples were subjected to non-reducing (left) and reducing (right) SDS-PAGE and visualized by autoradiography. The positions of HA-ubiquitin, the ubiquitin-USE1 conjugate (E2~HA-ubiquitin) and the ubiquitin-UBA6 conjugate (E1~HA-ubiquitin) are indicated at the left. The experiments shown are one out of three experiments performed with similar outcome.

To further characterize the interaction between FAT10 and USE1 *in vivo*, co-immunoprecipitation experiments were performed in transiently transfected HEK293 cells. A higher molecular weight conjugate between HA-FAT10 and 6His-USE1 could be detected by western blot analysis under either reducing or non-reducing conditions, which was not observed when the catalytic site Cys¹⁸⁸ of USE1 was mutated to alanine (Figure 5.4a). When His-USE1 and HA-ubiquitin were co-expressed, a His-USE1~HA-Ub conjugate could be readily detected under non-reducing conditions but, in contrast to the His-USE1-HA-FAT10 conjugate, it could be cleaved readily with β -mercaptoethanol consistent with thioester linkage (Figure 5.4b). As a control we performed similar experiments with UbcH8 which can serve as a conjugating enzyme both for ISG15 and ubiquitin *in vitro* (Durfee et al., 2008; Kim et al., 2004; Zhao et al., 2004). In contrast to USE1, UbcH8 did not form a conjugate with FAT10, but only with ubiquitin under non-reducing conditions (Figure 5.4c). When these samples were treated with reducing agents, the interaction disappeared. Therefore, FAT10 cannot be transferred to UbcH8, but to USE1 forming a covalent and non-reducible bond *in vivo*.

5.2.3 Formation of a USE1-FAT10 conjugate in cytokine stimulated cells

To test if a USE1-FAT10 conjugate can also be found in the absence of overexpression, we treated HEK293 cells with TNF- α and IFN- γ to induce FAT10 and then performed immunoprecipitation with a rabbit monoclonal anti-USE1 antibody followed by western blot analysis with either a newly generated FAT10 specific mAb (designated 4F1, Figure 5.5) or a USE1 specific polyclonal antibody (Figure 5.6) under reducing conditions (10% 2-ME).

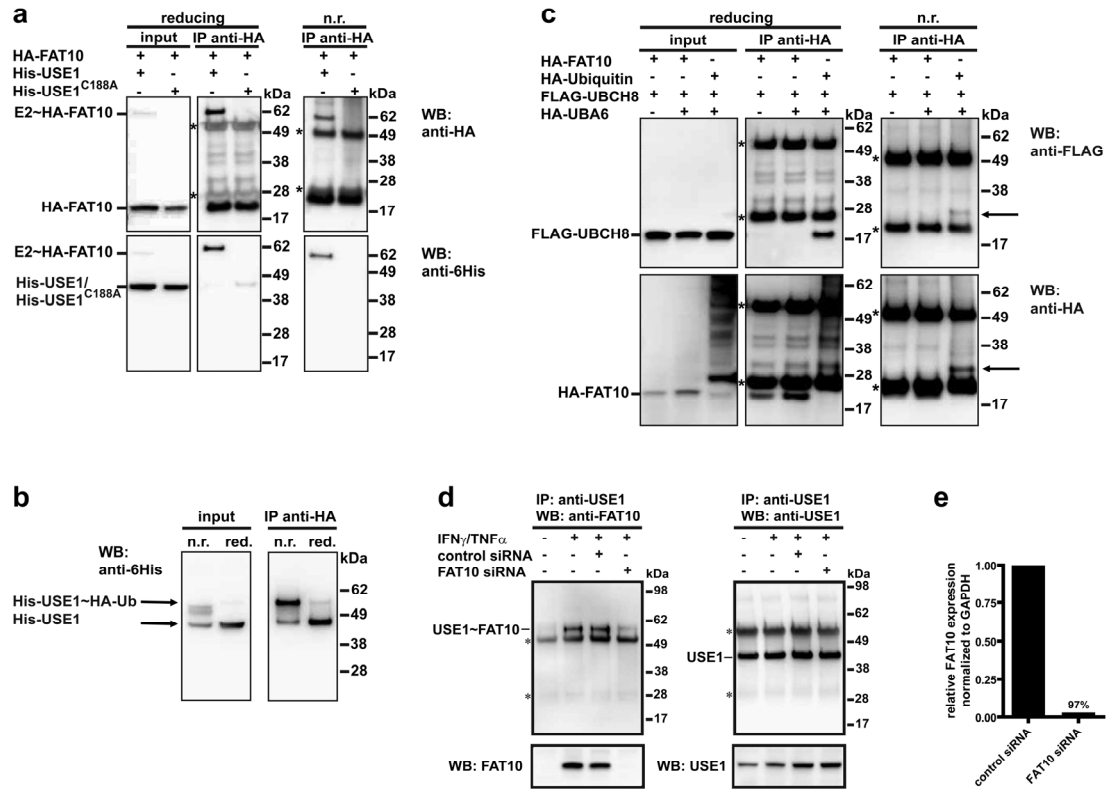


Figure 5.4 Conjugate formation of FAT10 and USE1 *in vivo*. (a) Co-immunoprecipitation of HA-FAT10 and His₆-USE1. HEK293 cells were transiently transfected with pcDNA3.1-HA-FAT10 and pcDNA3.1/His-A-Xpress-USE1 as indicated. After immunoprecipitation against the HA-tag of FAT10, samples were subjected to western analysis with a peroxidase-conjugated polyhistidine-reactive antibody or a HA-reactive mAb. Samples were analysed under non-reducing (n.r.) and reducing conditions. (b) Co-immunoprecipitation of HA-ubiquitin and His₆-USE1. HEK293 cells were transfected with pcDNA3.1-HA-ubiquitin and pcDNA3.1/His-A-Xpress-USE1. After immunoprecipitation against the HA-tag of HA-ubiquitin, samples were subjected to western blot analysis with a peroxidase-conjugated polyhistidine-reactive antibody. Samples were analysed under non-reducing (n.r.) and reducing (red.) conditions. (c) Co-immunoprecipitation from HA-FAT10, HA-ubiquitin (Ub), FLAG-UbcH8 or HA-UBA6 expressing cells, which were transiently transfected with pcDNA3.1-HA-FAT10, pcDNA3.1-HA-Ub, pcDNA3-FLAG-UBCH8 or pcDNA3.1-HA-UBA6 as indicated. After immunoprecipitation with anti-HA-Sepharose, reduced and non-reduced (n.r.) samples were subjected to western blot analysis with HA-reactive and FLAG-reactive mAb. Asterisks indicate the heavy and light chains of the HA-reactive antibody used for the immunoprecipitation. Arrows indicate the FLAG-UbcH8-HA-Ub conjugate. Representative experiments out of three experiments with similar outcome are shown. (d) Co-immunoprecipitation of endogenous USE1 and FAT10 from HEK293 cells. Cells were transfected twice with a negative control siRNA or with a mixture of four siRNAs against FAT10 and subsequently treated with 200U/ml IFN γ and 400 U/ml TNF α . After 3 days, cells were lysed and USE1 was immunoprecipitated using a USE1-reactive rabbit mAb. Subsequently, proteins were subjected to western blot analysis using a FAT10-reactive mAb (left panel) or a USE1-reactive mAb (right panel) under reducing conditions. Loading controls are shown in the bottom panels. (e) The knockdown of FAT10 was confirmed by real time RT-PCR. The relative FAT10 mRNA expression level in IFN γ /TNF α treated HEK293 cells transfected with control siRNA was set to unity and the RNA levels were normalized to the expression of GAPDH. The experiments have been repeated three times with similar outcome. Asterisks indicate the heavy and light chains of the HA-reactive antibody used for the immunoprecipitation.

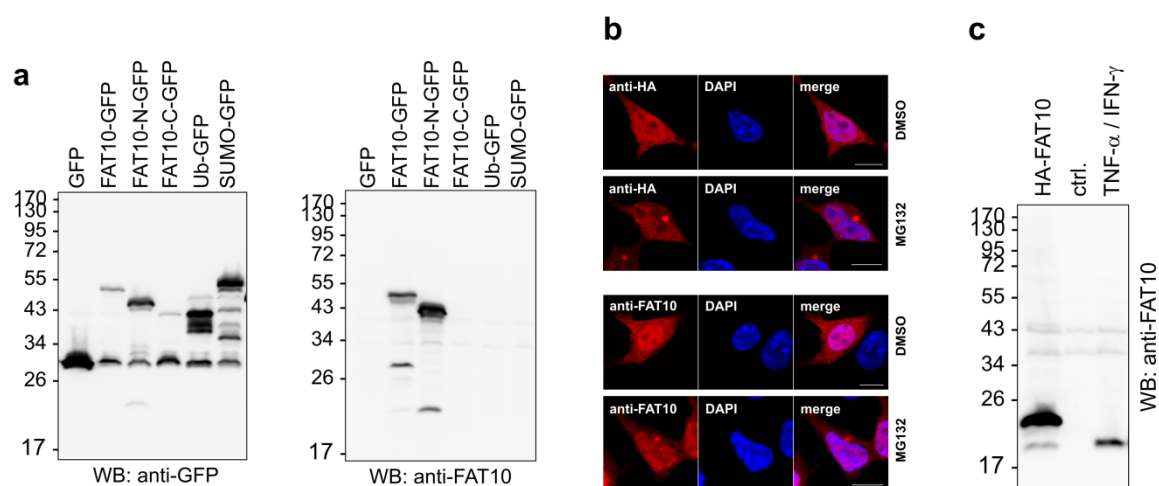


Figure 5.5 Characterization of a FAT10-specific monoclonal antibody which recognizes the N-terminal UBL-domain of human FAT10. (a) HEK293T cells were transiently transfected with GFP or N-terminal GFP fusions of full length FAT10, its isolated UBL-domains (FAT10-N and FAT10-C), Ubiquitin (Ub) or SUMO. Cell lysates were then analysed by western blot with an anti-GFP antibody (Sigma, G1544) as a transfection control (left panel) or with a 50ng/ml dilution of the FAT10-specific clone 4F1 monoclonal antibody (right panel). (b) HEK293T cells were transiently transfected with HA-FAT10, treated with 5 μ M MG132 for 6 hours to induce the formation of aggresomes and subsequently fixed with 4% PFA. Cells were then stained with a 6.8 μ g/ml dilution of anti-HA clone HA-7 (Sigma, top panel) or a 1 μ g/ml dilution of the FAT10-specific mAb 4F1 (bottom panel) followed by an Alexa Fluor 546-coupled secondary goat-anti-mouse antibody. Confocal fluorescence microscopy images are shown. (c) HEK293T cells were either transiently transfected with HA-FAT10, left untreated (ctrl.) or treated with 100U/ml of TNF- α and IFN- γ for 16h. Cell lysates were then analysed by western blot with an 100ng/ml dilution of the FAT10-specific mAb 4F1.

The blots shown in Figure 5.4d reveal that the endogenous FAT10-USE1 conjugate was detectable in the presence but not in the absence of FAT10 induction by pro-inflammatory cytokines (anti-FAT10 blot, lane 1 and 2). Consistently, the endogenous conjugate was strongly diminished when FAT10 was downregulated by FAT10 specific siRNA but not when cells were treated with a negative control siRNA (anti-FAT10 blot lanes 3 and 4). The successful knockdown of FAT10 mRNA by about 97% as compared to treatment with control siRNA was verified by real time RT-PCR (Figure 5.4e). When USE1 was immunoprecipitated with USE1 specific mAb followed by western analysis with the same USE1 specific mAb (Figure 5.4d, right panel) or a USE1 specific pAb (data not shown) we failed to visualize the endogenous USE1-FAT10 conjugate in most experiments as this detection system was not sensitive enough for this purpose.

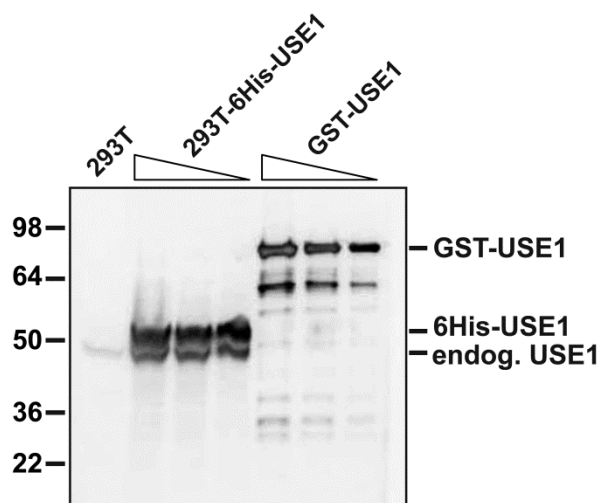


Figure 5.6 Characterization of USE1 specific polyclonal antibody. Rabbits were immunized with a USE1₂₃₁₋₂₄₂ peptide conjugated to keyhole limpet hemocyanin and the resulting immune serum was used for the displayed western blot at a dilution of 1:2000. Blotted are total lysates of wild type HEK293T cells (100µg) and HEK293T cells transiently transfected with a 6His-USE1 expression construct (100µg, 50µg, 25µg) as well as 200ng, 100ng, and 50ng of recombinant GST-USE1 protein.

To address the question of the relevant efficiency of charging USE1 with ubiquitin versus FAT10, a semi-endogenous immunoprecipitation experiment was performed where upon transfection of HEK293 cells with expression constructs for FLAG-His-USE1 and HA-ubiquitin, FAT10 expression was induced with IFN- γ and TNF- α . As shown in Figure 5.7, the USE1-FAT10 conjugate was clearly formed next to the USE1-Ub conjugate (IP, lane 4). In cells which expressed HA-ubiquitin, the USE1-FAT10 conjugate was still readily detectable indicating that endogenous FAT10 could readily compete with ubiquitin for conjugation to USE1 (IP lane 5 versus 4). Under reducing conditions, the USE1-ubiquitin conjugate was completely reduced whereas again the USE1-FAT10 conjugate again was not (data not shown).

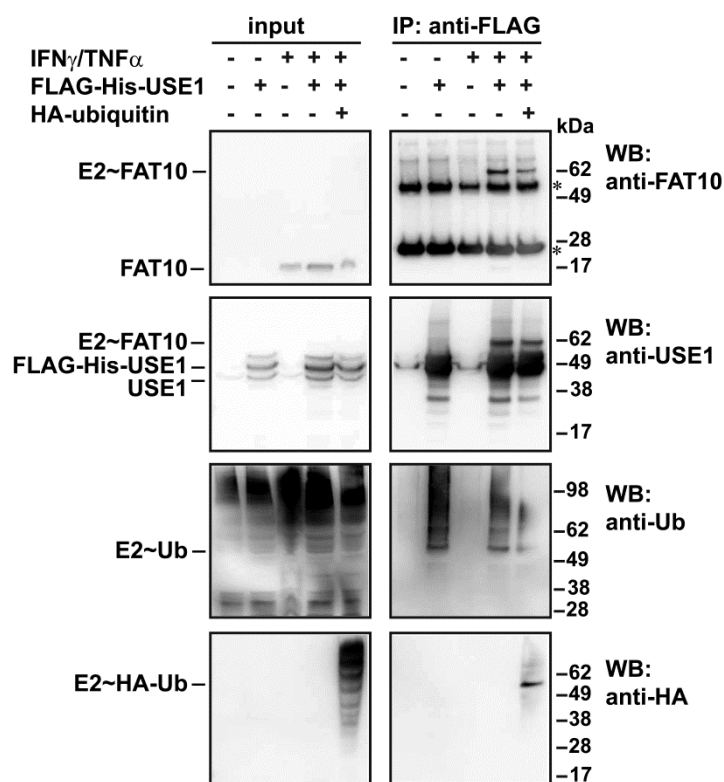


Figure 5.7 Endogenous FAT10 competes with ubiquitin for USE1 conjugation. HEK293 cells were transfected with an expression construct encoding FLAG-His-USE1 or additionally transfected with an HA-ubiquitin expression construct and subsequently stimulated for 24 hours with IFN- γ and TNF- α , as indicated. An anti-FLAG immunoprecipitation was performed and the precipitates were analysed under non-reducing conditions on a western blot using a monoclonal anti-FAT10 antibody, a polyclonal anti-USE1 antibody, a polyclonal anti-ubiquitin or a polyclonal anti-HA antibody to evaluate USE1 conjugate formation with FAT10, ubiquitin or HA-ubiquitin. The asterisks denote reactivity of the second stage Ab with the IgG heavy and light chain of the FLAG specific mAb. The plasmid content in all transfections was balanced with empty expression vector. One out of three experiments with similar outcome is shown.

5.2.4 Silencing of USE1 leads to a reduction of FAT10 conjugates

To determine if USE1 plays a central role in FAT10 conjugate formation *in vivo*, we tested whether targeted knockdown of USE1 with siRNA leads to a significant reduction of FAT10 conjugates in HEK293 cells transfected with a FLAG-His-FAT10 expression construct. As a control, knockdown of UBA6 or UBA6 and USE1 together was performed. Immunoprecipitation experiments in Figure 5.9a showed that the down-regulation of endogenous USE1 by about 90%, as measured by real time RT-PCR (Figure 5.9b), compared to HEK293 cells transfected with control siRNA, led to a strong decrease in the amount of FAT10-conjugates that was comparable to the decrease of FAT10

conjugates observed with a 90% down-regulation of UBA6 mRNA. This effect was slightly enhanced when both, USE1 and UBA6 were down-regulated simultaneously. As a control, cells were transfected with the diglycine mutant of FAT10 (FLAG-His-FAT10 Δ GG) that did not show FAT10 conjugation. As a loading control for the prepared lysates, β -actin was used for western blot analysis and the levels of UBA6, USE1 and FLAG-His-FAT10 proteins in the lysates used for the immunoprecipitation were monitored by western blot analysis (Figure 5.9a, lower panels). The UBA6 antibody (Enzo Lifesciences) is highly sensitive as determined by its reactivity for endogenous UBA6 in HEK293T cells (Figure 5.8). Since transfection of the cells with siRNA lowered the total amount also of monomeric FAT10 in the cells, we quantified the ECL signals with Quantity One Software (BioRad) and calculated a coefficient of conjugated-FAT10 : mono-FAT10 which was 1.0 for lane 2 (no siRNA), 0.96 for lane 3 (control siRNA), 0.4 for lane 4 (UBA6 siRNA), 0.7 for lane 5 (USE1 siRNA), 0.5 for lane 6 (UBA6 and USE1 siRNA) and 0.04 for lane 7 (diglycine mutant of FAT10), clearly showing the reduction of the FAT10 conjugates after down-regulation of USE1 or UBA6. To exclude off-target effects by using a mixture of 4 different USE1 siRNAs, each single siRNA was used separately to downregulate USE1 in HEK293 cells, which also led to a clear reduction of the FAT10 conjugates (Figure 5.10) supporting further the results shown in Figure 5.9a.

In a second approach, the USE1 knockdown experiment was performed under completely endogenous conditions. Therefore the cells were again transfected with siRNA against USE1 and subsequently endogenous FAT10 expression was induced by stimulation of the cells for 24 hours with IFN γ /TNF α . As shown in Figure 5.9c, knockdown of USE1 mRNA by about 89% as compared to control siRNA transfected cells led to a strong reduction of FAT10-conjugates relative to FAT10 monomer expression under completely endogenous conditions with coefficients of conjugated-FAT10 : mono-FAT10 of 1 for lane 3 (no siRNA), 0.3 for lane 4 (control siRNA) and 0.1 for lane 5 (USE1 siRNA).

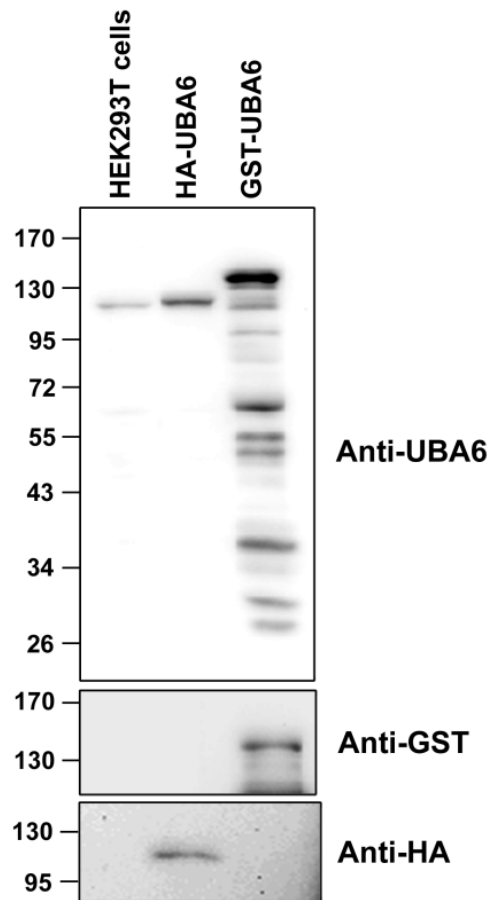


Figure 5.8 Characterization of UBA6 specific polyclonal antibody. Shown is a western blot of cell lysates from HEK293T cells, either untransfected (left lane) or transiently transfected with HA-UBA6 expression construct (middle lane), as well as recombinant GST-UBA6 (200ng, right lane). The serum from immunized rabbits was used at a dilution of 1:1000 in western blot. Anti-HA and anti-GST western blots are shown as control.

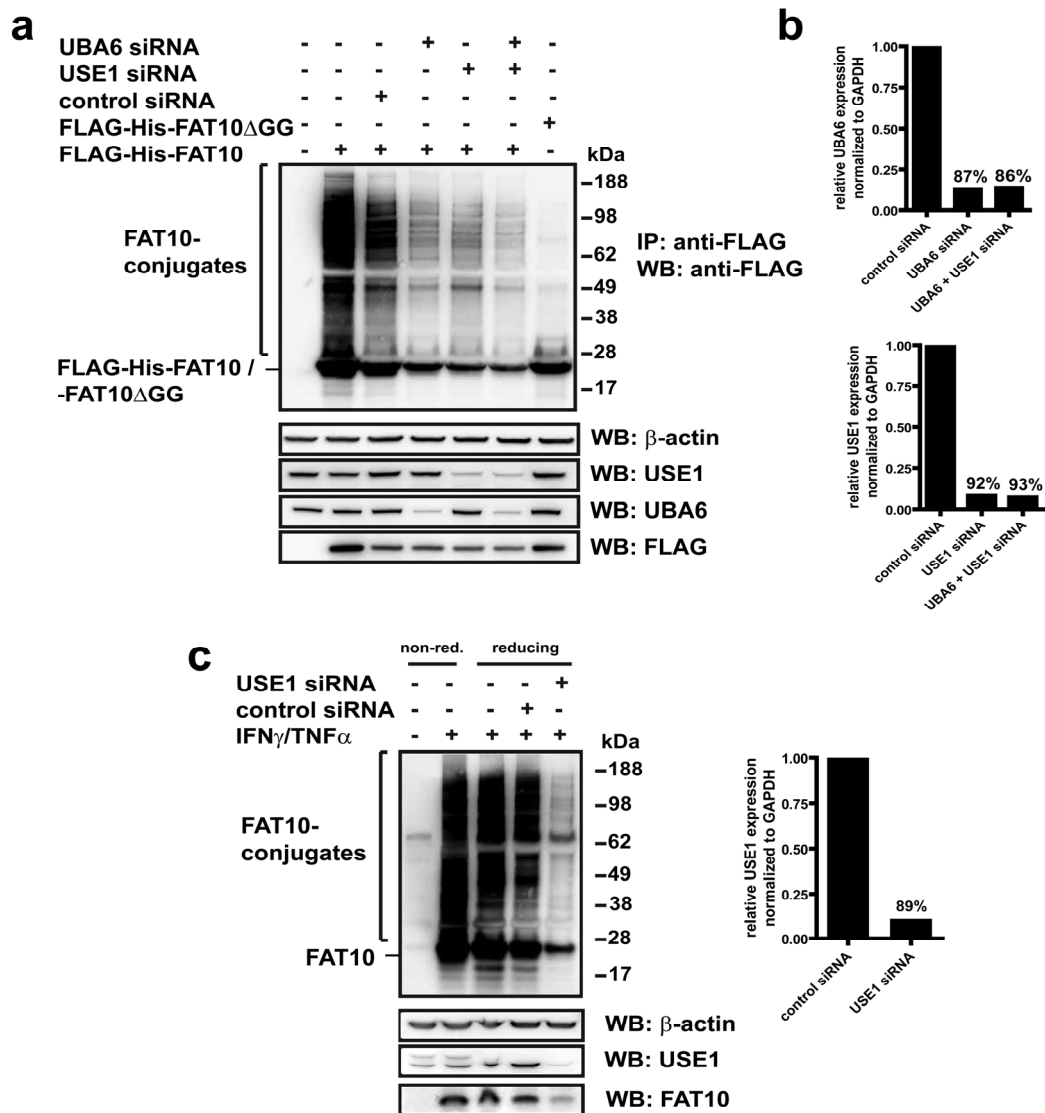


Figure 5.9 Knockdown of USE1 leads to impaired FAT10 conjugate formation. (a) HEK293 cells were transfected or not with a mixture of four different siRNAs against UBA6, USE1, both together, or with a control siRNA and subsequently transfected with an expression plasmid encoding FLAG-His-FAT10 or its diglycine mutant FLAG-His-FAT10 Δ GG. After four days an anti-FLAG immunoprecipitation was performed and the precipitates were analysed by western blot using a directly peroxidase-linked anti-FLAG mAb to evaluate FAT10 conjugate formation. Untransfected HEK293 cells or HEK293 cells transfected with FLAG-His-FAT10 or its mutant form FLAG-His-FAT10 Δ GG served as positive and negative control for the FAT10-conjugate formation, respectively. In the upper panel the western blot against FLAG-FAT10 after the immunoprecipitation of FLAG-FAT10 conjugates is shown. The lower panels show the expression levels of USE1, UBA6 and FAT10, respectively; β -actin served as a loading control for the western blot. (b) The knockdown of USE1 and UBA6 was confirmed by real time RT-PCR. The relative UBA6 or USE1 mRNA expression level in HEK293 cells, transfected with control siRNA and FLAG-His-FAT10 was set to unity and the RNA levels were normalised to the expression of GAPDH. A representative experiment out of three with similar outcome is shown. (c) HEK293 cells were transfected twice with siRNA against USE1 and subsequently treated for 24 hours with IFN γ /TNF α to induce endogenous FAT10 expression, followed by an immunoprecipitation using a FAT10 specific mAb. FAT10 conjugates were analysed on a western blot using a FAT10 pAb (left panel). Knockdown of USE1 (right panel) was confirmed by real time RT-PCR as described in (b). One out of three experiments with similar outcome is shown.

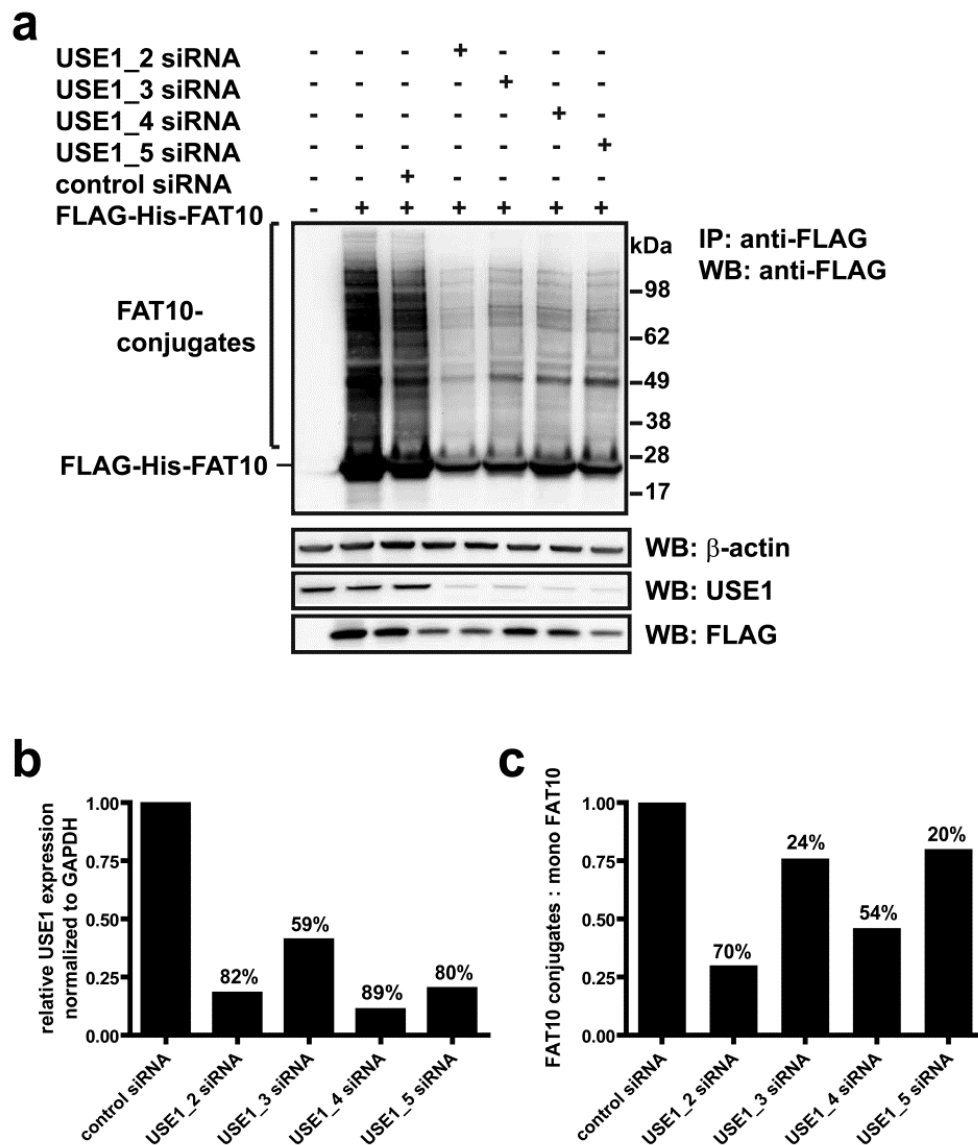


Figure 5.10 Knockdown of USE1 by single USE1 siRNAs downregulates the amount of FAT10 conjugates in HEK293 cells. (a) HEK293 cells were transfected or not with four different siRNAs against USE1 or with a control siRNA and subsequently transfected with an expression plasmid encoding FLAG-His-FAT10. After four days an anti-FLAG immunoprecipitation was performed and the precipitates were analysed by western blot using a directly peroxidase-linked anti-FLAG monoclonal antibody to evaluate FAT10 conjugate formation. Untransfected HEK293 cells or HEK293 cells transfected with FLAG-His-FAT10 serve as a negative or positive control for FAT10-conjugate formation, respectively. In the upper panel a western blot against FLAG-FAT10 after the immunoprecipitation of FLAG-FAT10 conjugates is shown. The lower panels show the expression levels of USE1 or FLAG-FAT10 with an anti-USE1 or anti-FLAG antibody, respectively. β -actin serves as a loading control for the western blot. (b) The knockdown of USE1 was confirmed by real time RT-PCR. The relative USE1 mRNA expression level in HEK293 cells, transfected with control siRNA and FLAG-His-FAT10 was set to unity and the RNA levels were normalised to the expression of GAPDH. (c) Since transfection of the cells with siRNA lowered the total amount of mono-FAT10 in the cells as well, the ECL signals were quantified with Quantity One Software (BioRad) and the coefficient of conjugated-FAT10 to monomeric FAT10 was set to 1 for lane 3 (control siRNA). Down-regulation of USE1 by the different USE1 siRNAs USE1_2, USE1_3, USE1_4 or USE1_5 decreased the amount of FAT10 conjugates by about 70%, 24%, 54% or 20%, respectively. A representative experiment out of three with similar outcome is shown.

5.2.5 USE1 auto-FAT10ylates itself in *cis* but not in *trans*

In view of the fact that the USE1-FAT10 conjugate could not be reduced, we hypothesized that USE1 auto-FAT10ylates itself. To investigate this hypothesis, we generated expression constructs for HA-USE1 and an HA-tagged USE1 mutant in which all 11 lysines were replaced by arginines. Transient co-transfection of HEK293T cells with FLAG-His-FAT10 and either HA-USE1 wild type or HA-USE1 11K-11R mutant followed by combined immunoprecipitation and western blot analysis revealed that both wild type USE1 and the 11K-11R mutant formed a conjugate with FLAG-His-FAT10 under non-reducing conditions, thus indicating that the mutant was functional for thioester formation (Figure 5.11). In contrast to the conjugate formed with the USE1 wild type protein, the FLAG-His-FAT10~HA-USE1-11K-11R could be largely reduced suggesting that the two proteins are mainly thioester linked. Whether the residual complex was due to FAT10ylation of the USE1 N-terminus or whether residues other than lysine were FAT10ylated or whether some thioester bonds resisted boiling in β -ME remains to be determined.

To investigate whether auto-FAT10ylation occurs in *cis* or in *trans*, HEK293 cells were transfected with HA-FAT10 and with either His₆-tagged USE1 wildtype or FLAG-His tagged [C¹⁸⁸A]USE1 mutant alone or in combination with both together. The outcome of the immunoprecipitation assays analysed by western blot was that His₆-USE1 wild-type only transferred FAT10 onto itself either in the condition with HA-FAT10 alone or in the set-up where the FLAG-His-USE1-[C¹⁸⁸A] mutant was co-transfected. The inactive mutant did not form a conjugate when it was co-expressed with just HA-FAT10 and also not when active and inactive USE1 and HA-FAT10 were co-expressed (Figure 5.12). Therefore, auto-FAT10ylation of USE1 occurs in *cis* but not in *trans*.

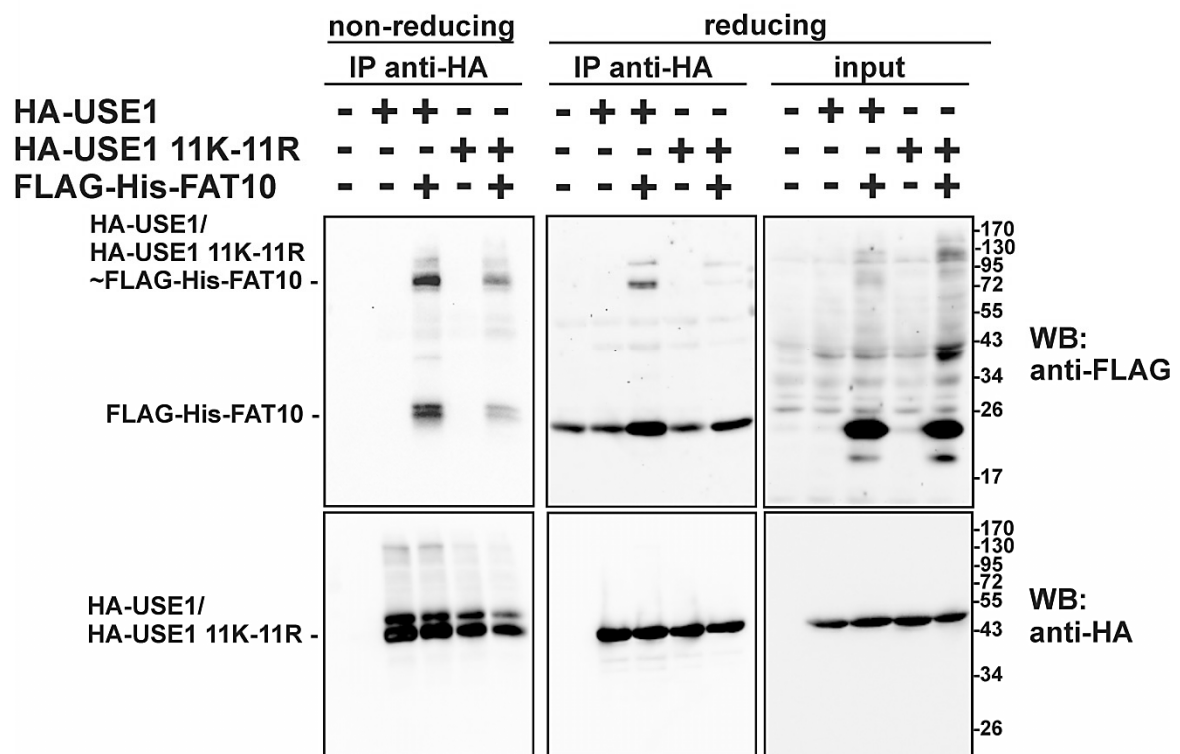


Figure 5.11 Co-immunoprecipitation of HA-USE1 wild type or HA-USE1 11K-11R mutant with 3xFLAG-His-FAT10. As indicated above the panels, HEK293 cells were transiently co-transfected with pcDNA3.1-3xFLAG-His-FAT10 and either pcDNA3.1-HA-USE1 encoding USE1 wild-type or pcDNA3.1-HA-USE1 11K-11R encoding a USE1 mutant in which all lysines were replaced by arginines. After anti-HA immunoprecipitation against USE1, samples were separated on SDS-PAGE and subjected to western blot analysis with either FLAG-reactive antibody conjugated to horseradish peroxidase (HRP) (top panels) or HRP conjugated HA-reactive antibody (bottom panels). Samples were analysed under non-reducing (left panels) and reducing conditions (right panels). A representative experiment out of three with similar outcome is shown.

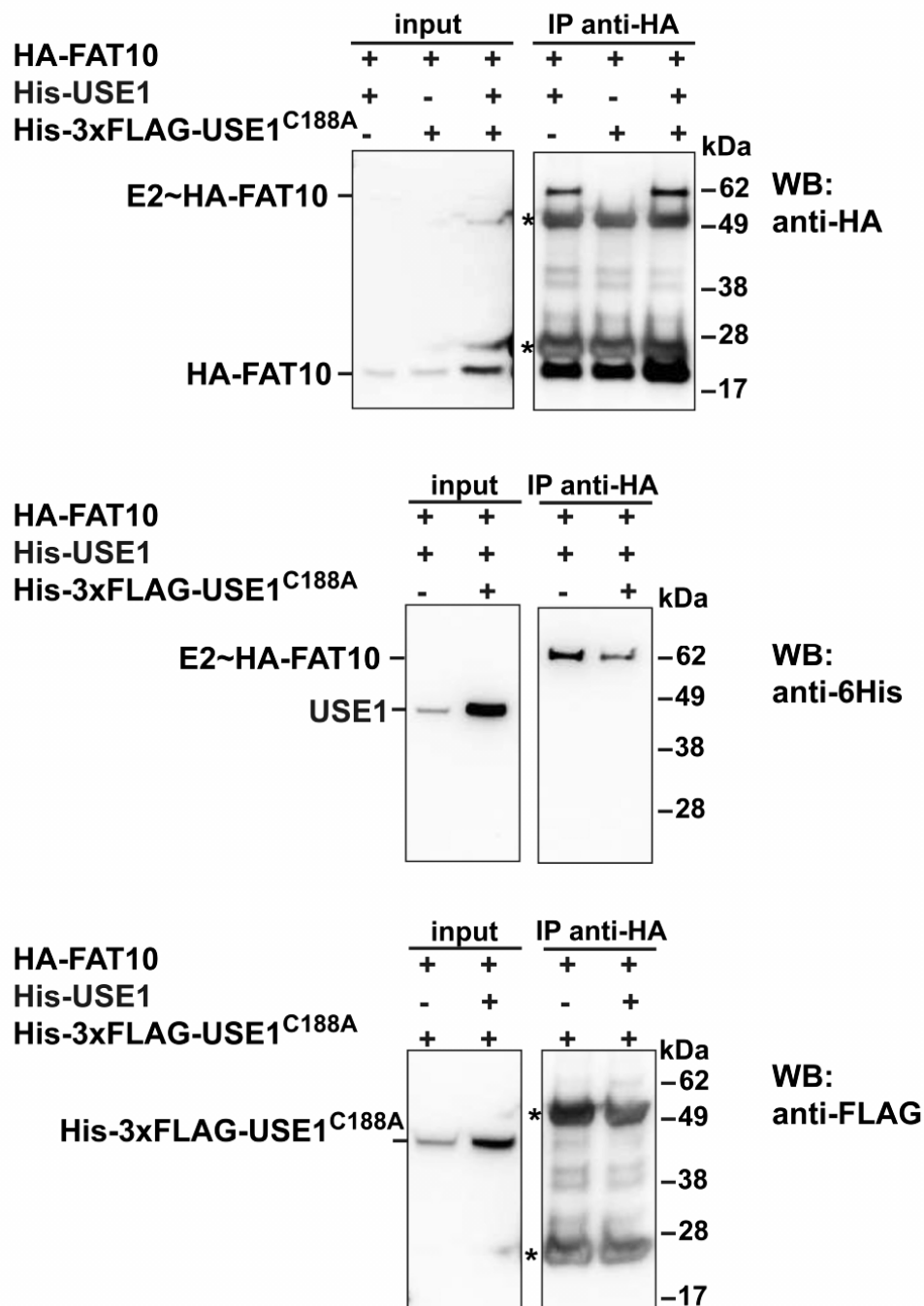


Figure 5.12 USE1 transfers FAT10 in *cis* but not in *trans*. Co-immunoprecipitation of HA-FAT10 and His₆-USE1 and 3xFLAG-His₆-[C¹⁸⁸A]USE1 expressing HEK293 cells. Cells were transiently transfected with pcDNA3.1-HA-FAT10 and pcDNA3.1/His₆-Xpress-USE1 or pcDNA3.1-3xFLAG-His₆-[C¹⁸⁸A]USE1 as indicated above the top panel. After immunoprecipitation against the HA-tag of FAT10, samples were separated on 4-12% bis-Tris SDS gels and subjected to western blot analysis with an HA-reactive monoclonal antibody (top panel), a peroxidase-conjugated polyhistidine reactive antibody (middle panel), or a FLAG M2-reactive monoclonal antibody (bottom panel). Asterisks indicate the heavy and light chains of the HA-reactive antibody used for the immunoprecipitation. The positions of the USE1~HA-FAT10 conjugate (E2~HA-FAT10), HA-FAT10, His-USE1, and FLAG- [C¹⁸⁸A]USE1 are indicated at the left. A representative experiment out of three experiments with similar outcome is shown.

5.3 DISCUSSION

FAT10 is a very young member of the ubiquitin family proteins, young in terms of evolution as it is found only in mammals, and young in terms of the history of investigation, beginning with its discovery in the MHC class I locus in 1996 (Fan et al., 1996). In this study, we identify an E2 enzyme for FAT10 as well as the first substrate of FAT10 conjugation and show that it is one and the same protein: USE1.

In order to identify new interaction partners of FAT10 we performed a yeast two hybrid screen using a human thymus cDNA library as FAT10 was found to be most highly expressed in the thymus (Lukasiak et al., 2008). Our screen identified 11 different bait-dependent clones, but USE1 was the only E2 enzyme found among these putative FAT10-interacting proteins. A priori one would not need to postulate a high affinity binding between a modifier and a cognate E2 enzyme. Nevertheless, it has recently been shown for SUMO-1 that it binds to its cognate E2 enzyme UBC9 not only via a thioester linkage but also non-covalently, and that this non-covalent interaction promotes the formation of short SUMO-1 chains (Knipscheer et al., 2007). Also for ubiquitin, a non-covalent interaction with the heterodimeric E2 enzyme Mms2/Ubc13 has been shown (VanDenmark et al., 2001). A weak non-covalent interaction between FAT10 and USE1 was therefore a reasonable basis for investigating USE1 as a potential E2 enzyme for FAT10. Another striking argument for USE1 as a potential E2 enzyme for FAT10 was that USE1 was identified as the only E2 enzyme, which could receive activated ubiquitin exclusively from UBA6 and not from UBE1 (Jin et al., 2007). Moreover, it was shown that depletion of UBA6 but not of UBE1 reduced the charging of USE1 with activated ubiquitin in intact cells. While these data convincingly document a functional cooperation between UBA6 and USE1, an interaction between these E1 and E2 enzymes has not been shown. By yeast two hybrid screen we show that UBA6 and USE1 indeed interact and that this interaction is specific and did not occur with UBE1 or UBE1L (Figure 5.2). Combined with the identification of UBA6 as the E1 enzyme of FAT10 (Chiu et al., 2007), it was therefore clearly warranted to investigate USE1 as an E2 enzyme for FAT10 conjugation.

By *in vitro* FAT10 transfer assays we showed that radioactively labeled FAT10 was activated by UBA6. In the presence of USE1, the amount of UBA6~FAT10 was reduced

and instead a complex of radioactive FAT10 bound to USE1 appeared (Figure 5.3a). These data show that purified FAT10, UBA6, and USE1 are sufficient to generate FAT10 charged USE1 *in vitro*. The enzymatic transfer of FAT10 both to UBA6 and to USE1 *in vitro* relied on the C-terminal diglycine motif of FAT10 consistent with formation of a thioester bond between the C-terminus of FAT10 and the active site cysteine residues of UBA6 and USE1. Whether the active site cysteine of USE1 was indeed required for formation of a FAT10-USE1 conjugate was then investigated in co-immunoprecipitation experiments (Figure 5.4a). Co-expression of FAT10 with wild type USE1 resulted in the formation of a USE1-FAT10 conjugate in intact cells but this was not the case when the [C¹⁸⁸A]USE1 active site mutant was used. Formation of such a conjugate was specific for USE1 as a similar co-expression experiment performed with UbcH8, which is a bi-specific E2 enzyme for ubiquitin and ISG15 (Kim et al., 2004; Zhao et al., 2004), formed a conjugate with ubiquitin but not with FAT10 (Figure 5.4c). In order to investigate if USE1 is in fact required for FAT10 conjugation to target proteins, USE1 expression was down-regulated by siRNA transfection of HEK293 cells transiently expressing FLAG-FAT10 and also in TNF- α /IFN- γ stimulated HEK293 cells. Using our newly generated FAT10 reactive mAb for immunoprecipitation we could show for the first time that endogenously expressed FAT10 forms a broad high molecular weight smear of bona fide FAT10 conjugates which were only observed after FAT10 induction by pro-inflammatory cytokines. Interestingly, siRNA-mediated reduction of USE1 mRNA expression by 92% led to a strong decrease in the intensity of conjugate bands (Figure 5.9). A pre-treatment with siRNA in general reduced the amount of transiently expressed FAT10 which was probably due to a reduced transfection efficiency (Figure 5.9a). The siRNAs specific for UBA6 or USE1 strongly reduced FAT10 conjugates but, unexpectedly, also to some extent monomeric FAT10. We repeatedly observed that silencing of UBA6 and USE1 caused increased cell death in FAT10 expressing cells which may have caused a selective loss of cells with high FAT10 expression. Nevertheless, the relative quantities of FAT10 conjugates compared to monomeric FAT10 were strongly decreased both under ectopic and endogenous FAT10 expression. We conclude from these experiments that USE1 is a major E2 enzyme involved in FAT10 conjugation. Whether the few residual putative FAT10 conjugates in the siRNA-treated cells are due to incomplete silencing of USE1 or whether other E2 enzymes for FAT10 may exist will need to be addressed in use1-deficient mice in the future.

Thioester bonds between ubiquitin-like modifiers and their E1, E2 or E3 enzymes are normally cleaved by boiling in reducing Laemmli sample buffer as we have also seen in this study for USE1~ubiquitin and UbcH8~ubiquitin conjugates (Figure 5.4b, c). It was therefore quite unexpected that the USE1-FAT10 conjugates in transfected HEK293 cells completely resisted boiling in the presence of 10% β -mercaptoethanol and/or 0.5M DTT. Since formation of this conjugate relied on the active site cysteine of USE1, we reason that USE1 uses activated FAT10 in its active site to form an isopeptide bond between the FAT10 C-terminus and one of its 11 lysine residues. This notion was confirmed in cells expressing a lysine-less USE1 which also bound to FAT10 but in a reducible manner (Figure 5.11). Such an auto-modification of USE1 with FAT10 is reminiscent of E3 ligases of ubiquitin, which also auto-ubiquitylate themselves, and for the auto-SUMOylation reported for Ubc9 (Knipscheer et al., 2008). Moreover, E2 enzymes of the ubiquitin system have been shown to monoubiquitylate proteins, which contain different types of ubiquitin interaction domains without the involvement of E3 enzymes (Hoeller et al., 2007). It is hence feasible that USE1 transfers activated FAT10 onto itself in the absence of an E3 enzyme. Along these lines we investigated whether FAT10 conjugation by USE1 can occur only in *cis* or also in *trans*. In Figure 5.12 we show that the co-expression of FAT10 together with a catalytically inactive USE1 mutant and wild type USE1 only led to FAT10 conjugation of the wild type enzyme thus demonstrating that USE1 can only FAT10ylate in *cis* but not in *trans*. Currently we are investigating what the consequences of auto-FAT10ylation of USE1 may be. As linkage of FAT10 to long lived proteins has been shown to target them for degradation by the proteasome in intact cells (Hipp et al., 2005) and *in vitro* (Schmidtke et al., 2009), it is possible that FAT10 self-conjugation by USE1 accelerates the degradation of the modifier-charged E2 enzyme which could constitute a negative feedback mechanism to limit the availability of activated FAT10 in thioester linkage to USE1. Alternatively, FAT10 modification of USE1 could affect its activity as a conjugating enzyme for ubiquitin or FAT10 or it may lead to the association of USE1 with FAT10 binding proteins like HDAC6 (Kalveram et al., 2008) or NUB1L (Hipp et al., 2004) (Figure 5.13).

Taken together, we show here that one E1 enzyme (UBA6) specific for the two modifiers FAT10 and ubiquitin can transfer both thioester-linked modifiers to one and the same bi-specific E2 enzyme (USE1). This is an unprecedented case in the conjugation of ubiquitin family proteins and raises the question at what stage it will be decided whether a substrate

protein will become ubiquitylated or FAT10ylated. The identification of FAT10-specific E3 enzymes may shed light on this interesting question.

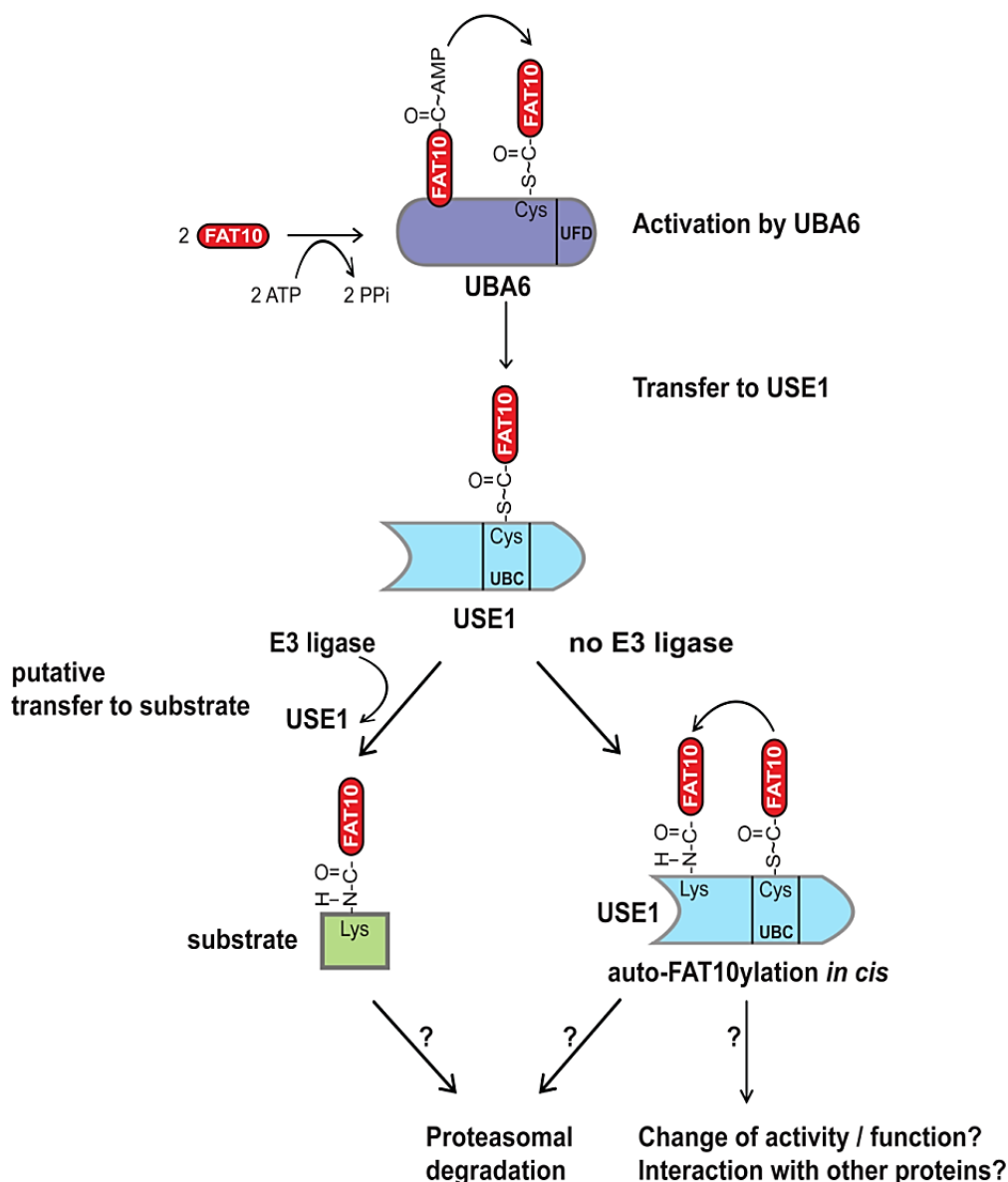


Figure 5.13 Model of the regulation of USE1. FAT10 becomes activated with ATP by the FAT10-specific E1 enzyme UBA6 which adenylates the FAT10 C-terminal carboxyl group, thus forming a FAT10~AMP intermediate. The intermediate is then transferred onto the active site cysteine of UBA6 where it forms a thioester linkage. By a transthioesterification reaction, FAT10 becomes transferred to the active site cysteine of USE1. With the help of a so far unidentified E3 ligase, FAT10 becomes conjugated via an isopeptide bond to substrates, which might mark them for proteasomal degradation. USE1 undergoes auto-FAT10ylation *in cis* and forms non-reducible isopeptide bonds with FAT10. This could lead to USE1-FAT10 degradation by the proteasome thus limiting the availability of activated FAT10 in thioester linkage to USE1 in case a putative E3 ligase or substrates are not available. Alternatively, auto-FAT10ylation of USE1 could change its enzymatic properties or mediate the interaction with FAT10 binding proteins such as HDAC6 or NUB1L. Abbreviations are: UFD: ubiquitin-fold domain; UBC: ubiquitin conjugating enzyme E2 domain.

5.4 METHODS

5.4.1 Primers and constructs

USE1 was identified as a FAT10-interacting protein in the yeast two-hybrid screen. The isolated cDNA was lacking 259nt at the 5' end (pACT2-USE1short). To generate constructs containing full length USE1 cDNA, the plasmid pDEST17-USE1 (Jin et al., 2007) was used as a template for PCR amplification. To generate the yeast two-hybrid plasmid pACT2-USE1 as well as the human expression plasmid pcDNA3.1-HisA-Xpress-USE1, USE1 cDNA was PCR amplified using 5'-ccg gaa ttc gaa tgg cgg aga gtc cga ctg agg-3' as forward and 5'-ccg ctc gag tca aac cct cag gct ccc atg aag g-3' as reverse primer and cloned into pACT2 (BD Clontech) or pcDNA3.1/His-A (Invitrogen), respectively, using EcoRI and XhoI restriction sites. The yeast two-hybrid plasmid pLexA-N-USE1 was generated using the forward primer 5'-ccg gaa ttc gag atg gcg gag agt ccg act gag g-3' and the reverse primer 5'-cgg ggt acc tca aac cct cag gct ccc atg aag g-3' with an EcoRI and KpnI site, respectively. pcDNA3.1/His-A-USE1C188A was generated by site directed mutagenesis of pcDNA3.1/His-A-Xpress-USE1 using the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene) with the primer 5'-cta ccg caa tgg gaa agt cgc ctt gag tat tct agg tac atg g-3' as described by the manufacturer.

For generation of 3x-FLAG-6His-USE1 and 3x-FLAG-6His-USE1C188A, primers 5'-ccg gaa ttc atg gcg gag agt ccg act gag gag g-3' as forward and 5'-ata gtt tag cgc ggc cgc gtc aaa ccc tca ggc tcc cat gaa ggt ctg-3' as reverse primer were used with pcDNA3.1/His-A-USE1 and pcDNA3.1/His-A-USE1C188A as templates, respectively, and cloned via EcoRI and NotI into the plasmid pcDNA3.1-3xFLAG-His-FAT10 (Chiu et al., 2007). E1 enzymes UBE1 and UBA6 were cloned into pACT2 using primers 5'-cgg gat ccg aat gtc cag ctc gcc gct gtc c-3' and 5'-cgg gat cct cag cgg atg gtg tat cgg ac-3' as forward and reverse primer for cloning of UBE1 via BamHI, and primers 5'-cat gcc atg gaa gga tcc gag cct gtg g-3' and 5'-ccg ctc gag tta atc agt gtc atg act gaa gta g-3' as forward and reverse primer for cloning of UBA6 via NcoI and XhoI. UBE1L was cloned into pACT2 by cutting out UBE1L cDNA from pcDNA3.1/His-UBE1L by EcoRI and XhoI and ligation into EcoRI / XhoI digested pACT2. For the generation of pLexA-N-ISG15, primer 5'-cgg ggt acc atg ggc tgg gac ctg acg gtg-3' as forward and 5'-cgg ggt acc tca gcc tcc ccg cag gcg cag att c-3' as reverse primer were used to PCR amplify ISG15 cDNA.

The amplicon was cloned into the KpnI restriction site of pLexA-N (Dualsystems Biotech, Zurich, Switzerland). All constructs were verified by sequencing (Microsynth AG, Balgach, Switzerland, or GATC Biotech, Konstanz, Germany). pET23a-UbcH8 was used as template to generate UbcH8 with BamHI and XhoI restriction sites by PCR. As forward and reverse primers respectively, 5'-gcg gga tcc atg gcg agc atg cga gtg gt-3' and 5'-gcg ctc gag tta gga ggg ccg gtc cac t-3' were used. The PCR product was then cloned into pcDNA3-FLAG-NEDD8. Human UBA6 was PCR amplified from the pcDNA3.1-HA-UBE1L2 (Pelzer et al., 2007) expression construct with forward (5'-aga gct agc atg gaa gga tcc gag cct gt-3') and reverse (5'-gcg gct agc tta atc agt gtc atg act ga-3') primers and cloned into the XbaI site of pGEX-2TKs. pBI-HA-NUB1L was generated as described (Hipp et al., 2004).

The exchange of all lysine to arginine codons in use1 was performed with the QuickChange® Multi Site-Directed Mutagenesis Kit from Stratagene, according to the instructions provided by the supplier. We used a maximum of 3 primers for each step of mutagenesis. The primers were selected with the help of software from Stratagene and read:

K1: 5'-GCA GTG TCT ACT CCG GAT CAG GCG GGA TAT CAT-3'

K2: 5'-GCG GGA TAT CAT GTC CAT TTA TAG GGA GCC TCC TCC-3'

K3: 5'-CTG ATA CTG TTG ACA TGA CTA GGA TTC ATG CAT TGA TCA CAG G-3'

K4: 5'-CCA CCC ACC TCG GGT CAG ACT GAT GAC AAC G-3'

K5: 5'-CAA CTT CTA CCG CAA TGG GAG AGT CTG CTT GAG TAT-3'

K6: 5'-GAG ACA TCC AGG AGA CAG CAG AAA CTA TAA TGA ATG TAT CCG-3'

K7: 5'-CTG TGA CAT GAT GGA AGG AAG GTG TCC CTG TCC-3'

K8: 5'-CGA GGG GTG ATG GAG AGG TCC TTT CTG GAG TAT-3'

K9: 5'-CGA GGT GGC CTG CAG AGA TCG CCT GC-3'

K10: 5'-GCA GGA CCC TTT TGG AGA GAG GCG GGG CCA-3'

K11: 5'-GCC TGG GAC TGA TAC GTC AGA GAG TGC TGG AGA-3'

The PCR program was adjusted as recommended by the supplier. Primers K1 to K6 were used for the N-terminal lysines, the remaining primers on a different vector with the same cDNA for the mutagenesis of the C-terminal lysines. After each step the mutations were

verified by sequencing. After the mutation of all N- and C-terminal lysines the N-terminal part was amplified with the primers Nfor: 5'-CGA AGA GGT ACC AAT GGC GGA GAG TCC GAC TGA-3' and Nrev: 5'-GAG CCC AGC CCA GAG CAT CTC CTC AGT GCT-3', and the C-terminal part was amplified with the primers Cfor: 5'-GAG CAC TGA GGA GAT GCT CTG GGC-3' and Crev: 5'-GCC AGA CTC GAG CTA AAC CCT CAG GCT CCC ATG-3'. The two PCR products were purified and joined as described (Schmidtke et al., 1996) with the primers Nfor and Crev. The assembled full-length lysine-less USE1 cDNA was digested with KpnI and XhoI and cloned into pcDNA3.1 vector containing an HA-tag.

5.4.2 Yeast two hybrid Screen

Full-length *fat10* cDNA was cloned into the plasmid pLexA-DIR (pLexA-FAT10) (Dualsystems Biotech, Zurich, Switzerland) as previously described (Hipp et al., 2004) and used as a bait construct to screen a human thymus cDNA library (Thymus MATCHMAKER cDNA library, Clontech). Briefly, the bait construct was transfected into the yeast strain NMY51 (*MATa his3Δ200 trp1-901 leu2-3,112 LYS2::(lexAop)4-HIS3 ura3::(lexAop)8-lacZ (lexAop)8-ADE2 GAL4* (Dualsystems Biotech, Zurich, Switzerland)) by a standard LiCl transformation method and the absence of self-activation was tested with an X-Gal filter assay on selection plates lacking tryptophan. For the yeast two hybrid screen, the human thymus library was transformed into these cells as described by the DUALhybrid kit instructions. Two independent screenings were performed and a total number of 101 clones which grew on selection plates lacking tryptophan, leucine and histidine, supplemented with 1 mM 3-amino-1,2,4-triazole (3-AT), were positively tested by an X-Gal test. For this, yeast cells were grown for 4 days on selection plates lacking tryptophan, leucine and histidine in the presence of 1mM 3-AT. Whatman filters, which were used to generate replicates of the plates, were frozen in liquid nitrogen, subsequently soaked with PBS containing 100 μg/ml X-Gal and incubated at room temperature. Clones that turned blue were grown on new selection plates and subjected to a second X-Gal test. The prey plasmids of the remaining 53 clones were isolated using a standard method with glass beads and were re-transformed into NMY51 together with either pLexA-FAT10 or with pLexA-LaminC or pLexA-p53, respectively, to test for bait-independency. The identity of the remaining 46 clones was determined by sequencing.

5.4.3 Immunoprecipitation

HEK293 cells were transfected using FuGene6 (Roche) or TransIT-LT1 transfection reagent (Mirus) as described in the manufacturer's instructions. After 24 hours, cells were harvested, lysed and immunoprecipitation was performed as previously described (Pelzer et al., 2007) using 30µl of monoclonal anti-HA-agarose conjugate HA-7 (Sigma) or EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma). Proteins were separated on 4-12% gradient Bis-Tris NuPage SDS-gels (Invitrogen) and subjected to western blot analysis using monoclonal anti-polyhistidine-peroxidase conjugated Ab (clone His-1), monoclonal anti-FLAG (M2), monoclonal anti-HA-7 (all from Sigma) or a ubiquitin specific, peroxidase conjugated mAb (clone FK-2, Enzo Life Sciences). For immunoprecipitation of endogenous FAT10, USE1 or FAT10-USE1 conjugate, HEK293T or HEK293 cells were stimulated for 24 hours with 200 U/ml IFN-γ and 400 U/ml TNF-α (both from Peprotech). Cells were harvested, lysed and immunoprecipitation was performed with a FAT10 reactive mAb (Figure 5.5) or a USE1 reactive rabbit mAb (kindly provided by B. Amidon, Millennium Pharmaceuticals) bound to protein A sepharose as previously described (Pelzer et al., 2007). Proteins were separated on 4-12% gradient bis-TRIS NuPage SDS-gels (Invitrogen) and subjected to western blot analysis using a USE1 specific pAb (Figure 5.6), a USE1 specific mAb (Millennium Pharmaceuticals), a FAT10 specific pAb (Hipp et al., 2005) or a FAT10 specific mAb (Figure 5.5).

5.4.4 *in vitro* conjugation assay

To generate ³⁵S-labelled FLAG-His-FAT10, FLAG-His-FAT10ΔGG and HA-ubiquitin proteins, *in vitro* transcription and translation T7 coupled reticulocyte lysate system (Promega) was used and the procedures were performed as recommended by the supplier. The translated proteins were purified from the reticulocyte lysate by immunoprecipitation using EZview™ Red ANTI-FLAG® M2 Affinity Gel or anti-HA-sepharose (Sigma) for 2 hours at 4°C. After washing the gel three times, 100µl reaction mixtures were prepared. 20mM Tris pH 7.6, 50mM NaCl, 10mM MgCl₂, 5mM ATP, 1mM DTT, 10U/ml inorganic pyrophosphatase were mixed with the FLAG-His₆-FAT10- or HA-ubiquitin bound beads. Where indicated, 250ng GST-UBA6 and 5µg His₆-USE1 of purified proteins were added. The reactions were incubated at room temperature for 30 minutes

followed by one wash with reaction buffer. 50µl of sample buffer containing 4M Urea were added to the beads and incubated at 30°C for 15 minutes. Samples were subjected to 8-15% gradient SDS-PAGE and detected by autoradiography.

5.4.5 USE1 knockdown and real-time PCR

HEK293 cells were transfected twice with a pool of four different human USE1 specific siRNAs (Hs_FLJ13855_4, Hs_UBE2Z_5, Hs_UBE2Z_3, Hs_UBE2Z_2, Qiagen) or a pool of four different human UBA6 specific siRNAs (Hs_UBE1L2_1, Hs_UBE1L2_2, Hs_FLJ10808_4, Hs_FLJ10808_2, Qiagen), both pools together or with a control siRNA (AllStars Negative Control siRNA, Qiagen) or with the single USE1 siRNAs separately using X-tremeGENE siRNA Transfection Reagent (Roche). At day one, cells were transfected with a total amount of 400nM siRNA, at day two with a total amount of 200nM siRNA. On the third day, cells were transfected with FAT10 expression plasmids pcDNA3.1-3xFLAG-His-FAT10 or pcDNA3.1-3xFLAG-His-FAT10ΔGG using TransIT-LT1 transfection reagent (Mirus). Cells were harvested at the fourth day for an anti-FLAG immunoprecipitation using EZviewTM Red ANTI-FLAG® M2 Affinity Gel (Sigma) Western Blot analysis was performed with a polyclonal anti-USE1 antibody (Figure 5.6), a polyclonal anti-UBA6 antibody (Enzo Life Sciences), a direct peroxidase-labeled anti-FLAG-M2-HRP (SIGMA) or as loading control with an antibody directed against β-actin (AbCam). Knockdown of USE1 and UBA6 was verified with real-time RT-PCR on a Applied Biosystems 7900-HT Fast Real-Time PCR Cyclor using the QuantiTect SYBR®Green PCR kit (Qiagen) with specific Primers for USE1 and UBA6 (QuantiTect Primer Assays Hs_FLJ13855_1_SG and Hs_UBA6_1_SG, QIAGEN) and normalised to the expression of GAPDH which served as housekeeping gene. Specific Primers for GAPDH (Hs_99999905_m1) were used with TaqMan Fast Universal PCR Master Mix (both from Applied Biosystems). For siRNA experiments with endogenous FAT10, FAT10 expression was induced at day three with IFN-γ/TNF-α and at day four subjected to an immunoprecipitation using a FAT10 mAb (Figure 5.5).

5.4.6 Expression and purification of proteins

The plasmids pGEX2TKs-UBA6 and pDEST17-USE1 were used for expression of GST-UBA6 and His-USE1. pGEX2TKs-UBA6 was transformed into BL21-CodonPlus(DE3)-

RIPL (Stratagene), pDEST17-USE1 into the strain B834(DE3)pLysS (Novagen). Expression of the proteins was induced with 0.1mM IPTG. Purification of GST-UBA6 was carried out using Glutathione-SepharoseTM 4B (GE Healthcare) as recommended by the supplier. His-USE1 was purified with Protino® Ni-IDA Resin (Macherey-Nagel) according to the manufacturer's protocol.

5.4.7 Generation of polyclonal USE1 and UBA6 antibodies

Two peptide sequences corresponding to residues 231-242 and 274-287 of the USE1/UBE2Z protein sequence (UniProt Accession Number Q9H832) and amino acid residues 1034-1052 of human UBA6 (Accession number A0AVT1) with the addition of a terminal cysteinyl residue were synthesised and purified by reverse-phase high performance liquid chromatography and analyzed by mass spectrometric analysis. The peptides were separately conjugated to keyhole limpet haemocyanin (KLH) using maleimidobenzoic acid N-hydroxysuccinimide ester. New Zealand white rabbits (three *per* peptide) were immunized with conjugates in Freund's complete adjuvant and sera were screened against the immunising peptides using ELISA. Harvest bleed sera were screened (dilution 1:2000) utilizing western blot with HRP-coupled goat-anti-rabbit IgG (dilution 1:5000) as second stage reagent and chemiluminescence detection for reactivity against endogenous, recombinant and transfected USE1 and UBA6. Both antisera raised against the two peptide conjugates yielded very similar results.

5.4.8 Generation of a monoclonal FAT10-reactive antibody

The monoclonal antibody was generated in essence as previously described (Yokoyama et al.). Briefly, two FAT10^{-/-} mice (Canaan et al., 2006) were each immunized with 100µg of recombinant human GST-FAT10 in lipopeptide (EMC Microcollections). After three rounds of booster immunizations, the animals were sacrificed, their spleens were harvested and a single cell suspension was prepared. Spleen cells were then fused with SP2/0-Ag14 myeloma cells at a 1:1 ratio and plated into flat bottom 96-well plates. After selection with HAT medium, the surviving cells were screened by ELISA for the production of GST-FAT10 specific antibodies. A GST ELISA was performed to exclude those hybridomas which produced antibodies with anti-GST reactivity. Clone 4F1 could be stabilized after two rounds of subcloning and was determined to be of the IgG2a isotype using the Mouse Immunoglobulin Isotyping ELISA kit from BD Biosciences.

After expansion, the antibody was purified from hybridoma supernatants using a protein G column (GE Healthcare).

5.4.9 Immunofluorescence and confocal microscopy

Immunofluorescence and confocal microscopy were performed exactly as previously described (Kalveram et al., 2008).

5.5 ACKNOWLEDGEMENTS

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5.6 NOTES

UBA6 is specifically interacting with USE1 in yeast two-hybrid assay

UBA6, but not UBE1, transfers ubiquitin specifically to the E2 enzyme USE1 (Jin et al., 2007). To determine whether binding of human USE1 and UBA6 can occur in *S. cerevisiae*, USE1 was cloned into the bait vector, which was used in a yeast two-hybrid protein interaction test with different E1 enzymes, expressed as prey proteins. Figure 1A illustrates that USE1 specifically and directly interacts with UBA6 in a yeast two-hybrid assay. When the different constructs were co-transformed into yeast, only UBA6 together with USE1 gave a positive result in the X-Gal filter assay demonstrating the interaction with USE1. UBE1 or UBE1L together with USE1 did not show activation of the *lacZ* gene (Figure 5.2a, right panel). In addition to growth on selection plates lacking histidine,

leucine and tryptophan, indicating a positive protein-protein interaction, yeast NMY51 cells have an *ADE2* reporter gene. This gene is not transcribed when protein-protein interaction does not occur, resulting in growth of red coloured colonies. However, *ADE2* gene is transcribed when interaction takes place resulting in a colour change to faint pink or white. White colonies indicate a stronger interaction between the proteins. Growth of co-transformed USE1 and UBA6 on 3-amino-1,2,4-triazole (3-AT) selection plates showed lighter pink to white colour, also indicating the interaction of USE1 and UBA6 (Figure 5.2a, left panel). Self-activation of the yeast two-hybrid constructs could be excluded because expression of the different E1 enzymes or USE1 alone did not result in *lacZ* or *ADE2* activation as seen in the control experiments (Figure 5.2b,c). These results demonstrate a cognate interaction of USE1 with UBA6 but not with the most highly conserved human E1 type enzymes UBE1 and UBE1L.

6. Generation of Monoclonal and Polyclonal Antibodies Specific for Mouse FAT10

Neha Rani and Marcus Groettrup

6.1 INTRODUCTION

Antibodies are produced by B cells in response to antigens (or immunogens), which are recognized as foreign by the immune system. They are important tools in biological research and medicine. Animals can be used to produce antibody against a certain protein of interest. The intensity of the response depends on several factors like the size of the immunogen, its chemical properties, and how distinct it is from the host protein. To enhance the immune response, immunogen is combined with chemical agents called adjuvants. Hybridoma technology has been extensively used to generate monoclonal antibodies.

Here, we generated polyclonal and monoclonal antibodies against mouse FAT10 in rabbit and FAT10 deficient mice, respectively. Earlier, we generated antibodies against human FAT10 in rabbit (Hipp et al., 2005) and mouse (Aichem et al., 2010). These antibodies have been used in various experimental applications but, since the polyclonal antibody showed some unspecific binding in western blotting and the monoclonal antibody did not show any reactivity against mouse FAT10, we pursued to raise an antibody against mouse FAT10. This tool could be utilized in mouse experiments, e.g., to study the expression of FAT10 in different tissues of mouse or in different mouse models. Although, in our experiments, both antibodies recognized endogenous mouse as well as human FAT10, irrespective of the tag, but unfortunately, they showed slight cross-reactivity for another ubiquitin-like modifier, interferon stimulated gene 15 (ISG15). Since, ISG15 and FAT10 have a molecular weight of 17 kDa and 18 kDa respectively, it was hardly possible to differentiate between these proteins on western blot. Further work will be required to generate specific monoclonal antibody but as evident from results presented here there could be a difficulty in obtaining a specific antibody against mouse FAT10.

6.2 RESULTS AND DISCUSSION

For the production of monoclonal antibody, two FAT10 deficient mice were immunized with the recombinant mouse GST-FAT10 protein and four rounds of booster immunization resulted in high titer of antibody in the serum of mice. Since untagged FAT10 protein has a high tendency to precipitate, we utilized GST-tagged FAT10 protein

for the immunization. The fusion of myeloma cells SP2/0-Ag14 with spleen cells was achieved by using the hybridoma technology. The first round of screening for positive clones was performed by executing ELISA for the reactivity against GST-FAT10 and excluding the reactivity against GST. This step yielded two positive clones which were further stabilized by two rounds of subcloning. Further experiments revealed that the 7A5 clone was the better one amongst the two clones.

For the generation of a polyclonal antibody, two rabbits were immunized with GST-FAT10 protein and booster immunized four times. After analysis of antibody titers in the serum, further experiments were performed. Antibody from the rabbit 1023 worked better than the other.

6.2.1 Monoclonal and polyclonal antibodies recognizes human as well as mouse FAT10, irrespective of the tag

The antibody generated against human FAT10 (4F1) displayed no reactivity for mouse FAT10 (Figure 6.1A). Therefore, we decided to generate an antibody against mouse FAT10 (mFAT10) which would be helpful in future experimental applications. Two FAT10-deficient mice were immunized with recombinant GST-mFAT10, and thereby, after implying hybridoma technology, positive clones were selected by performing ELISA for GST-FAT10 and GST alone, which eliminated the possibility of the antibody being reactive for GST. To determine the specificity of the antibody, we performed western blotting with an antibody concentration of 1 µg/ml. Whole cell lysate from *E. coli* expressing recombinant His-mFAT10 was used for detection. The antibody (designated as 7A5) specifically recognized only His-mFAT10 but not GST alone (Figure 6.1B). We could also show this by using recombinant FAT10 protein with GST-tag used at different concentrations (Figure 6.1C). This demonstrated the reactivity of the antibody for FAT10, irrespective of the tag.

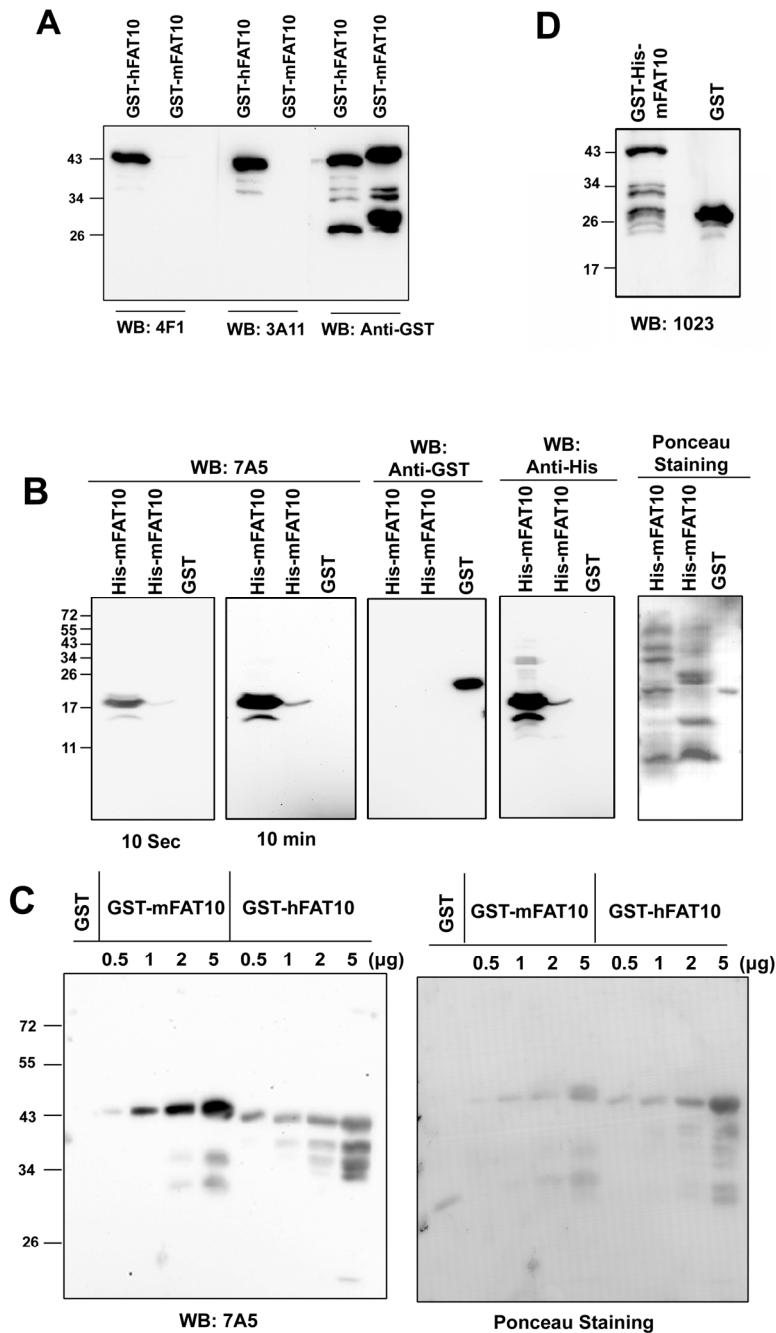


Figure 6.1 Monoclonal and polyclonal antibodies against mouse FAT10 recognize recombinant human as well as mouse FAT10. (A) Human FAT10 monoclonal antibodies (3A11 and 4F1) were tested for their reactivity towards recombinant GST-hFAT10 (human) and GST-mFAT10 (mouse) in western blot. An anti-GST western blot served as loading control. (B) Recombinant His-mFAT10 and GST proteins were used to test the monoclonal antibody generated against mouse FAT10 (7A5). The western blot was exposed for either 10 sec (left panel) or 10 min (second panel). Anti-GST or anti-His blots served as loading controls. A ponceau staining (right panel) of the blot showed the total protein extract loaded on the blot. (C) Detection of purified recombinant GST-mFAT10 and GST-hFAT10 protein, loaded at different concentrations (0.5, 1.0, 2.0 and 5.0 μ g), by the monoclonal antibody, 7A5 (right panel). Ponceau staining served as loading control (left panel). (D) Western blot showing the reactivity of polyclonal antibody (1023, raised against mouse FAT10) for recombinant GST-His-mFAT10 as well as GST protein.

We also generated rabbit polyclonal antibody against FAT10 (rabbits immunized with GST-mFAT10) and analyzed its specificity in western blotting. The antibody (designated as 1023) recognized purified recombinant GST-His-mFAT10, but on the other hand, it also recognized GST alone (Figure 6.1D), as expected, due to immunization of rabbits with GST-mFAT10.

6.2.2 Monoclonal and polyclonal antibodies detect human as well as mouse FAT10 in transiently transfected HEK293T cells

Next, we analyzed the specificity of antibodies for FAT10 protein extracted from cells. HEK293T cells were transfected with human or mouse HA-FAT10. After 24 hours of transfection, cells were harvested, lysed and subsequently a portion of lysates was analyzed for the expression of the protein. The remaining lysate was used for immunoprecipitation with protein G beads immobilized with anti-HA antibody. Monoclonal antibody, when used at a concentration of 1 μ g/ml, recognized HA-hFAT10 but not HA-mFAT10 (Figure 6.2, left panel) which could be due to very low expression of HA-mFAT10, as determined by anti-HA blotting (Figure 6.2, right panel). Upon raising the antibody concentration to 3 μ g/ml, the intensity of detection of HA-mFAT10 protein was improved (Figure 6.2, middle panel). Similarly, polyclonal antibody also presented remarkable reactivity for HA-tagged human and mouse FAT10 protein derived from HEK293T cells (Figure 6.3B).

6.2.3 Monoclonal and polyclonal antibodies display cross-reactivity with ubiquitin-like modifier, ISG15

To examine the cross-reactivity of these antibodies with other ubiquitin-like proteins, having certain homology to FAT10, HEK293T cells were transfected either with empty vector, HA-Ub-GFP, FLAG-mISG15/HA-mISG15, HA-SUMO-GFP, HA-hFAT10, HA-mFAT10 or HA-NEDD8. Lysates from transfected cells were subjected to immunoprecipitation with anti-HA antibody and thereafter, blotted with anti-HA antibody or anti-mouse FAT10 antibodies. Monoclonal mouse FAT10 antibody showed weak cross-reactivity with HA-mISG15 (Figure 6.3A). Polyclonal antibody also recognized FLAG-mISG15 (Figure 6.3B). The structure of ISG15 protein (Narasimhan et al., 2005) is quite similar to the predicted structure of FAT10 (Groettrup et al., 2008), although the

amino acid sequence similarity is not very significant (Figure 6.3C). Possibly, there is a similar hydrophobic region exposed on the surface of FAT10 and ISG15, which is easily accessible by the antibody.

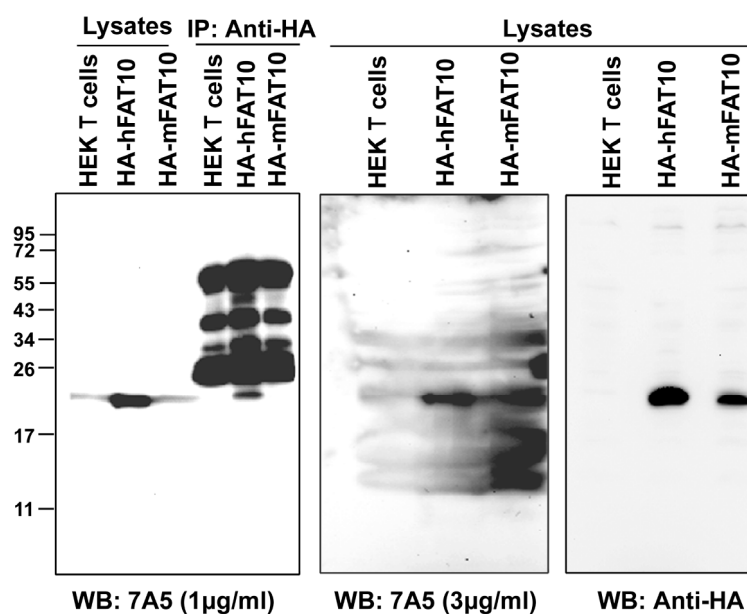


Figure 6.2 The monoclonal antibody recognizes FAT10 expressed in HEK293T cells in western blot. HEK293T cells were transiently transfected with human or mouse HA-FAT10 (HA-hFAT10 or HA-mFAT10) and cell lysates were analyzed by western blot with either anti-HA antibody (right panel) or 7A5 at a working concentration of 1µg/ml (left panel) or 3µg/ml (middle panel). The left panel also shows the reactivity of the antibody for lysates immunoprecipitated with anti-HA antibody.

6. Generation of Monoclonal and Polyclonal Antibodies Specific for Mouse FAT10

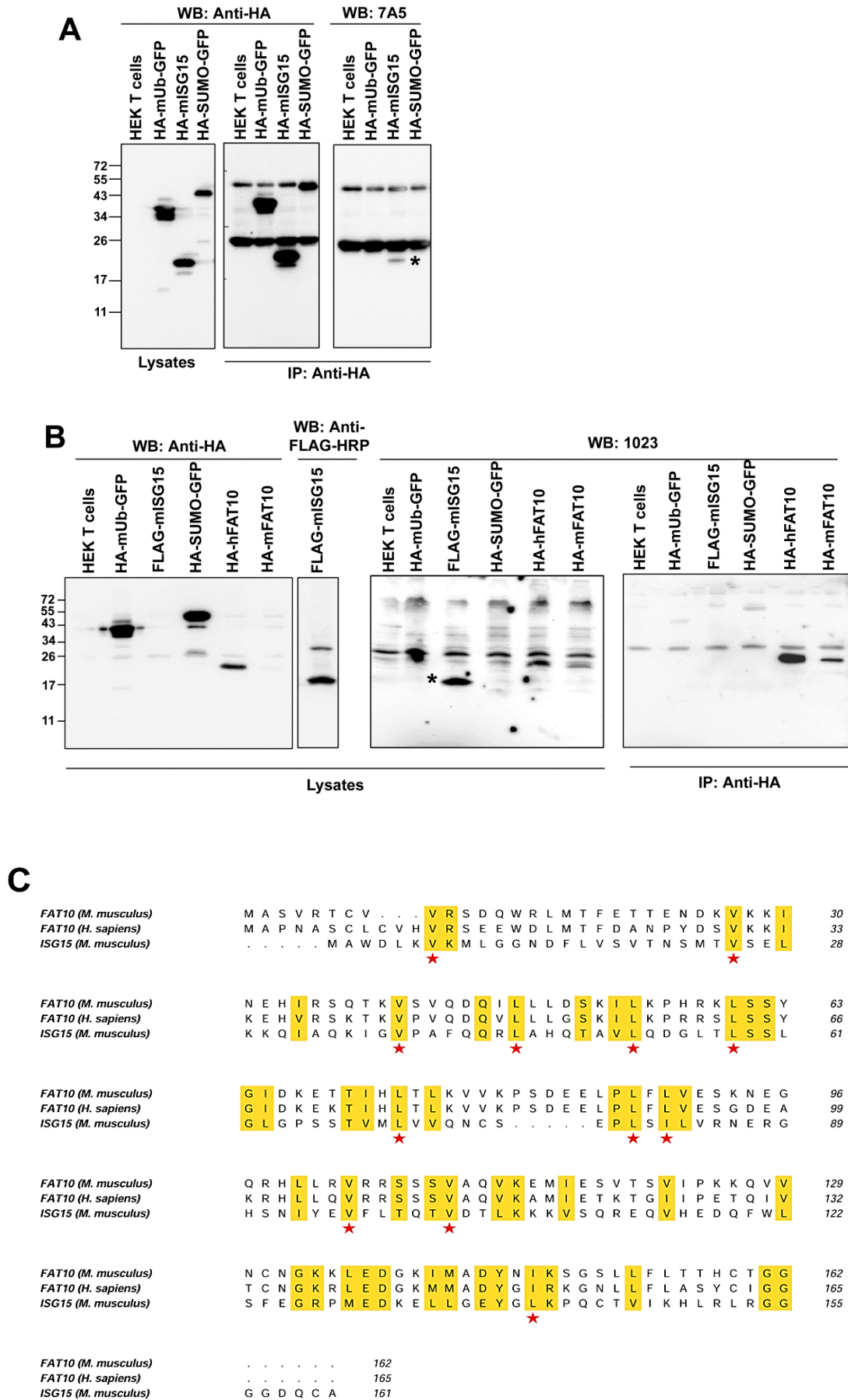


Figure 6.3 Monoclonal and polyclonal antibodies show cross-reactivity for ISG15. (A) Left panel: Cell lysates from transiently transfected HEK293T cells were analyzed by western blot with anti-HA antibody for the expression of protein of interest. Middle panel: Lysates were immunoprecipitated with anti-HA antibody and subsequently analyzed by western blotting with anti-HA antibody. Right panel: Immunoprecipitated proteins were analyzed for the reactivity of 7A5 antibody with mouse ubiquitin (HA-mUb-GFP), mouse ISG15 (HA-mISG15) or SUMO (HA-SUMO-GFP). (B) Left panel: Cell lysates from transiently transfected HEK293T cells were analyzed with anti-HA antibody or anti-FLAG-HRP antibody, similarly as above. Middle panel: Analysis of cell lysates in western blot with the polyclonal antibody (1023), showing the cross-reactivity of the antibody with mouse ISG15 (FLAG-mISG15) protein. Right panel: Analysis of anti-HA immunoprecipitated proteins with the polyclonal antibody. Note: The asterisk represents the cross-reactivity of antibody for ISG15. (C) Multiple sequence alignment of human and mouse FAT10 with mouse ISG15 generated using the software CLUSTAL W and ALINE. The percentage identity between ISG15 and human or mouse FAT10 is only 16% as calculated by CLUSTAL W. The highly conserved hydrophobic residues are indicated by a red star.

6.2.4 The isotype of the FAT10-reactive monoclonal antibody is IgG2a with kappa light chain

The FAT10-reactive monoclonal antibody was purified from the supernatant of hybridoma cells, by employing a protein G column. Coomassie staining demonstrated the purity of the antibody (Figure 6.4A). The isotyping of the monoclonal antibody was performed by using a mouse immunoglobulin ELISA kit (BD Pharmingen). After performing indirect ELISA, we determined the isotype of the antibody as IgG2a antibody with kappa (κ) light chain (Figure 6.4B).

6.2.5 Monoclonal as well as polyclonal antibodies recognizes FAT10 in the thymus of the mouse

It has been reported earlier that FAT10 mRNA expression is highest in the thymus (Lukasiak et al., 2008), which was not analyzed on the protein level due to the lack of a reliable sensitive antibody. With our newly generated antibodies we examined the expression of FAT10 in the thymus of mice. Thymus from 4 weeks old mice were lysed, and the whole protein lysate was analyzed for the presence of FAT10 after blotting with monoclonal antibody (7A5) or polyclonal antibody (1023). The protein lysate from the thymus of FAT10 deficient mouse served as negative control. After loading about 10 μ g of the total lysate, it was possible to detect a band corresponding to the size of FAT10 (Figure 6.5A, B). But, unfortunately, this band was very faintly visible in the lysate of thymus from FAT10-deficient mice. We suspect this unspecific band to be ISG15 protein

(which runs at approx. same size on SDS-gel, 17 kDa) because of its cross-reactivity with antibodies.

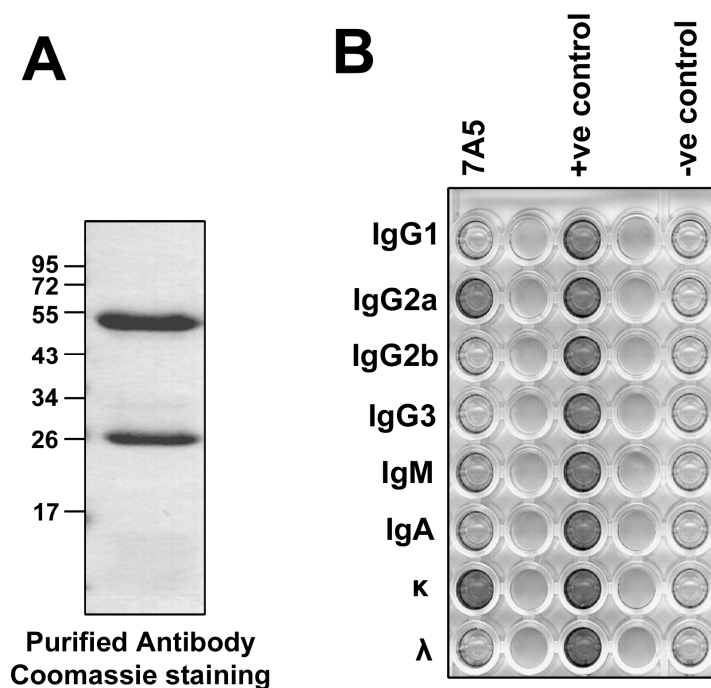


Figure 6.4 The isotype of monoclonal antibody is IgG2a. (A) Coomassie staining displays the high purity of monoclonal antibody with heavy and light chain. (B) An ELISA plate was coated with each Ig isotype, as marked on the left side. 7A5 antibody showed reactivity for IgG2a and κ light chain (shown in dark color). Positive (dark) and negative (light) controls show the validity of the experiment.

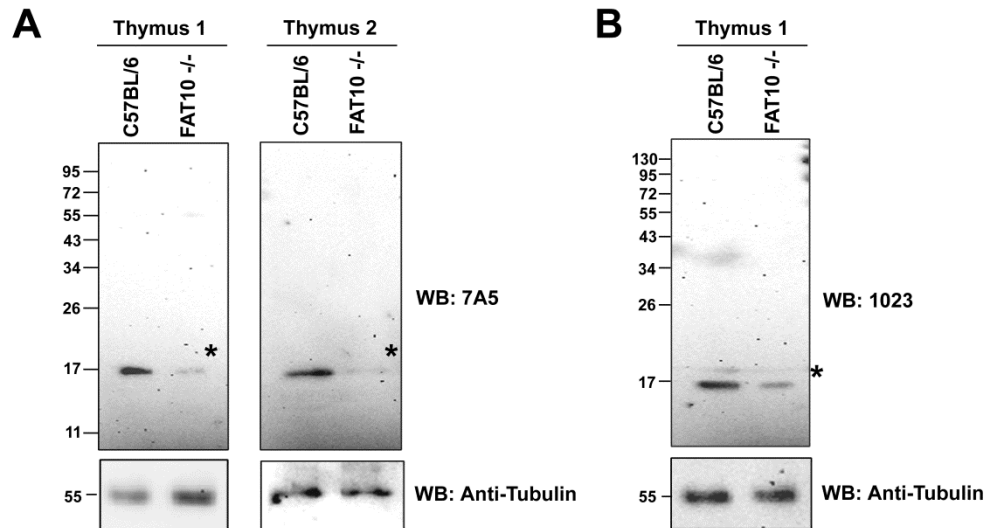


Figure 6.5 Monoclonal and polyclonal antibodies recognize endogenous FAT10 in the thymus of C57BL/6 mouse. Total protein was isolated from the thymus of C57BL/6 mouse and FAT10^{-/-} mouse and analyzed by western blot with (A) monoclonal antibody (7A5) or (B) polyclonal antibody (1023). An anti-tubulin blot served as a loading control. Note: Asterisks mark unspecific binding of antibodies.

6.3 MATERIALS AND METHODS

6.3.1 Expression constructs

Murine *fat10* was amplified and cloned into the pGEX2TKS vector via EcoRI and XbaI restriction sites or in pQE30 vector via SacI and HindIII restriction sites. HA-FAT10, HA-Ubiquitin-GFP, HA-SUMO-GFP (Hipp et al., 2005), as well as GST-FAT10 (Hipp et al., 2004) have been described previously. HA-mISG15 was a kind gift from Prof. Dr. M. Scheffner (University of Constance, Germany).

6.3.2 Purification of human and mouse recombinant FAT10 protein

Human GST-FAT10 protein was expressed and purified as described earlier (Hipp et al., 2004). Mouse FAT10 was expressed at 30°C with 0.1 mM IPTG for 5 hours in *E.coli* strain *B834(DE3)pLysS*. The purification was performed using Glutathione Sepharose 4B beads (GE Healthcare) and subsequent steps were performed according to the manufacturer's protocol. The buffer of purified protein was exchanged to PBS by dialysis for 24 hrs at 4°C.

6.3.3 Generation of monoclonal antibody in the mouse

Two FAT10 deficient mice were immunized four times with the purified GST-mFAT10 protein. 100 µg of protein in 0.9% NaCl (w/v) was injected in each mouse for the immunization. After a pre-check of antibody titer in the blood serum of mice by ELISA, they were sacrificed and their spleens were harvested and a single cell suspension was prepared under sterile conditions. Myeloma cells SP2/0-Ag14 were mixed with spleen cells in a 1:1 ratio using polyethylene glycol (Roche) and plated in 96-well flat-bottomed plates and incubated in tissue culture incubator at 37°C. The method for fusion was adapted from the article “Production of Monoclonal Antibodies” (Yokoyama, 2001). After selection with HAT/HT (Sigma) medium, hybridomas were screened for the antibody production against FAT10 by indirect ELISA. A control ELISA with GST protein was also performed to eliminate hybridomas reactive for GST. The best clones were subcloned further to stabilize them and again checked for antibody production by ELISA. The clone with the desired reactivity was further expanded and the antibody was purified by subjecting the supernatant to the protein G column (GE Healthcare) using a standard protocol for antibody purification.

6.3.4 Generation of polyclonal antibody in rabbits

Polyclonal antibody was generated by immunizing two rabbits with 200 µg GST-FAT10 by immunization with three booster doses. After the fourth immunization, the antibody titer in the serum of both rabbits was analyzed by indirect ELISA.

6.3.5 ELISA

After the third and fourth immunizations, serum from two mice or rabbits was screened by ELISA as described earlier (Hornbeck et al., 2001). Eventually, the supernatant from the hybridoma was also tested by coating the Microlon ELISA plates (Greiner) with 20 µg/ml of recombinant protein. Hybridoma supernatants were diluted 1:2 in blocking buffer. The reactive antibody was detected by alkaline phosphatase coupled protein G (Calbiochem) diluted 1:2500 in blocking buffer and developed with freshly prepared 3mM *p*-nitrophenyl phosphate (Fluka). The absorbance after substrate hydrolysis was measured at 405 nm wavelength with a Tecan microplate reader. The antibody isotype

was identified by using a mouse immunoglobulin ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

6.3.6 Tissue culture and transfection

HEK293T cells were cultured in DMEM supplemented with 10% FCS and 100µg/ml penicillin/streptomycin (Invitrogen). Cells were transiently transfected using Fugene 6 (Roche) according to manufacturer's instructions.

6.3.7 Preparation of lysates and immunoprecipitation

After harvesting the transfected cells, they were lysed in 20mM Tris/HCl, pH 7.8 containing 0.1% Triton X-100, 1 µM pepstatin, 10 µM leupeptin, 5 µg/ml aprotinin and 100 µM PMSF for 30 min on ice. Protein from thymus was isolated by lysing tissue in RIPA buffer (50mM Tris/HCl, pH 7.5, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche) with the aid of a tissue homogenizer. This was followed by sonication and centrifugation at 20,000×g for 15 min. Cleared lysates were either used directly for western blotting or subjected to protein G affinity gel immobilized with anti-HA antibody and incubated overnight at 4°C. Immunoprecipitates were washed 5 times in lysis buffer and analyzed by SDS-PAGE and western blotting.

6.3.8 Multiple protein sequence alignment

The amino acid sequences were obtained from Protein Database on NCBI (<http://www.ncbi.nlm.nih.gov/protein/>) and were aligned using CLUSTAL W (Thompson et al., 1994) (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and formatted using Jalview and ALINE softwares. The percent identity was calculated using CLUSTAL W.

6.4 ACKNOWLEDGEMENTS

We thank Prof. Dr. M. Scheffner (University of Constance, Germany) for providing HA-mISG15 and FLAG-mISG15 constructs. We would also like to thank Christine Wuensch for some technical support in the work.

7. Discussion

The discovery of the “Ubiquitin-Proteasome System” was a major breakthrough in the field of biology. Due to the enormous number of ubiquitin substrates and its involvement in various processes, discoverers of ubiquitin: Avram Hershko, Aaron Ciechanover and Irwin Rose, were awarded Nobel Prize in Chemistry in the year 2004. Since the discovery of ubiquitin in 1975, several ubiquitin-like proteins have been identified, including SUMO, NEDD8, ISG15, FAT10 and Hub1.

For about 30 years, ubiquitin was considered as the only protein involved in the “clearance” of other proteins. In 1996, FAT10 was discovered and later, its function, analogous to ubiquitin’s function in proteasomal degradation of conjugated proteins, was identified. The first question that comes to mind is why nature has evolved two proteins with similar function of tagging the other proteins for proteasomal degradation? This could be explained in several ways: first, FAT10 was evolved in higher eukaryotes only (Groettrup et al., 2008), so it might fulfill some specialized functions and probably target some unique proteins for proteasomal degradation, which cannot be regulated by ubiquitin; secondly, the exclusive feature of an up-regulation of FAT10 mRNA by proinflammatory cytokines, as compared to ubiquitin (Lee et al., 2003; Lukasiak et al., 2008; Raasi et al., 1999), suggests a possible role of FAT10 in alleviating certain diseases by a different mechanism; thirdly, FAT10 deficient mice are viable and displays hypersensitivity to lipopolysaccharide (Canaan et al., 2006), suggesting its possible link to bacterial pathogenesis; and fourthly, FAT10 is not “ubiquitous” in expression but highly expressed in mature dendritic cells, B cells and lymphoid organs like thymus, lymph nodes and spleen (Bates et al., 1997; Lee et al., 2003; Lukasiak et al., 2008), which again points towards its specialized role in the immune system. Like ubiquitin, FAT10 bears a free diglycine motif at the C-terminus, which mediates the conjugation to the target proteins. Moreover, FAT10 has conserved lysines corresponding to K27, K33, K48 and K63 in ubiquitin but the evidence for multi-FAT10 chain formation is still lacking.

The proteasomal degradation of targeted substrates by ubiquitin is known to be mediated by its binding to the UIM domains of the hRpn10 subunit (Beal et al., 1998; Deveraux et al., 1994). Several UBL-UBA domain containing proteins are also known to bind the hRpn10 subunit of the proteasome. Yet, another layer of regulation of proteins is provided by FAT10 via its interaction with the proteasome ((Schmidtke et al., 2006) and Chapter 2). NUB1 also interacts with hRpn10 subunit but its functional relevance was not examined (Tanji et al., 2005). Initially, NUB1 was identified as a NEDD8 interacting

protein that diminishes the level of NEDD8 and its conjugates by targeting them for the proteasomal degradation (Kamitani et al., 2001; Tanaka et al., 2003). But later, Hipp et al., (2004) argued for NUB1L being a negative regulator of FAT10 and its conjugates rather than NEDD8 and its conjugates, as they could not detect interaction of NUB1L with NEDD8.

Despite the fact that FAT10 and its conjugates are degraded by the proteasome independently of ubiquitin, its docking site on the 26S proteasome was not determined. Furthermore, other major questions were: is the ubiquitin-proteasome system similar to the “FAT10-proteasome” system, how NUB1L accelerates the degradation of FAT10 and is the function of NUB1L analogous to that of ubiquitin receptors like, Rad23, Dsk2 and Ddi1? The interaction of purified FAT10 or NUB1L with hRpn10 subunit, and furthermore, the glycerol gradient analysis indicated the direct binding of FAT10 and NUB1L to the proteasome (Chapter 2). Moreover, FAT10 and NUB1L dock on the same domain in hRpn10, i.e., the VWA domain, as determined by GST-pull down assays. This was an unexpected observation for two reasons: first, FAT10 consists of two ubiquitin-like domains, and functions similarly like ubiquitin, which suggests the possibility that it could bind to the loosely packed α -helical UIM domains like ubiquitin does; secondly, since NUB1L accelerates the degradation of FAT10, we expected different docking sites for NUB1L and FAT10 on the proteasome. Therefore, some other most likely binding candidates were also analyzed: Rpn13 (ubiquitin receptor), Rpt5 (ubiquitin receptor) or Rpn1 (UBL domain protein receptor). As expected, GST-pull down assay demonstrated that NUB1L can dock on another subunit of the proteasome, Rpn1, but not on the Rpt5 subunit (Birte Kalveram, unpublished results) or on the Rpn13 subunit (Chapter 2). This supported the earlier finding, which showed that the UBL domain proteins can bind the leucine-rich-repeat-like (LRR) domain in the Rpn1 subunit (Elsasser et al., 2002).

The expression of FAT10 was stable in *rpn10 Δ* *S. cerevisiae* strain and its degradation was dependent on the VWA domain, as shown by the cycloheximide chase experiments. One could argue that the VWA domain is not incorporated into the yeast proteasome and it is the free cytosolic fraction of the VWA domain that assists in the degradation process. To investigate this, the *PDR5* gene was knocked out from the wt and the *rpn10 Δ* yeast strain because the multi-drug transporter Pdr5, exports MG132 out of the cells and renders the cells resistant to the drug. The *rpn10 Δ* or *pdr5 Δ rpn10 Δ* strain was reconstituted with the VWA domain and the yeast proteasome was inhibited with

MG132. Additionally, the degradation of FAT10 was observed in the *pre1-1* yeast strain, which is sensitive to stress conditions and shows accumulation of ubiquitin-conjugates due to an abolished proteasome function. In both cases, the cycloheximide chase for 6 h showed that the FAT10 expression was stabilized, which all together, suggested a novel function of the VWA domain in protein degradation (Chapter 2).

By determining the three-dimensional structures of hRpn10 and NUB1L computationally, several possible interacting amino acids were identified, but none of these residues seemed to be crucial for the interaction (Chapter 3). Asp11 residue in the MIDAS motif of the VWA domain has been shown to be crucial for the degradation of the ubiquitin-fusion degradation pathway substrates, probably due to the requirement of the acidic residue to form the salt bridge, which maintains the structural integrity of the VWA domain (Fu et al., 2001). The degradation of FAT10 appears to be dependent on the Asp11 residue in the MIDAS motif of the VWA domain but this residue is not involved in the direct interaction with FAT10 (Chapter 2). Further studies are ongoing to crystallize the VWA domain protein and determine its three-dimensional structure. Since the VWA domain has a very compact structure with α -helices and β -sheets, and the UIM domains have a loosely packed α -helical structure, one could argue against the binding of FAT10 and NUB1L to the VWA domain. To support our finding, we analyzed the amino acid sequence alignment of N- and C-terminal FAT10 and ubiquitin, and this clearly showed that Lue8, Ile44 and Val70 residues in ubiquitin, which are crucial for the interaction of ubiquitin with the hRpn10 subunit, do not align with the corresponding amino acid residues in FAT10 (Chapter 2).

The three UBA domains of NUB1L bind the N-terminal UBL domain of FAT10 whereas the VWA domain of hRpn10 bind the C-terminal UBL domain of FAT10 (Chapter 2). But surprisingly, the docking of NUB1L to the proteasome, but not the binding to FAT10, is essentially required for its function as a negative regulator of FAT10 (Schmidtke et al., 2006). How could NUB1L enhance the degradation of FAT10 even if it is not binding FAT10 but the proteasome? Probably hRpn10 serves as an indirect link for the association between FAT10 and NUB1L because NUB1L is unable to accelerate the degradation of FAT10 in the absence of hRpn10, as shown by the cycloheximide chase experiments (Chapter 2), or probably there is a weak interaction of FAT10 with the newly identified putative UBA domain in NUB1L (Chapter 3) that was ignored in the earlier GST-pull down experiments (Schmidtke et al., 2006) and this weak interaction was

enough for NUB1L to accelerate the degradation of FAT10. Another possibility could be a conformational change in the proteasome mediated by the docking of NUB1L to it, which allows a faster degradation of FAT10 and its conjugates.

We expected that NUB1L could accelerate the degradation of FAT10 and its conjugates probably because of its binding to the Rpn1 subunit, which occupies the position at the base of the 19S RP of the proteasome. This could allow a direct targeting of proteins to the proteasomal ATPases, which enables the unfolding of proteins. But surprisingly, NUB1L can accelerate the degradation of FAT10, only when it docks to the hRpn10 subunit, as suggested by the stabilization of FAT10 for 6h of cycloheximide chase in the *rpn10Δ* yeast strain ectopically expressing NUB1L. Therefore, it appears that the major route of protein degradation by the “FAT10-proteasome” pathway is via the hRpn10 subunit (Chapter 2). The Rpn1 subunit seems to be incapable of taking over the function of the Rpn10 subunit.

These studies raised another question: why does NUB1L interact with the Rpn1 subunit (functional relevance)? Our hypothetical model suggests two different possibilities: first, NUB1L binds to the Rpn1 subunit and could act like a “FAT10-receptor”, and transfer FAT10 and may be its conjugated substrates to the hRpn10 subunit of the proteasome to be degraded faster; secondly, NUB1L could induce some conformational changes in the hRpn10 subunit, which allows FAT10 and its conjugated substrates to be degraded faster (Chapter 2). Then another obvious question is why NUB1L is required if FAT10 itself is able to dock to the hRpn10 subunit and thereby, degraded by the proteasome? The explanation for this could be that NUB1L provides a specificity layer in the regulation of certain proteins to be degraded by the FAT10-conjugation pathway. It could be possible that NUB1L recognizes only certain substrates conjugated to FAT10, which require a fast turn-over, whereas monomeric FAT10 is degraded directly by its binding to the hRpn10 subunit. Therefore, it still needs to be investigated if the enhanced protein degradation effect of NUB1L is substrate specific or a general effect. The functional studies by mutating the NUB1L-binding sites on the Rpn1 subunit could support these hypotheses.

In addition to the role of FAT10 in the proteasomal degradation, the localization of FAT10 in aggresomes and its non-covalent association with HDAC6 under proteasome inhibition suggested the possible role of FAT10 in another route of protein degradation, i.e., autophagy (Kalveram et al., 2008). But, till now there is no strong evidence to

support this. One of the factors for the formation of aggregates is misfolded protein stress and this encouraged us to determine the expression of FAT10 mRNA by inducing misfolded protein stress in cells using canavanine, thapsigargin or proteasome inhibitor, and we could not detect an up-regulation of FAT10 mRNA, as determined by real-time RT-PCR (Chapter 3). In the earlier study, the proteasome was inhibited artificially in cells in tissue culture (Kalveram et al., 2008), and therefore the localization of FAT10 in aggresomes can be speculated to be a general phenomenon that is observed with most of the proteins under stress. Since FAT10 was found to be associated with HDAC6, we expect it to be an important functionally relevant phenomenon. Despite the fact that FAT10 mRNA expression is least in the un-inflamed brain (Lukasiak et al., 2008), we determined FAT10 expression in mouse models of neurodegenerative diseases by real-time RT-PCR. Interestingly, the mRNA expression of FAT10 was up-regulated more than 550 fold in the brain of mice infected intracranially with LCMV as compared to the uninfected mice (Chapter 4). This up-regulation co-related with the induction of proinflammatory cytokines, IFN- γ and TNF- α . Upon further analysis in mouse models of neurodegenerative diseases, like Huntington's disease and Parkinson's disease, a similar conclusion was derived. Additionally, FAT10 could be detected in aggregates in such diseases, as determined by western blotting and immunohistochemical staining (Chapter 4). This clarified a major point that it is not a mere accumulation of FAT10 in aggregates (as could be expected after proteasome inhibition) but an induction of FAT10 at the transcription level. Probably, FAT10 is thereby transported to aggregates when the proteasome is functionally inhibited, probably via its non-covalent interaction with HDAC6, which can be protective by eliminating the so-far unidentified target proteins. It is very unlikely that FAT10 is directly involved in autophagy, therefore, we are currently investigating the localization of FAT10 in the different cell types (astrocytes, neurons and microglia cells) in the brain of mouse after induction with LPS. The most favorable possibility is the presence of FAT10 in microglial cells. This would be consistent with the function of FAT10 in the brain during such diseases.

Ubiquitin is involved in the removal of protein aggregates, organelles and intracellular pathogens in the context of autophagy (Kirkin et al., 2009). Endogenous cellular cargo is usually targeted to autophagosomes, and subsequently degraded by the lysosome, by interaction between polyubiquitin (usually K63 and K27 linkages) and adaptor proteins such as p62, NBR1 (Neighbor of BRCA1 gene 1), LC3 and NDP52 (nuclear dot protein

52) (Levine et al., 2011). But it not yet clear if ubiquitylation of proteins directly involved in autophagy occurs. HDAC6 can regulate the retrograde transport of aggregate-containing inclusion bodies that have to be degraded (Iwata et al., 2005). Additionally, HDAC6 is also required for Parkin/p62-mediated clearance of damaged mitochondria (Lee et al., 2010). Recently, p62 was recognized as being covalently modified by FAT10 (Annette Aichele et al., manuscript submitted). Moreover, FAT10 was found to be co-localized with the immunoproteasome subunits (LMP2, LMP7 and MECL-1) around the mitochondria of liver cells (French et al., 2011). The above findings provide a clue for the possible role of FAT10 in mitochondria clearance during such diseases.

Another study demonstrated that NUB1 interacts with synphilin-1, an α -synuclein interacting protein involved in the pathogenesis of Parkinson's disease (Tanji et al., 2007; Tanji et al., 2006). Endogenous NUB1 localized to the synphilin-1 positive aggregates and this interaction initiates the proteasomal degradation of synphilin-1. Therefore, it is feasible that FAT10 could also interact with synphilin-1. Possibly, FAT10 initiates the degradation of synphilin-1 and NUB1 further accelerates the degradation. Upon analyzing this possibility by co-immunoprecipitation experiments, we could not detect a covalent interaction of FAT10 and synphilin-1 (data not shown) under normal conditions in HEK293T cells. Further experiments can be established using different conditions, like proteasome inhibition, or including some other factors like E1 and E2 enzymes for FAT10.

The characterization of UBA6 as an E1 enzyme for FAT10 and ubiquitin (Chiu et al., 2007; Pelzer et al., 2007) encouraged us to further identify the enzymes in the cascade of the conjugation pathway. The yeast two hybrid assay revealed that the USE1 enzyme interacting with UBA6. Furthermore, USE1 was characterized as an E2 enzyme that accepts FAT10 and ubiquitin as substrates, as determined by *in vitro* and *in vivo* studies. USE1 forms a stable conjugate with FAT10, which is non-reducible with β -mercaptoethanol whereas ubiquitin forms a reducible thioester linkage with USE1. The USE1-FAT10 conjugate was abolished when the catalytic Cys188 of USE1 was mutated to alanine. Interestingly, endogenous FAT10 could compete with ubiquitin for the conjugation to USE1. To further confirm the finding, knock down of USE1 was performed, which clearly showed the reduction in FAT10-conjugates. This pathway is probably regulated by the auto-FAT10ylation of USE1 in *cis* by an isopeptide bond formation leading to the degradation of USE1 (Chapter 4). USE1 enzyme identification

raised new questions in the conjugation pathways as ubiquitin and FAT10 share the same E1 and E2 enzymes. How is the specificity for these two conjugation pathways determined? This question can be resolved probably by identifying the E3 ligases for FAT10, which would determine the specificity for substrates of FAT10 and ubiquitin.

Rassi et al., (2001 and 1999) suggested a role of FAT10 in apoptosis due to the lack of survival of Hela cells overexpressing human FAT10, and the cell death in murine fibroblast cell line stably expressing wt-FAT10 as compared to the cells expressing FAT10 Δ GG. This suggested the possibility that FAT10 form conjugates with apoptosis related proteins. In an effort to search for conjugates of FAT10, we employed an advanced technique - two-dimensional differential gel electrophoresis (2D-DIGE) – that is based on the direct labeling of lysine residues on proteins with cyanine dyes before isoelectric focusing (Tannu and Hemby, 2006). This technology has an advantage of labeling 2-3 samples with different dyes and electrophoresing all the samples on the same 2D gel. The analysis of thymi from wild type C57BL/6 mouse and FAT10^{-/-} mouse on the 2D gel demonstrated several proteins which were up-regulated as well as down-regulated in the thymus of FAT10^{-/-} mouse (Appendix, Figure 1). The mass spectrometry analysis revealed that the programmed cell death 6 protein (also known as apoptosis-linked gene 2 (ALG2)) was highly up-regulated in FAT10^{-/-} thymus. ALG2 is a member of Ca²⁺-binding proteins required for apoptosis induced by Fas, T-cell receptors and glucocorticoids. It is most abundant in the thymus and liver (Jung et al., 2001). But unfortunately, further biochemical analysis by performing pulse chase experiments and co-immunoprecipitating FAT10 and ALG2, could not support the notion that the degradation of ALG2 is directly dependent on FAT10. But may be an indirect association of FAT10 with some other factors is involved in this regulation, which can have an influence on the expression of ALG2 (Appendix, Figure 2).

Some evidences direct the function of FAT10 towards defense mechanism against bacterial and viral pathogens. The hypersensitivity of FAT10^{-/-} mice for LPS argues for its possible role during bacterial invasion (Canaan et al., 2006). The evidences for the association of FAT10 with the antiviral activity includes: the up-regulation of FAT10 mRNA by proinflammatory cytokines, its up-regulation in some organs, including liver, by LCMV infection (Chapter 3) and involvement of FAT10 in apoptosis in renal epithelial cells infected with HIV (Ross et al., 2006). Moreover, among all the ubiquitin-like proteins, FAT10 and ISG15 show close resemblance to each other as they share

similar structural folds, and are also involved in processes related to the immune system. ISG15 is involved in antiviral activities by interacting with several antiviral effector proteins like MxA, protein kinase R and RNaseL (Zhao et al., 2005) and this suggests a possible role of FAT10 in similar antiviral activities.

One more clue for the involvement of FAT10 in the antiviral activity was provided by the identification of a 35-kDa protein in the murine fibroblast cell line stably transfected with FAT10 (Raasi et al., 2001). The mass spectrometry analysis confirmed the presence of FAT10 and a small fragment of large T-antigen in the 35-kDa band, which was absent in cells stably expressing FAT10 Δ GG protein. Furthermore, co-immunoprecipitation experiments confirmed the covalent interaction of FAT10 with a fragment of large T-antigen in the same cell line, and this was also dependent on the C-terminal diglycine motif (Christiane Pelzer, unpublished results). It was intriguing that only a small fragment of large T-antigen covalently attached to FAT10, and therefore, we changed the system to a human cell line to verify this finding. Upon further investigation in HEK293T cells (immortalized with SV40 large T-antigen), it was observed that full-length large T-antigen can interact covalently with FAT10 and not with FAT10 Δ GG (Appendix, Figure 3). This provides an evidence for the involvement of FAT10 during viral infection. Probably, FAT10 targets large T-antigen for proteasomal degradation or somehow interferes with the replication of virus, and supports the host in overcoming the infection. Since this is an artificial system, and may not have a direct physiological relevance, it would be helpful to identify other viral proteins, involved in human pathogenesis, with an amino acid sequence homologous to the amino acid sequence of SV40 large T-antigen. Successively, viral infections can be carried out in C57BL/6 and FAT10 knock-out mice and differences in the viral clearance or viral replication can be determined. Although the MHC class I presentation remains unaltered after the infection of LCMV or MCMV in murine cell line stably transfected with FAT10 (Raasi et al., 2001), this possibility cannot be ruled out for other viruses.

A recent investigation to identify FAT10 conjugates unveiled several proteins with diverse cellular functions, mainly involved in DNA/RNA binding proteins (transcription factors and polymerases), apoptosis, cancer and E3 ligases (Annette Aichele, manuscript submitted).

Taking together, the conjugation pathway and the proteasome pathway studies provide a platform to identify new conjugates for FAT10 and to better understand the biological importance of FAT10. These studies provide a link of FAT10 in neurodegenerative diseases and viral infections. Furthermore, a mechanism of the regulation of FAT10 by two different receptors: NUB1L (non-proteasomal) and hRpn10 (proteasome-associated), has been identified. The work presented here has opened several new aspects in the field of proteasome research, by the identification of the VWA domain as a novel docking site for ubiquitin-like proteins, and also by determining an “unusual” function of the VWA domain of hRpn10. The FAT10- and the ubiquitin-conjugation pathway appear to be similar due to the identification of the same E1 and E2 enzymes, but we identified a major difference in the ubiquitin-proteasome system and the “FAT10-proteasome” system. This could explain the importance of FAT10 in targeting specific proteins, which are not regulated by the ubiquitin-conjugation pathway. Further understanding the mechanism of the degradation of FAT10 will be important to define the conditions when NUB1L plays its role in the “FAT10-proteasome” pathway.

Appendix

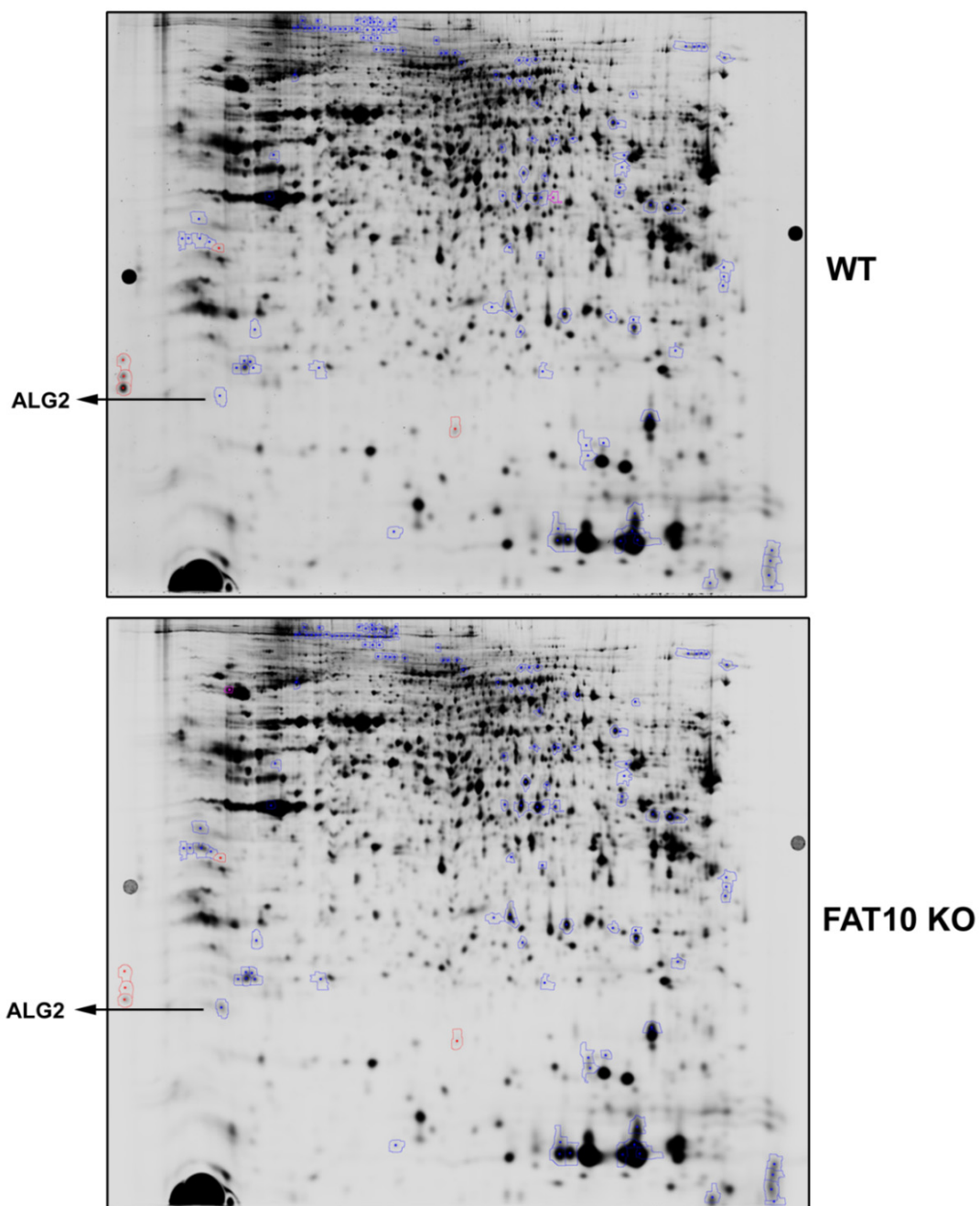


Figure 1: Up-regulation of ALG2 in the thymus of $FAT10^{-/-}$ mouse. Total protein lysate, extracted from the thymus of C57BL/6 and $FAT10^{-/-}$ mice, was labeled with Cy3 (red) and Cy5 (blue) dye, respectively. Proteins were separated on 10% Tris-glycine gel (IEF pH 4-7). The up-regulated proteins in the $FAT10^{-/-}$ thymus sample are marked in blue and the down-regulated proteins are marked in red. The experiment was performed with a standard protocol as described earlier (Tannu and Hemby, 2006). The data was obtained in collaboration with Prof. Dr. Janssen Ottmar, University Clinic Schleswig Holtstein, Campus Kiel, Germany.

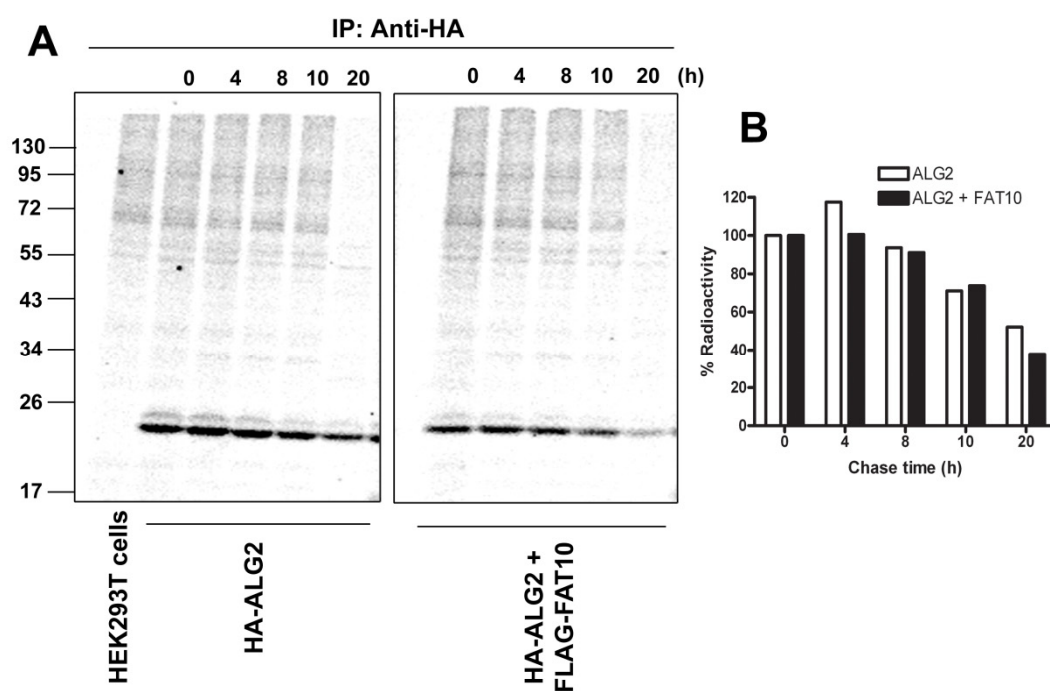


Figure 2: The degradation of ALG2 is not directly dependent on FAT10. (A) HEK293T cells were transfected with either HA-ALG2 (kind gift from Prof. Martin Berchtold, University of Copenhagen, Denmark) alone or together with FLAG-FAT10. The cells were labeled with [^{35} S] methionine, and thereafter, lysed and harvested at the indicated time points. Proteins were immunoprecipitated with the anti-HA antibody and analyzed by autoradiography. The untransfected HEK293T cells served as a negative control. (B) Quantitation of the amount of ALG2 left in (A) after the pulse chase.

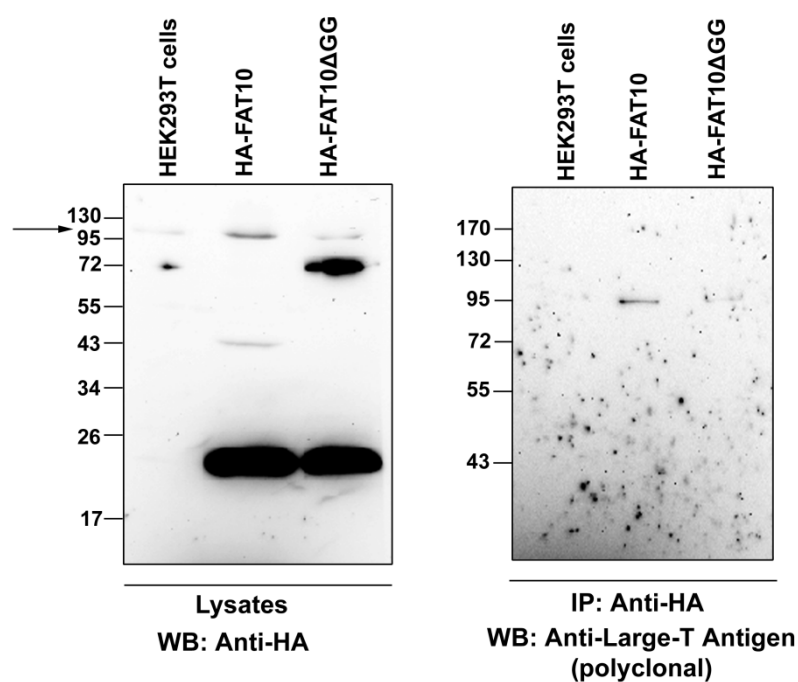


Figure 3: Co-immunoprecipitation of large T-antigen with FAT10, *in vivo*. HEK293T cells were transfected with HA-FAT10 or HA-FAT10ΔGG or left untransfected. Cells were lysed and separated on the SDS-gel. The large-T antigen is approx. 78 kDa protein. The arrowhead represents the conjugate of HA-FAT10 and large-T antigen. Left panel: The cell lysates were blotted on the membrane and probed with anti-HA antibody. Right panel: The cell lysates were also immunoprecipitated with anti-HA antibody and probed with anti-large T-antigen polyclonal antibody (kind gift from Prof. Wolfgang Deppert).

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Abbreviations

AD	Alzheimer's disease
AIPL1	Aryl hydrocarbon receptor-interacting protein-like 1
AMP	Adenosine monophosphate
3-AT	3-amino-1,2,4-triazole
ATG	Autophagy related
AP1	Aminopeptidase 1
ATP	Adenosine-5'-triphosphate
BD	Binding domain
CD	Circular dichroism
CNS	Central nervous system
CP	Core particle
cTECs	Cortical thymic epithelial cells
°C	degree Celsius
DTT	Dithiothreitol
DRiPs	Defective ribosomal products
DUB	Deubiquitinating enzyme
E6-AP	E6-associated protein
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ERAD	Endoplasmic reticulum associated degradation
FAT10	HLA-F locus adjacent transcript 10
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione
GST	Glutathione-S-transferase
h	Hour
HD	Huntington's disease
hrs	Hours
HA	Haemagglutinin
HAT	Hypoxanthine aminopterin thymidine
HDAC	Histone deacetylase
HECT	Homologous to E6-AP C-terminus
HEK	Human embryonic kidney
HIV	Human immunodeficiency virus
HPRT	Hypoxanthine guanine phosphoribosyltransferase
Hsp	Heat shock protein
Htt	Huntingtin
i.c.	Intracranial
IL	Interleukin
IFN- γ	Interferon gamma

IPTG	Isopropyl- β -D-1-thiogalactopyranoside
ISG15	Interferon stimulated gene 15
JAK	Janus kinase
kDa	Kilodalton
LB	Lewy bodies
LC3	Light chain 3
LCA	Leber congenital amaurosis
LCMV	Lymphocytic choriomeningitis virus
LMP	Low molecular-weight protein
LRR	Leucine-rich-repeat
mAb	Monoclonal antibody
MAD2	Mitotic arrest deficient 2
MHC	Major histocompatibility complex
μ g	Microgram
ml	Milliliter
μ M	Micromolar
mM	Millimolar
mRNA	messenger ribonucleic acid
NEDD8	Neuronal precursor cell-expressed developmentally down-regulated 8
NEMO	NF-kappa-B essential modulator
NUB1	NEDD8 ultimate buster-1
NUB1L	NEDD8 ultimate buster-1 long
ODC	Ornithine decarboxylase
PA	Proteasome activator
pAb	Polyclonal antibody
PAC	Proteasome assembling chaperones
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDB	Protein data bank
PDR	Pleiotropic drug resistance
Pfu	Plaque forming unit
Pgk1	Phosphoglycerate kinase 1
PHD	Plant homeodomain
PINK	PTEN-induced putative kinase
pI	isoelectric point
PLIC	Protein linking IAP to the cytoskeleton
Pru	Pleckstrin-like receptor for ubiquitin
RING	Really Interesting New Gene
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Regulatory particle
RTEC	Renal tubular epithelial cells
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Skp1-Cullin-F-box protein

SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Sec	Seconds
SEN	SUMO-specific protease
Sic1	Substrate inhibitor of cyclin-dependent protein kinase
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
Tg	Transgenic
TGF	Transforming growth factor
TNF- α	Tumor necrosis factor alpha
Ub	Ubiquitin
UBA	Ubiquitin associated
Ubc	Ubiquitin conjugating
UBD	Ubiquitin domain
UBL	Ubiquitin-like
UBP	Ubiquitin-specific protease
UCH	Ubiquitin C-terminal hydrolases
UDP	Ubiquitin domain protein
UIM	Ubiquitin-interacting motif
ULP	UBL specific protease
Ulp1	Ubiquitin-like protein 1
UMP	Ubiquitin maturation protein
UPS	Ubiquitin-proteasome system
UV	Ultraviolet
Vpr	Viral protein R
VWA	von Willebrand factor A
WT	Wild type
Y2H	Yeast two hybrid
YPD	Yeast Peptone Dextrose

List of Amino Acids

Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	K
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophane	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Record of Contributions

Chapter 2: RPN1 and the VWA domain of RPN10 bind the UBL-UBA protein NUB1L and the ubiquitin like modifier FAT10 for proteolytic targeting to the 26S proteasome

I performed and established all the experiments except for Figure 2.2d and wrote the manuscript. This manuscript is submitted to Nature Communications.

Chapter 3: Studies on the Structure of the hRpn10 Subunit of the 26S Proteasome and the UBL-UBA Domain Protein NUB1L

I performed and established experiments for the purification of VWA and hRpn10 proteins as well as GST-pull down assays. All the experiments were performed by me except for Figure 3.5b, c and Figure 3.6. The three-dimensional structure was obtained and analyzed by me with the support of Prof. Dr. Tancred Frickey. All the alignments were performed by me.

Chapter 4: Investigating the Role of FAT10 in Neurodegenerative Diseases

All the experiments were performed by me except the immunohistochemical stainings (Figure 4.6).

Chapter 5: USE1 is a Bispecific Conjugating Enzyme for Ubiquitin and FAT10 which FAT10ylates itself in *cis*

I characterized the UBA6 antibody (Figure 5.8) and also performed isotyping experiments on FAT10 monoclonal antibody. I wrote the section describing UBA6 antibody characterization. This chapter was published in Nature Communications 1, 13 (2010).

Chapter 6: Generation of Monoclonal and Polyclonal Antibodies Specific for Mouse FAT10

I generated and characterized the polyclonal and monoclonal antibodies. All the experiments were performed by me except for Figure 6.1c, which was performed under my direct supervision.