

# The Search for Deconjugating Enzymes of FAT10

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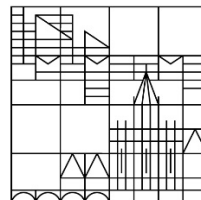
## Dissertation

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## Abbreviations

Abx	antibiotic
AIP1	aryl hydrocarbon receptor interacting protein-like 1
AMC	7-amino-4-methylcoumarin
AMSH	associated molecule with the SH3 domain of STAM
AMV	avian myeloblastosis virus
APC	anaphase promoting complex
ATG15	autophagy related protein 15
ATP	adenosine triphosphate
BAP1	BRCA1 associated protein-1; a UCH family of DUB
BRCA	breast cancer 1
BSA	bovine serum albumin; also known as BSA or "Fraction V"
Cas	caspase
CBL	casitas B-lineage lymphoma, an E3 ligase
CCHFV	ceimean congo haemorrhagic fever
cDNA	complementary DNA
ChT	chymotrypsin
CHX	cyclohexamide
ciAP	cellular inhibitor of apoptosis
CM	complete media
COP9	constitutive photomorphogenesis 9
CRL	cullin-RING-ligases
CSN	COP9 signalosome complex
CTD	C-terminal domain
cTEC	cortical thymic epithelial cells
Cul	cullin
CYLD	cylindromatosis
DMSO	dimethyl sulfoxide
DMSO	dimethyl sulfoxide
DN	double negative
DP	double positive

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DUBA	deubiquitinating enzyme A
DUBs	deubiquitinating enzymes
EDTA	ethylene-diamine-tetraacetic-acid
EGF	epidermal growth factor
EMBL	European Molecular Biology Laboratory
ESCRT	endosomal sorting complex required for transport
FAT10	HLA-F adjacent transcript 10
FBS	fetal bovine serum
FCS	fetal calf serum
FOXO4	forkhead box O-class 4
FOXP	forkhead box P3
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
GMPS	Guanosine monophosphate synthetase
GRAIL	gene related to anergy in lymphocytes
GSB	gel sample buffer
GST	glutathione-S-transferase
HA	hemagglutinin, is used as protein tag in expression vectors
HAUSP	herpes associated USP
HCMV	human cytomegalovirus
HDAC6	histone deacetylase 6
HECT	homologous to E6-AP carboxyl terminus
HEK293	human embryonic kidney 293 cells
HERC	HECT domain and RCC1-like domain-containing protein 5,
HGF	hepatocyte Growth Factor
HIVAN	HIV associated neuropathy
HLA	human leukocyte antigen
HRP	horseradish peroxidase
Htt	huntingtin
ICP0	human herpes virus (HHV) infected cell polypeptide 0
IFN- $\gamma$	interferon gamma
IMDM	iscoves modified dulbeccos medium

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IP	immunoprecipitation
ISG15	interferon stimulated gene 15
IκK	inhibitor of NFκB kinase
Jak/STAT	janus kinase/ signal transducer and activator of transcription
JAMM	Jab1/Mov34/Mpr1 Pad1 N-terminal+ (MPN+) domain proteases
kDa	kilo Dalton
LB	luria-bertania medium
LPS	lypoploysaccharide
mAb	monoclonal antibody
MAD2	mitotic arrest-deficient 2
MCPIP	monocyte chemotactic protein-induced protein
Mdm2	mouse double minute 2 homolog, an E3 ligase
MERS	middle east respiratory syndrome
MESNa	2-mercapto-ethane-sulfonic acid
MG132	is a potent, reversible, and cell-permeable proteasome inhibitor
MHC	major histocompatibility complex
MJDs	machado-josephin domain proteases
MNSF-β	monoclonal nonspecific suppressor factor-β
mTEC	medullary thymic epithelial cells
MTT	tetrazolium dye
MVB	multivesicular bodies
NEDD8	neural precursor cell expressed, developmentally down-regulated 8
NEMO	NF-kappa-B essential modulator
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NIK	NF-kappa-B-inducing kinase
NMJs	neuro muscular junction
NP40	detergent, nonyl phenoxy-polyethoxyl-ethanol 40
NTD	N-terminal domain
NUB1	NEDD8 ultimate buster 1, a negative regulator of NEDD8
NUB1L	NEDD8 ultimate buster-1 long
OD	optical density
OTU	ovarian tumor domain
pAb	polyclonal antibody

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PBS	phosphate buffered saline
PGPH-L	caspase like activity of 20S proteasome, Cas-L
PHD	plant homeodomain
PPPDE	after Permuted Papain fold Peptidases of DsRNA viruses&Eukaryotes
PRR	pattern recognition receptor
PTMs	post translational modifications
RING	really interesting new gene
RNF4	RING finger protein 4, an E3 ubiquitin-protein ligase RNF4
RP	regulatory particle
Rpm	revolution per minute
RPN	regulatory particle non ATPase
RPT	regulatory particle triple A
RT	room temperature
RTEC	renal tubular epithelial cells
SAMPs	ubiquitin-like small archaeal modifier proteins
SARS	severe acute respiratory syndrome
SCA	spinocerebellar ataxia type 3, is a medical condition
SCF	skp1-Cul1-F-box protein
SENP	SUMO specific protease
SIAH	seven in absentia homologue 1
siRNA	small interfering RNA
SMAD	small body size mothers against decapentaplegic
SNP	single nucleotide polymorphism
SOC	super optimal broth (SOB) with glucose (catabolic repressor) media
SP	single positive
SQSTM1	sequestosome-1, also called p62 an autophagy receptor
SUMO	small ubiquitin-like modifier
TAE	Tris base, acetic acid and EDTA containing buffer
TAK	TGF $\beta$ activated kinase
TAMRA	carboxy tetramethyl rhodamine
TAP	transporter associated with antigen processing
TBST	Tris buffered saline and 0.05% Tween 20
TCR	T-cell receptor

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T-DNA	transfer DNA
TMR	tetramethylrhodamine
TNF $\alpha$	tumor necrosis factor alpha
TRABID	an OTU family of DUB, also known as ZRANB1
TRAF2	TNF receptor- associated factor 2
Tregs	regulatory T cells, CD4+CD25+Foxp3+ T cells
TRIM	tripartite motif containing protein 11, an E3 ligase
U/ml	unit per milliliter
Ub	ubiquitin
UBE1	ubiquitin-activating enzyme E1, also known as UBA1
UBLs/ULM	ubiquitin-like modifiers
UBP	ubiquitin processing protein
UCH	ubiquitin C-terminal hydrolase
UDB	ubiquitin domain protein
UFD	ubiquitin fold domain
UHRF2	ubiquitin-like with PHD and ring finger domains 2, E3 Ub-ligase
UPS	ubiquitin protease system
USE1	UBA6-specific E2 enzyme
USP	ubiquitin specific protease
VCIP135	valosin containing protein p97/p47 complex interacting protein1
YOD1	ovarian tumor family of DUB, also known as OtuD2 or DUBA8
YUH	yeast ubiquitin hydrolase

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## Summary

FAT10 (HLA-F locus adjacent transcript 10) is an 18 kDa polypeptide. It consists of two ubiquitin-like domains and bears a free diglycine-motif at its C-terminus through which it covalently conjugates to target proteins e.g. p62, UBE1, USE1, p53, Huntingtin, UBA6 etc. More substrates of FAT10 are still being identified. The *fat10* gene is encoded in the MHC-I locus and is synergistically inducible by the proinflammatory cytokines IFN- $\gamma$  and TNF- $\alpha$ . FAT10 is the only one among ubiquitin-like modifiers which directly targets conjugated proteins for degradation via the 26S proteasome. FAT10 is upregulated in tumor microenvironments (i.e. colon carcinoma, bladder cancer, hepatocellular carcinoma or uterus carcinoma) and various oncogenes and apoptotic inhibitors might interact with FAT10. The thymus has the highest level of FAT10 expression, especially in mTECs [96]. Conjugated ubiquitin can be removed from its substrates by selective proteases called deubiquitinating enzymes. Since some oncogenes and apoptosis inhibitors are among the substrates of FAT10 conjugation, the enhancement of their FAT10ylation through inhibition of putative deconjugating enzymes may be a rewarding therapeutic approach.

In the first part of the thesis, our goal was to uncover some of the unknown facets of FAT10 biology and its potential deconjugating enzymes. For most modifiers (Ubiquitin, SUMO, NEDD8, ISG15 etc.) deconjugating enzymes have so far been reported. We have demonstrated that USP7 is a FAT10 interacting deconjugating enzyme from the USP family. Moreover, USP7 interacts with FAT10 through its C-terminus.

Overexpression of p53 has been shown to downregulate FAT10 [95]. Therefore, in the second part of the thesis, the effect of overexpression of p53 on FAT10 transcription and protein stability was investigated. We found with increasing p53 overexpression in p53 null cells endogenous FAT10 was not downregulated.

USP7 have been shown to deubiquitinate not only Mdm2 and p53 but it also deubiquitinates and stabilize the transcription factor Foxp3 in regulatory T cells. Therefore, in the third part of the thesis, we investigated the interaction of FAT10 and Foxp3. Overexpressed Foxp3 interacts with FAT10 in HEK293 cells.

Ubiquitinated proteins have been shown to activate the proteasome by 20S gate opening. In the final part of this thesis, the mammalian 26S proteasome activation status was investigated in the presence of FAT10, NUB1L or FAT10 conjugates. In the presence of any of these proteins, the proteasome activities designated as trypsin-like, caspase-like or the chymotrypsin-like activity were not stimulated.

In conclusion, USP7 is a novel FAT10 interacting protein. Overexpression of p53 cannot downregulate endogenous FAT10 in p53 null cells. Neither FAT10 alone, nor FAT10 conjugates can stimulate any of the three different catalytic activities of the 26S proteasome.

## Zusammenfassung

FAT10 (HLA-F locus adjacent transcript number 10) ist ein 18 kDa grosses Protein. Es besteht aus zwei Ubiquitin-ähnlichen Domänen und trägt am C-terminalen Ende ein Diglycinmotiv. Durch dieses Motiv kann FAT10 kovalent an Zielproteine wie: p62, UBE1, USE1, p53, Huntingtin, UBA6 usw. gebunden werden. Es werden ständig neue Substrate von FAT10 beschrieben. Das FAT10 exprimierende Gen liegt im MHC-Klasse I Bereich und kann synergistisch mit den entzündungsstimulierenden Zytokinen Interferon Gamma (IFN- $\gamma$ ) und Tumornekrosefaktor Alpha (TNF- $\alpha$ ) stimuliert werden. FAT10 ist einzigartig unter den Ubiquitin-ähnlichen Modifizierern, da es zum direkten Abbau der Substrate durch das 26S Proteasome führt. FAT10 ist in der Tumor Microumgebung (wie z.B. Darmkrebs, Blasenkrebs, Leberkarzinom und Gebärmutterkarzinom) verstärkt exprimiert, und verschiedene Onkogene sowie Apoptoseinhibitoren können mit FAT10 interagieren. Der Thymus zeigt die höchste Expressionsrate für FAT10, besonders in den mTECs. Ubiquitin wird durch spezielle Proteasen, genannt Deubiquitinasen, von seinen Substratproteinen entfernt. Da einige Onkogene und Apoptoseinhibitoren zu den FAT10 Substraten zählen, wäre eine Erhöhung der FAT10-Modifizierung dieser Proteine durch gezielte Inhibition potentieller FAT10 abspaltender Dekonjugierungsenzyme ein vielversprechender therapeutischer Ansatz. Im ersten Teil der Arbeit wurden einige unbekannt Details der FAT10 Biologie und seiner potentiellen Dekonjugierungsenzyme untersucht, da solche Enzyme bisher für fast alle modifizierenden Proteine (Ubiquitin, SUMO, NEDD8, ISG15 usw.) beschrieben wurden. Zusammenfassend konnte gezeigt werden das USP7 ein mit FAT10 interagierendes Enzym der USP Familie ist. USP7 bindet FAT10 mit seiner C-terminalen Domäne.

Der zweite Teil der Arbeit befasste sich mit dem Effekt der verstärkten Expression von p53 auf die Transkription des FAT10 Gens sowie auf die Stabilität des FAT10 Proteins. Die Überexpression von p53 in p53-defiziente Zellen hatte keinen Einfluss auf das endogene FAT10 Level.

USP7 kann nicht nur Mdm2 und p53 deubiquitinylieren, es deubiquitinyliert und stabilisiert auch den Transkriptionsfaktor FoxP3 in Tregs. Im dritten Teil der Arbeit wurde die Interaktion von FAT10 und FoxP3 näher charakterisiert. Überexprimiertes FoxP3 bindet an FAT10 in HEK-Zellen.

Im letzten Teil der Arbeit wurde der Aktivitätsstatus des 26S Proteasomes unter Einfluss von FAT10, NUB1L und von FAT10-Konjugaten untersucht, da Ubiquitin-Konjugate das Proteasome über „gate opening“ aktivieren können. Keines der untersuchten Proteine war in der Lage eine der drei proteasomalen Aktivitäten (Chymotrypsin-, Trypsin- oder Caspase-ähnliche Aktivität) zu beeinflussen.

Zusammenfassend konnte gezeigt werden das USP7 ein neues FAT10 bindendes Protein ist. Verstärkte Expression von p53 hat keinen Einfluss auf endogenes FAT10 in p53-defiziente Zellen. Weder FAT10 alleine noch seine Konjugate beeinflussen die peptidolytischen Aktivitäten des Proteasomes.

# 1. Introduction

Previously it was believed that proteins are degraded in cells through lysosomes, the membrane-bound organelles containing proteases and acid pH which digest aged, damaged protein or if protein needs recycling. In 1977, it was reported by Alfred Goldberg, that in cells lacking lysosomes (reticulocytes) a second intracellular protein degradation mechanism exists, which is ATP-dependent [1]. The biochemists Dr. Avram Hershko, Dr. Aaron Ciechanover, and Dr. Irwin, shared the 2004 Nobel Prize in chemistry for the discovery of ubiquitin-mediated protein degradation.

## 1.1 Ubiquitin proteasome system

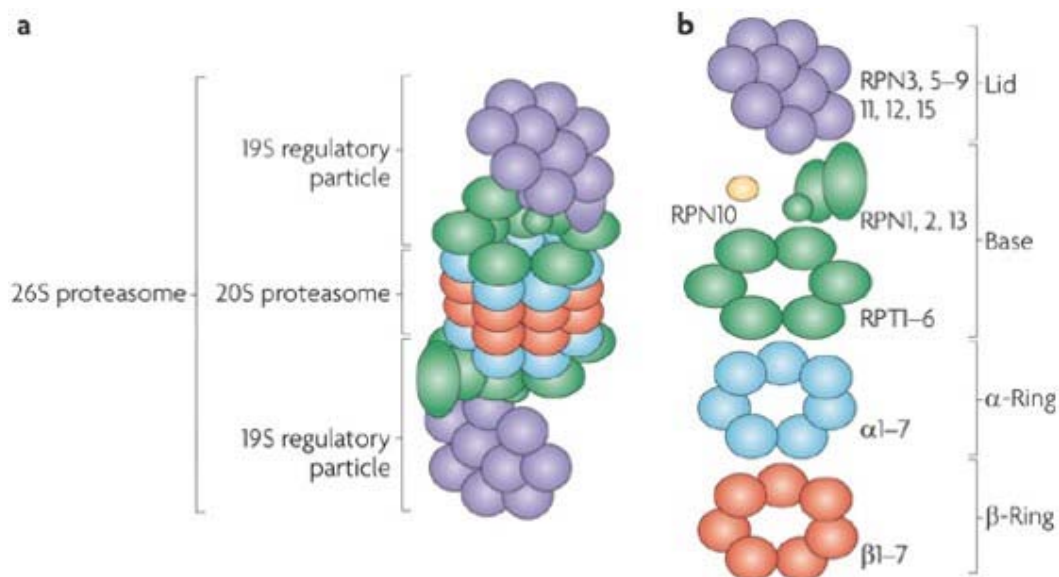
The ubiquitin-proteasome system (UPS), is a nonlysosomal intracellular protein degradation pathway consisting of proteasome holoenzyme, ligation cascade, and deubiquitinases [2]. The UPS controls many cellular processes either by proteolytic or non-proteolytic means.

### 1.1.1 Proteasome

In eukaryotic cells two protein degradation systems exist; the proteasome and the lysosome. The ubiquitin protease system mostly degrades intracellular, soluble proteins, but sometimes degrades transmembrane proteins only when they are transported from the membrane into the cytosol. In addition, ubiquitinated substrates are also ferried to VCP/p97/Cdc48; a multi-subunit and best-characterized type-II AAA-ATPase-complex responsible for ubiquitin-dependent degradation, mitotic spindle assembly and vesicle fusion [3][4]. On the other hand, the lysosome degrades most membrane proteins, endocytosed proteins and digests cytosolic proteins through autophagy [5]. The 26S proteasome is a large protease complex with a molecular weight of  $2.4 \times 10^6$  Dalton. In mammalian cells, most proteins are degraded by the 26S proteasome and this is an ATP-dependent process. The large cylindrical 26S proteasome is involved in degradation of unwanted or damaged proteins and is present in eukaryotes, archaea, and some eubacteria. In cells, they are present at the kinetochores, cytoplasm, and nucleus. The 26S proteasome is composed of a 20S core particle, within which proteins are digested. The 20S core is capped by one or

two 19S complexes (PA700) that bind and help to unfold, and translocate ubiquitinated proteins into the 20S catalytic tunnel (**Fig.1**) [6][7].

The 20S proteasome (700 kDa) is composed of four rings of seven subunits each (hetero-heptameric rings) i.e. two rings are composed of seven  $\alpha$ -subunits and two rings are composed of seven  $\beta$ -subunits. Each of the two internal rings ( $\beta$ -rings) contains the catalytic subunits ( $\beta$ 1,  $\beta$ 2, and  $\beta$ 5), whereas the outer rings ( $\alpha$ -rings) form a gated channel through which polypeptides enter the central tunnel where they are cleaved by multiple proteolytic sites. The N-terminal regions of the  $\alpha$ -subunits function as a gate, restricting entry of protein or peptide through it [8]. The 20S proteasomes with closed gates are catalytically inert [9]. The activation of the 20S core is achieved by regulatory protein binding such as proteasome activator PA28 or the 19S regulator; they bind to the outer  $\alpha$ -ring and releases the barrier by N-terminal extensions of the  $\alpha$ -subunits and thus allow access of substrate to the catalytic chamber [10].



**Figure 1. The 20S and 26S Proteasome**

**[a]**. The 26S proteasome is composed of the catalytic 20S proteasome (a barrel of 4 stacked rings; 2 outer alpha-rings and 2 inner beta-rings) and the 19S regulatory particle (RP, also known as PA700); **[b]**. Subunits in 26S proteasome. The regulatory particle is further subdivided into the base and the lid sub-complexes, which are composed of regulatory particle triple-A (RPT) and regulatory particle non-ATPase (RPN) subunits. The yellow color RPN10 is supposed to be located at the base-lid interface. The image is taken from [11].

The 19S regulator is composed of a base and a lid formed by at least 19 different protein subunits. The base is composed of 6 distinct ATPases (AAA+ ATPase subunits; Rpt1-Rpt6) and 3 non-ATPase subunits (Rpn1, Rpn2, and Rpn13) while the lid contains up to 9 non-ATPase subunits (Rpn3, Rpn5-9, Rpn11, Rpn12, and Rpn15). The six ATPases together form a hexameric ring and interact with the  $\alpha$ -ring of the 20S core particle, leading to the opening of the 20S gated pore and increased proteolytic activity. The 19S regulator contains ubiquitin receptors, deubiquitinating enzymes, and subunits with ATPase activity. The two subunits Rpn13 and Rpn10 (S5a) act as receptors for ubiquitin chains anchored on proteins [6]. In order to be degraded a substrate protein must be first deubiquitinated, unfolded, and then translocated through a gate channel into the 20S particle.

Classified according to the catalytically active subunits, 20S proteasomes can be of different types such as the constitutive proteasome, the immunoproteasome, or the thymoproteasome. Proteasomes constitutively expressed in most mammalian tissues have three different specialized catalytic subunits; the  $\beta$ 1 proteasomal subunit has caspase-like activity (PGPH-L or Cas-L) and cleaves peptides after acidic residues; the  $\beta$ 2 proteasomal subunit has trypsin-like activity (T-L) and cleaves peptides after basic residues and the  $\beta$ 5 proteasomal subunit has chymotrypsin-like activity (ChT-L) and cleaves peptides after hydrophobic residues.

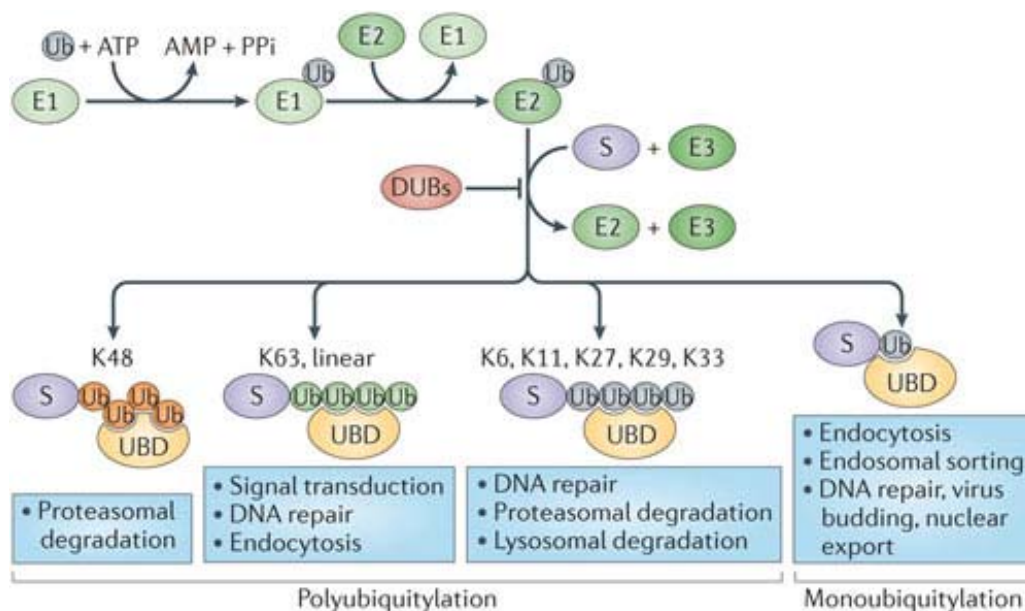
The 20S core exists in various types in higher eukaryotes. For example, stimulation with IFN $\gamma$  and /or TNF $\alpha$  leads to induction of three proteasomal 'immunoproteasomes';  $\beta$ 1i,  $\beta$ 2i, and  $\beta$ 5i which replaces the constitutive subunits of the proteasome. Therefore, while neosynthesis of the 20S core in cytokine-stimulated cells the constitutive proteasome subunits are replaced by immunoproteasomes resulting into immunoproteasome. The immunoproteasome and constitutive proteasome show different catalytic efficiencies [9]. The  $\beta$ 1,  $\beta$ 2, and  $\beta$ 5 subunits are highly homologous to the  $\beta$ 1i,  $\beta$ 2i and  $\beta$ 5i subunits of the immunoproteasome. The  $\beta$ 1i and  $\beta$ 5i subunits are encoded by genes in the major histocompatibility complex (MHC) class II regions near to genes coding for the transporter associated with antigen processing 1 and 2 while the  $\beta$ 2i gene has been found outside the MHC region. Constitutive proteasomes in the liver and other tissues are completely replaced by immunoproteasomes within 8 days after infection with bacteria, virus, and fungus. This replacement increases the production of peptides by the proteasome for presentation on MHC class I molecule. Dendritic cells and mTECs (medullary thymic epithelial cells) in the thymus express high amounts of the immunoproteasome helping them in negative selection and TCR

repertoire formation. The proteasome in cortical thymic epithelial cells (cTEC) in the thymus is a further variant called the thymoproteasome; which contains  $\beta 1i$ ,  $\beta 2i$  and the cTEC-specific proteasome subunit  $\beta 5t$  [12].

### 1.1.2 Ubiquitination

The polypeptide ubiquitin (Ub) is synthesized as precursors that must be proteolytically processed to release mature ubiquitin. Four different genes (*UBB*, *UBC*, *UBA52*, and *UBA80* (*RPS27A*)) encode for ubiquitin. The Ub polypeptide sequence is conserved among eukaryotic organisms with only two amino acid changes between yeast and human ubiquitin [13]. The small 76-amino acid polypeptide ubiquitin is 8.5 kDa in size, enriched with seven internal lysines. The N-terminal methionine (Met or M) and 7 different lysines (Lys or K) residues of ubiquitin are needed for chain formation and can form 8 different linkages via Met<sup>1</sup>, Lys<sup>6</sup>, Lys<sup>11</sup>, Lys<sup>27</sup>, Lys<sup>29</sup>, Lys<sup>33</sup>, Lys<sup>48</sup> and Lys<sup>63</sup>. In addition, mixed and branched chains can also be formed [14]. In regulating a broad range of physiological processes like proteasomal degradation, the DNA-damage response, transcription and membrane trafficking, ubiquitination plays a pivotal role. In order to be degraded a protein must contain, a minimum element within a protein that is sufficient for the recognition and degradation by a proteolytic apparatus are usually called degradation signal or degron [15]. Generally, proteasomal degradation requires the covalent attachment of a chain of four or more ubiquitin molecules [6]. Ubiquitin carboxyl-termini (C-termini) can be linked through isopeptide bonds to lysines of target proteins. The destination of substrate proteins varies with the type of linkage. Lys<sup>48</sup> or Lys<sup>29</sup>-linkages [16] act as degradation signal whereas Lys<sup>63</sup>- linkages [17] are frequent in signal-transduction cascades or can target proteins for lysosomal degradation (**Fig. 2**). Ubiquitin or members of the ubiquitin superfamily of proteins contain a similar 3D structure, called the  $\beta\beta\alpha\beta\beta$  fold, ubiquitin fold or  $\beta$ -grasp fold, even in the absence of primary sequence similarity [18]. The addition of ubiquitin to a substrate protein is called ubiquitination or ubiquitylation. Ubiquitination is a reversible covalent modification, catalyzed by three enzymatic steps [19]. The modification of a target substrate protein requires the concerted action of a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) enzyme cascade [20][21]. Occasionally, an E4 enzyme is required to catalyze multiubiquitin-chain assembly in collaboration with E1, E2, and E3 [22]. The ubiquitin-activating enzyme

(E1) catalyzes the formation of a reactive thioester bond via its active site cysteine residue and the C-terminal carboxylate of ubiquitin. Ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which in collaboration with ubiquitin ligases (E3) show substrate specificity. In this collaboration the C-terminus of ubiquitin is attached to the epsilon-amine of a lysine residue in the target protein, forming an isopeptide bond [20]. Alternatively, the C-terminus of ubiquitin when attached to the N-terminus of another ubiquitin resulting in regular peptide bond, is referred as linear-ubiquitin bond.



**Figure 2. Functions of different ubiquitin linkages**

Ubiquitination is a process involving the sequential action of three enzymes (E1, E2, and E3) catalyzing covalent attachment of ubiquitin to a target protein. The style of ubiquitin linkage formed over the substrate by the three enzymes, signals different functions. The well-studied K-48 linkage is a signal for degradation by the 26S proteasome. The image is taken from [23].

In mono-ubiquitination, a single ubiquitin molecule is conjugated to one or several lysines within substrate proteins. Mono-ubiquitination has been reported, to be required for internalizing cell-surface receptors (e.g. EGF receptor), in DNA repair and in regulating gene expression through histone modification [24][25]. Additionally, because ubiquitin itself carries lysines that act as sites of self-conjugation, multiple

ubiquitin (polyubiquitination) chains can form and be attached to proteins. Chains can either be linked homotypically through one specific residue in each module (Ub) or heterotypically through a selection of different residues in each module, including multiple residues per chain forming branched chain. There are approximately ~6 E1 like enzymes, ~53 E2 enzymes, and ~527 E3 enzymes estimated to encode by the human genome [26]. The specific cooperation of E2 and E3 enzymes determines the type of chain linkage formation as well as which protein substrate is modified. The Lys<sup>48</sup>-linked chains target proteins for proteasomal degradation [16][27], while chains having a function in DNA repair are Lys<sup>63</sup>-linked [28]. The N-terminally linked (linear) [29], Lys<sup>11</sup>, and Lys<sup>29</sup> chains have been shown to have functions in cell cycle control and signaling [20]. The functions of other linkages are still less clear and need extensive investigations. As recently reported, thiol (-SH) or hydroxyl groups (-OH) in cysteine (Cys or C), serine (Ser or S) or threonine (Thr or T) residues can also act as receptors for ubiquitin [30]. The attachment of ubiquitin to substrate proteins not only targets them for proteasomal degradation but also mediates non-degradative functions, such as protein trafficking, regulations of signal transduction events and protein-protein interaction [31].

### 1.1.3 E3 ligases

In the ubiquitination reaction, Ub is transferred via trans-thiolation reaction from an E1-enzyme to an E2-enzyme. Next, the E3-ubiquitin ligases bind the substrate protein and catalyze the transfer of ubiquitin from the Cys of an E2 to a Lys-residue on a substrate protein. E3 ligases are categorized into four major classes: HECT-type, RING-finger-type, U-box-type, and PHD-finger-type. RING E3s transfer Ub directly to the substrate. While the second type, HECT E3s do not directly carry and transfer activated Ub and require the presence of the E2 and often additional components (e.g. in the case of SCF complexes) for ubiquitination to proceed [32].

In mammals, there are ~30 HECT domain E3 ligases. In addition to their ligation function, they have additional roles in many signaling pathways involved in cell growth and proliferation, protein trafficking, and in the immune response [33]. The conserved 350 amino acid HECT domain is at the C-terminus, whereas the N-terminal domain is diverse and mediates substrate targeting [34]. The human genome encodes more than

600 potential RING finger E3 ligases [35]. The RING finger domain does not form a catalytic intermediate with ubiquitin, as the HECT domain does.

RING-finger type E3s are further divided into subclasses, including cullin-based E3s (the largest E3 class). There are seven cullin-based E3s including the SKP1-CUL1-F-box protein (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C) (reviewed in [36][37]). Members of RING finger E3 ligases function as monomers, dimers or multi-subunit complexes. Generally, dimerization occurs through the surrounding region or through the RING finger domain and results in homodimers (e.g. cIAP, RNF4, SIAH, and TRAF2) [38][39][40] or heterodimers (e.g. Mdm2 and MDMX) [41][34].

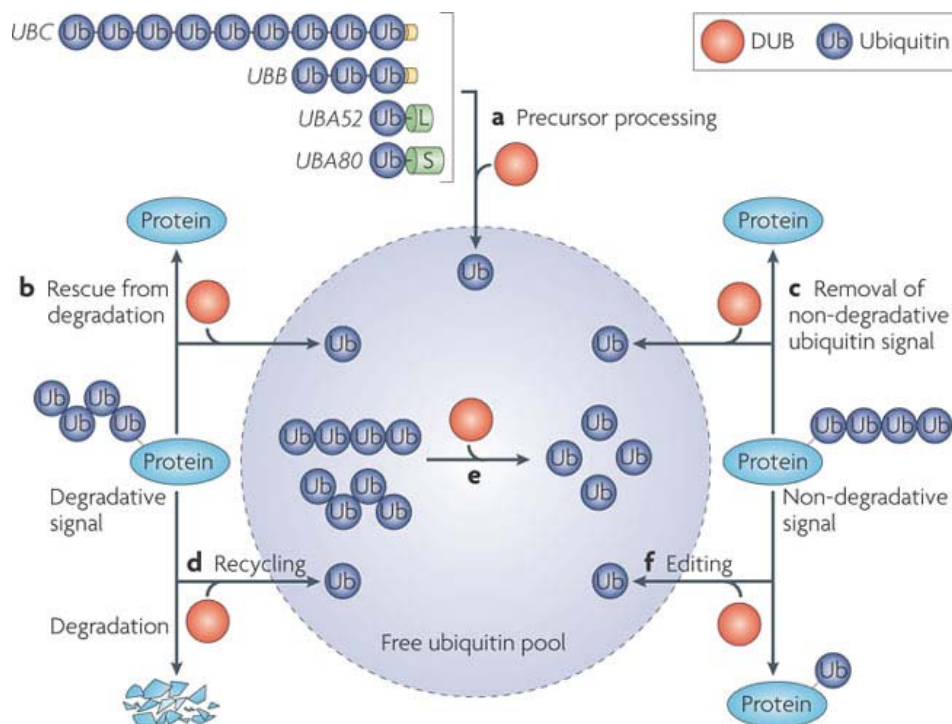
Sometimes DUBs and E3 ligase are present within the same functional complex. Within cells, approximately 500 - 1,000 different E3 ligases exist and these E3 ligases are involved in many biological processes so its deregulation can lead to cancer development. Thus, there are various drugs that target (inhibit) E3 ubiquitin ligases and are in various phases of clinical trials. Cereblon (CRBN) is the only approved drug, which inhibits a non-SCF E3 ligase [32]. Unlike Bortezomib, which inhibits the proteasome, targeting a specific E3 ligase would be advantageous in selectively stabilizing its target(s) and avoid unwanted off-target effects.

#### 1.1.4 Deubiquitination

The enzymatic removal of covalently attached ubiquitin from a substrate protein is called, deubiquitination. Protein ubiquitination is a post-translational modification which is both a dynamic and reversible process. Deubiquitinases, ubiquitin-processing-proteases or deubiquitinating enzymes (DUBs) catalyze the removal of ubiquitin from target proteins. DUBs also have additional roles like ubiquitin maturation, recycling and editing (**Fig. 3**) [13].

Proteases (peptidase or proteinases) catalyze the hydrolysis of amide bonds (peptide bond). They are abundant in animals, plants, bacteria and viruses. Based on their catalytic residues proteases are classified into seven types (**Table-1**) [42]. DUBs are cysteine proteases that cleave the isopeptide bond between the C-terminus of ubiquitin and the side chain of a lysine residue in another ubiquitin module or in a

substrate. Deubiquitinases can either remove ubiquitin or ubiquitin-like proteins from the target protein and thus have significant roles to reverse the ubiquitination or ubiquitin-like modification of target proteins [43]. Additionally, ubiquitin is always expressed as pro-protein i.e. ubiquitin attached to either ribosomal proteins or as linear polyubiquitin fusion proteins composed of multiple copies of mono-ubiquitin. DUBs process them to yield the mature ubiquitin monomer [44]. The polyubiquitin gene product also contains an additional residue at the C-terminus that is removed by DUBs to activate ubiquitin. Classification and functions of DUBs are further discussed in section 1.4.



**Figure 3. Functions of DUBs**

**[a]** DUBs generate free ubiquitin from ubiquitin precursor. Ubiquitin is encoded by four genes (UBC, UBB, UBA52, and UBA80). The ubiquitin gene is transcribed and translated as a linear fusion consisting of multiple copies of ubiquitin, or ubiquitin fused to the amino terminus of two ribosomal proteins, 40S ribosomal protein L40 (L) and 60S ribosomal protein S27a (S); **[b]** A protein can be rescued from 26S proteasome-mediated degradation by DUBs; **[c]** DUBs can also remove a non-degradative ubiquitin signal, additionally; **[d]** Recycling of ubiquitin is done by DUBs i.e. Ub homeostasis is maintained by DUBs by preventing degradation of ubiquitin together with substrates of the 26S proteasome and lysosomal pathways; **[e]** The release of free ubiquitin to maintain the Ub pool; **[f]** Some DUBs might function to edit ubiquitin chains and thereby help to exchange one type of ubiquitin signal for another; The image is taken from [45].

**Table 1. Different classes of Proteases** (adopted from reference [42])

Protease classes	Catalytic active site
aspartate proteases	uses an Aspartate-COOH group
cysteine proteases	uses a Cysteine-OH group
glutamic acid proteases	uses a Glutamate-COOH group
metalloproteases	uses a Metal, usually Zn
serine proteases	uses a Serine-OH group
threonine proteases	uses a Threonine-2°-OH group

## 1.2 Ubiquitin-like modifiers

Proteins having homology sequence to ubiquitin and a similar three-dimensional structure are called ubiquitin-like proteins (UBLs) [46]. Basically, UBLs are of two types: the ubiquitin-like-modifiers (ULMs), and the ubiquitin-domain-proteins (UDPs). For the regulation of eukaryotic cell function, reversible post-translational modifications (PTMs) of proteins play a major role. The PTMs involves small polypeptides as modifiers involve attachment of the small polypeptide ubiquitin or several of human ubiquitin-like modifiers e.g. SUMO, ATG8, NEDD8, ATG12, FAT10, ISG15, FUB1, URM1, and MNSF- $\beta$  [46][47]. Similar to the function of ubiquitin, the ULMs, also modify other proteins. ULMs exist in free form or, by specific conjugation enzymes they are covalently conjugated to other proteins via their C-termini. UBLs are conjugated to proteins by a sequential enzymatic cascade, similar to ubiquitination, and many of UBL conjugation components are the same as those involved in the ubiquitination pathway. Compared to a wide number of substrates known for ubiquitin, most ULMs have fewer substrates. Several poorly characterized UBLs are now assigned a function and participate in various cellular processes, e.g. Ufm1 in development, Hub1 in RNA splicing and SAMPs (archaea) in protein modification as well as sulfur transfer [46]. The presence of C-terminal diglycine motifs is a hallmark of almost all UBLs. As an exception, Hub1 does not have a C-terminal diglycine motif but still it is a UBL. Without having sequence similarity with ubiquitin both APG12 and URM1 are still considered as ULM because of the similar modification. Except for FAT10, APG12, and URM1 all UBLs and ubiquitin are expressed as inactive precursors; having extra amino acids or

polypeptides at their C-termini. These extra regions are removed by proteases leading to their activation [48]. There are many ULMs such as FAT10, small-ubiquitin-like protein (SUMO)-1/2/3, NEDD8, ISG15, ATG12 which become covalently conjugated to target proteins via the carboxyl group of their C-terminal glycine amino acid and form an isopeptide linkage with lysine of the target protein [46].

### 1.2.1 ISG15

Binding of interferon type-1 to the interferon receptor (IFNAR) initiates a signaling cascade, which leads to the induction of more than 300 interferon-stimulated genes (ISGs) [49]. Many ISGs are gene products encoding pattern-recognition receptors (PRRs), or transcription factors. Few of the ISGs, including ISG15 (IFN-stimulated protein of 15 kDa) have potential direct antiviral activity. ISG15 is a component of the innate immune response that has been known for 20 years [41]. ISG15 is a ubiquitin-like protein that is present in higher organisms possessing the IFN pathway, but it is absent in insects [50]. Recently, it was confirmed by many independent groups that it has antiviral activity against many virus types, including influenza A virus, herpes virus, human immunodeficiency virus, and Ebola [51][52][53]. ISG15 contains two ubiquitin-like (UBL) domains and the C-terminal UBL domain has a diglycine residue similar to Ubiquitin, FAT10, and other ULMs. ISG15 is expressed as a precursor of 165 amino-acids that is afterward processed to expose the C-terminal sequence LRLRGG. When the ubiquitin E1 enzyme (UBE1) was found unable to form a thiolester bond with ISG15 it was believed that ISGylation requires a parallel and distinct pathway [54]. In the meantime UBE1L (E1 like Ub activating enzyme) has been reported as the E1 enzyme for ISG15 [55]. In addition, the two E2 enzymes UBCH6 and UBCH8 were identified as ISG15 carriers [56]. Last but not least, two ubiquitin E3 ligases, HERC5, and TRIM25, have also been reported to conjugate ISG15 to substrate proteins [57] [58]. Thus, there seems to be a direct interplay between ubiquitylation and ISGylation.

DUBs can reverse ubiquitylation. Conjugation of ISG15 can also be reversed by several identified enzymes, including ubiquitin-specific protease 2, USP5, USP13, USP14 and USP18 (also known as UBP43) [59]. Proteases encoded by a number of viruses can de-ISGylate such as the SARS-associated coronavirus, equine arteritis virus, and Sindbis virus etc. [60]. Until now at least 158 putative ISG15 target proteins have been identified, ISGylation does not lead to degradation of the target protein (as occurs following K<sup>48</sup>-linked ubiquitin), but instead parallels the activating effects of

ubiquitination (occurs following K<sup>63</sup>-linked ubiquitination). In addition to its intracellular role, ISG15 is also secreted like a cytokine, to modulate the immune response [61].

### 1.2.2 SUMO

The small ubiquitin-like modifier (SUMO) is a ~12 kDa protein having essential roles in regulating the stability, intracellular localization or activity of hundreds of proteins in eukaryotic cells. Stress (e.g., heat stress, oxidative stress, insufficient blood supply etc.) and pathogen attack lead to global changes of SUMOylation in a cell. Failure of protein SUMOylation or dysregulation of SUMOylation is associated with diseases like cancer and heart failure [62]. Posttranslational modification with SUMO has been shown to regulate a number of cellular processes, including recombination, nuclear transport, chromosome segregation, cell-cycle progress, signal transduction, transcription in yeast and in the human. The function of many transcription factors is regulated by SUMOylation [63]. Similar to ubiquitin, SUMOs are synthesized as pro-peptides that require C-terminal cleavage to liberate their C-terminal di-glycine motifs prior to conjugation [64]. SUMO-specific E1, E2, and E3 ligases catalyze SUMOylation which is an ATP-dependent process giving rise to SUMO-chains (polySUMOylation) [62]. Conjugation between a C-terminal di-glycine motif of SUMO and epsilon-amino group in lysine within the target protein results in isopeptide bond formation. SUMO proteases are required for two tasks, SUMO processing (C-terminal hydrolase activity) and deconjugation (isopeptidase activity). They are termed ULM/UBL-specific proteases (ULP) in yeast [65] and Sentrin-specific-proteases (SENP) in mammals [66]. In mammals SUMO proteins are divided into two families consisting of SUMO-1 and SUMO-2/SUMO-3. Different independent groups discovered SUMO-1 (small ubiquitin-related modifier) giving rise to different names i.e. sentrin, DAP1, GMP1, PIC1, UBL1 and SMT3C [67]. Mature SUMO-2 is 92 and mature SUMO-3 is 93 amino acids long, respectively. Both of them are very similar in the human sharing 97% sequence identity. Mature SUMO-1 is only 47% similar to SUMO-2/3. Although, all SUMO proteins are not similar yet they are activated and conjugated by the same enzymes. The cysteine protease of three different types, ULP/SENP proteases, two members of the PPPDE super family (DeSI-1 and -2), and the USP family member USPL1 are reported to be SUMO-specific isopeptidases. In yeast, the SUMO-specific ULP/SENP family has two members; ULP1 is essential and ULP2 is not. Mammals have six proteins i.e. SENP-1, -2, -3, -5, -6, & -7. The ULP2 and SENP1/6/7 are nucleoplasmic

proteins; ULP1 & SENP2 are anchored to the nuclear pore complex, and SENP-3/5 are abundant in the nucleolus [62]. The deconjugating enzyme for SUMO1, SUMO-specific protease 1 (SENP1) plays an important role in the development of early T and B cells [68].

### 1.2.3 NEDD8

The protein NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) is a ubiquitin-like modifier which is encoded by the *nedd8* gene in humans. In *Saccharomyces cerevisiae*, this protein is known as Rub1. Of all the ULMs known up to now, Nedd8 has the highest similarity in sequence to ubiquitin (~58% identity). Nedd8 was originally identified from embryonic mouse brain, as one of the ten abundantly expressed Nedd genes [46]. Defects in the Nedd8 pathway do not have any impact in *S. cerevisiae* while in other organisms such as mice, *Drosophila melanogaster*, *A. thaliana*, *S. pombe*, *Caenorhabditis elegans* their defects have a lethal consequence [46]. Nedd8 has a carboxy-terminal tail and a globular ubiquitin-fold domain (UFD). The tail ends with a Gly-Gly sequence that becomes covalently conjugated to targets. The flexible tail adopts different structures upon interaction with NEDDylation and de-NEDDylation enzymes [47]. The globular domain is characterized by four  $\beta$ -sheets interspersed by one  $\alpha$ -helix and two  $3_{10}$ -helices. NEDD8 becomes covalently conjugated to a limited number of cellular proteins in a manner analogous to ubiquitination. The best-studied targets of neddylation are members of the Cullin (Cul) family. Cullins are scaffold proteins for the assembly of multicomponent RING E3 ligases (CRLs) that participate in ubiquitination and proteasomal degradation. But neddylation of other targets has been reported recently showing additional biological functions of NEDD8. The UCH-L3 (which also acts on Ubiquitin) or NEDP1 (DEN1, SENP8) have roles in processing the C-terminus of Nedd8. Covalent modification with Nedd8 required the sequential action of the Nedd8-activating enzyme (NAE), the E2 enzymes Ubc12 (Ube2M) or Ube2F, and a handful of E3 ligases [46].

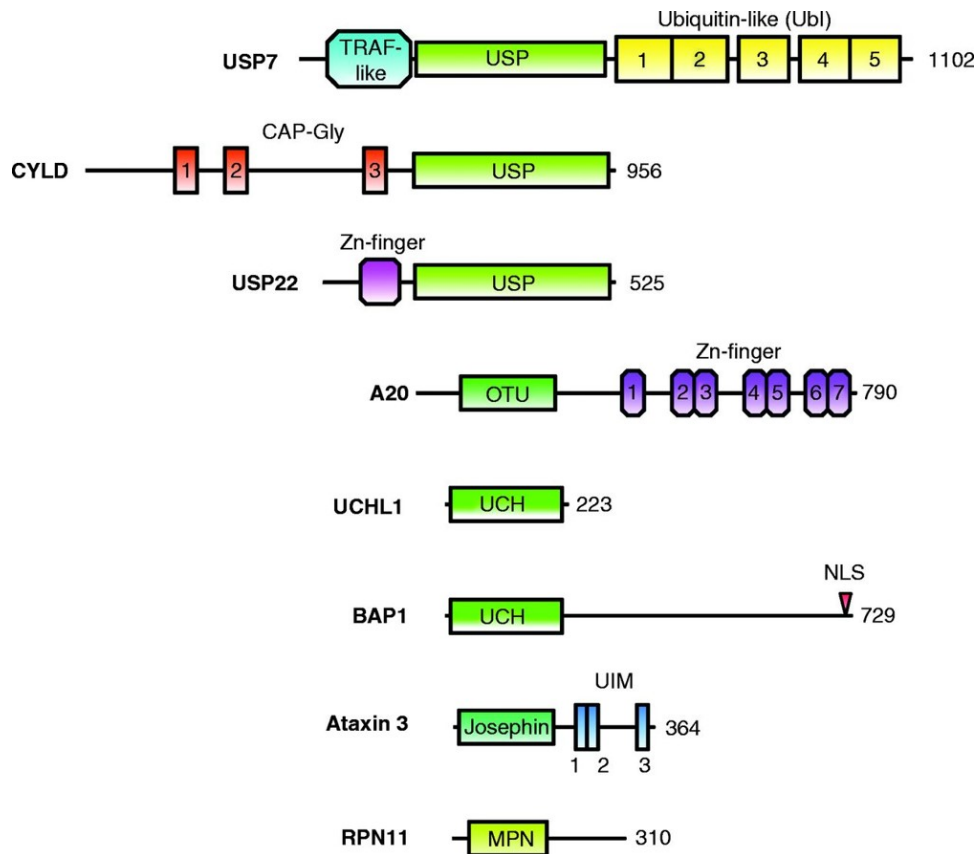
### 1.3 The ubiquitin-like modifier FAT10

FAT10 stands for "human leukocyte antigen F-adjacent transcript 10". It is also referred to as diubiquitin or ubiquitin D (UbD) [69][70]. The protein FAT10 has a molecular mass of ~18 kDa. There is a high degree of similarity between murine and human FAT10 sequences at both the DNA and protein levels. FAT10 was discovered when it was cloned for cDNA hybridization selection while analyzing the human HLA-F locus for novel genes [71]. It is an enigmatic ULM/UBL discovered to be encoded in the human major histocompatibility complex (MHC) class I locus [71]. FAT10 contains two tandem ubiquitin-like (UBL) domains with approximately 30% sequence similarity to ubiquitin. FAT10 with its C-terminal diglycine (Gly-Gly) can form a covalent linkage with lysine (Lys) on its substrates [72]. The ULM FAT10 is conjugated to its substrates by the same mechanism as other ULMs. These modifiers have their own E1 activating enzyme, one or more E2 conjugating enzymes and often different numbers of E3 ligases to get covalently attached to their substrate proteins [73]. It is interesting to learn that both FAT10 and ubiquitin share the same E1 activating enzyme UBA6 (ubiquitin-like modifier activating enzyme 6) and the E2 conjugating enzyme USE1 (UBA6-specific E2 enzyme 1) [74][75][76][77]. Covalent attachment of FAT10 (FAT10ylation) leads to proteasomal degradation of proteins but without the involvement of ubiquitin in this process [69], [78], [79]. Examples of proteins targeted for degradation by FAT10 are the E2 conjugating enzyme USE1, and the ubiquitin-binding scaffold protein p62 (sequestosome-1; SQSTM1) [80][81]. Lists of proteins covalently-conjugated with FAT10 have been published and their number is still increasing. Proteins reported to form covalent conjugates with FAT10 are E1-L2 (UBA6) [76], USE1 [74], huntingtin [82], p53 [83], p62 [81], and covalent conjugation of the ubiquitin activating enzyme UBE1 with FAT10 targets it for proteasomal degradation [84]. FAT10 fused to the N-terminus of HCMV-derived pp65 antigen (HCMV, human cytomegalovirus) accelerated proteasomal degradation of the pp65 antigen enabling improved presentation of the HLA-A2-restricted pp65<sub>498-503</sub> epitope. FAT10-pp65 differed from Ub-modified pp65 in this respect [85]. FAT10 has been reported to non-covalently interact with proteins such as HDAC6 (histone deacetylase-6) [86], the mitotic spindle checkpoint protein MAD2 [87] and the aryl hydrocarbon receptor-interacting protein-like 1 (AIP1) playing an important role during eye development [88]. NUB1L non-covalently interacts with FAT10 with its three C-terminal ubiquitin-associated domains (UBA) and with the 26S proteasome via its N-

terminal ubiquitin-like (UBL) domain. NUB1L accelerates the proteasomal degradation of FAT10 and FAT10-linked proteins [89][90]. FAT10 decorates autophagy-targeted *Salmonella* and contributes to *Salmonella* resistance in mice [91]. FAT10 expression can be synergistically induced by the pro-inflammatory cytokines tumor necrosis factor (TNF)- $\alpha$  and interferon (IFN)- $\gamma$  in any type of cell [92][93] and is suppressed by tumor suppressor protein p53 (TP53) [94]. High basal level expression of FAT10 is found in organs of the immune system [92][87][95]. The highest expression of FAT10 is found in medullary thymic epithelial cells (mTECs) of thymus tissue [96] but constitutive expression is high in dendritic cells [97]. Mature B-lymphocytes and dendritic cells express FAT10 and thus were reported to have a functional role in antigen processing, presentation or the immune response [97].

Furthermore, FAT10 is highly upregulated in certain types of cancer suggesting a role of FAT10 in cancer pathogenesis [98][93]. A role of FAT10 in apoptosis is controversial, as in two reports FAT10 overexpression in cultured cells, is somehow associated with apoptosis [72][95] while in another report FAT10 expressed in cardiac myocytes protected myocytes from apoptosis by suppressing p53 and miR-34 and increased expression of Bcl2, hence FAT10 is a cardioprotective protein [99]. FAT10 has a role in cell cycle control, chromosomal segregation and MHC class-I restricted antigen presentation [100][85][101]. FAT10 has a role in NF- $\kappa$ B activation and subsequent expression of pro-inflammatory genes. Induction with pro-inflammatory cytokines leads to upregulation of various genes including FAT10. This is achieved by NF- $\kappa$ B signaling; NF- $\kappa$ B binds to the FAT10 promoter leading to its upregulation [102]. It is believed that the role of FAT10 is cell type specific. FAT10 knockout (FAT10<sup>-/-</sup>) mice grow normally and in unstressed condition exhibit minimal phenotypic differences suggesting that NF- $\kappa$ B-mediated signaling is intact in many or most cells. An earlier report suggested, HIV-1 strongly up-regulates FAT10 in renal tubular epithelial cells (RTECs). However, FAT10 is not expressed at appreciable levels in CD4<sup>+</sup> T-cells before or after infection with HIV-1 and not all cell types increase expression of FAT10 in response to TNF- $\alpha$  [103].

FAT10 and Htt (Huntingtin) bind directly and this is interesting, because, although ubiquitin seems to prefer cytoplasmic Htt, FAT10 binds to nuclear Htt as well. This suggests that FAT10, in addition to UPS and UHRF-2, may function as a dominant poly-glutamine (pQ) modifier and degradation signal in the nucleus [82].



**Figure 4. Domain architecture of selected DUBs**

In addition, to specialized catalytic domains (indicated as green) DUBs contain other functional domains. Different domains in some of the human DUBs: USP7, CYLD, USP22, A20, UCHL1, BAP1, ataxin 3, and RPN11 are depicted. DUBs generate free ubiquitin from ubiquitin precursor. The image is taken from [13].

High levels of FAT10 protein in cells lead to increased mitotic-nondisjunction (nondisjunction during meiosis can produce gametes with an extra or missing chromosome, or aneuploidy), chromosome instability, and this effect is mediated by an abbreviated mitotic phase and the reduction in the kinetochore localization of MAD2 (mitotic arrest deficient 2) during the pro-metaphase stage of the cell cycle [104]. FAT10 modulates growth and activation of B-cells and dendritic cells interacting with MAD2, where MAD2 is a protein having a role in the cell-cycle check-point for spindle

assembly during anaphase [87]. Disruption of the FAT10-MAD2 interaction significantly reduced FAT10-induced tumor growth *in-vivo*, suggesting a role of FAT10 in tumor progression and that it is critically dependent on its interaction with MAD2 [105]. FAT10 (ubiquitin D; UBD) expression is high in regulatory T cells, and its level decreases upon activation of T cells by anti-CD3/anti-CD28 antibodies and IL-2 [106]. FAT10 deficient mice are prone to spontaneous apoptosis of lymphocytes and are hypersensitive to endotoxin challenge with LPS [107] but the role of FAT10 in apoptosis is controversial. The targeted deletion of the *fat10 (ubd)* gene in mice reduces body fat and slowed down aging [108]. FAT10 is a diabetes susceptibility gene, and, therefore, one example of the interplay of multiple genes and environmental factors in type 1 diabetes susceptibility [109]. High-profile studies reported that FAT10 interacts with approximately more than 300 proteins in cells. This gives a strong impression that the possibilities for future research of FAT10 are exciting.

## 1.4 Deconjugating enzymes (DUBs)

The human genome encodes around ~90 DUBs, some of them with unknown functions [110][45]. DUBs can be broadly divided into two types according to their catalytic cleaving mechanism, the cysteine proteases, and the metalloproteases. The cysteine proteases (papain-type), contain a catalytic diad or triad with a reactive cysteine residue while the metalloproteases (classified as JAMM domain superfamily), use zinc to co-ordinate residues within their active site. Furthermore, cysteine proteases are divided into four subtypes based on the architecture of their catalytic domains; USPs, OTUs, UCHs, and Josephins [111].

The five human DUB classes:

USPs are the largest DUB class, followed by OTUs, JAMMs, UCHs and Josephins comprising 56, 16, 12, 4 and 4 members, respectively. Among these five USPs and six JAMMs are supposed to be inactive DUBs while one OTU is a pseudogene (Hin1L). Perhaps the exact number of active DUBs in the human is not clear, but some have multiple isoforms. As recently reported, MCPIP (monocyte chemotactic protein-induced protein) is an example of the fifth class of cysteine protease DUBs. The human

genome encodes seven MCPIP deconjugating enzymes but whether they possess true DUB activity is still unclear [14]. Discussed below are DUBs based on their abundance in the human genome.

#### 1.4.1 USP Domain DUBs

Ubiquitin-specific proteases (USPs/UBPs) (3.1.2.15) form the largest family of DUBs having two conserved motifs i.e. cysteine and histidine box, in their catalytic domain [43]. To date, ~60 proteases in human are reported, and the size of USPs ranges from ~50 kDa to 300 kDa. In yeast, the USPs are known as ubiquitin-specific-processing proteases (UBPs). As the non-catalytic domains of USPs are highly diverse in their amino acid sequence, they are believed to confer substrate specificity, mediating protein-protein interaction and regulation of catalytic activity. The human genome contains 53 USP genes and the mouse genome contains 54 USP genes encoding DUBs, albeit some might be non-functional [43]. The ubiquitin-binding domains, including the zinc finger ubiquitin-specific protease (ZnF-UBP) domain, the ubiquitin-interacting motif (UIM) and the ubiquitin-associated domain (UBA) are the predicted domains of USPs. Several ubiquitin-like domains (UBL) are also common, but they are not fully confirmed (**Fig. 4**). Additionally DUSP domain, TRAF/MATH domain etc. are also common (reviewed in [112] and [110]).

#### 1.4.2 OTU Domain DUBs

Ovarian tumor proteases (OTUs) are a class of deconjugating enzyme (EC 3.1.2.-) which consists of 24 members in the human genome, making them the second largest family of mammalian DUBs. In *Drosophila melanogaster*, the first OTU gene was identified having a role in the development of ovaries. Mammalian OTUs were discovered later on the basis of OTU-domain homology [113]. A20, DUBA, otubain-1 and Cezanne are well-studied members of the OTU family of DUBs. OTUD5 interacts with p53 in the yeast two-hybrid system, it deubiquitinates p53 and stabilizes it leading to activation of the p53 response [114]. A20 has a unique feature of acting both as a deubiquitinase and as an E3 ligase [115][116].

#### 1.4.3 JAMM or MPN+ Domain DUBs

The JAMM or MPN+ (Jab1/Mpr1 Pad1 N-terminal+/Mov34) are JAMM domain containing metalloproteases (EC 3.1.2.15). Recently, the JAMM/MPN+ family of DUBs was shown to have specificity towards cleavage of K<sup>63</sup>-linked polyubiquitin [117].

AMSH, another K<sup>63</sup>-selective DUB [118], participates in multivesicular body sorting [119]. The Rpn11(Poh1) subunit of 19S (PA700) regulator has deubiquitination function, with Zn<sup>2+</sup> dependent proteolysis [120][121][122]. Poh1 acts on K<sup>48</sup>-linked polyubiquitin chains [121]. Jab1, is another metalloprotease which is a component of the COP9 signalosome, a multi-subunit complex highly similar to the lid subcomplex of the 19S regulator [123]. Jab1 cleaves the ubiquitin-like protein Nedd8 from cullin subunits of the SCF family of E3 ligases [124]. FLJ14981 is also a JAMM/MPN+ protein whose function is unknown, but its sequence suggests that it is also an isopeptidase. Brcc36, metalloprotease specifically cleaves Lys<sup>63</sup>-linked polyubiquitin chains but does not show activity towards Lys<sup>48</sup>-linked polyubiquitin chains. Brcc36 is part of a nuclear complex that contains the BRCA1 protein and is targeted to DNA damage center after irradiation [125][126]. KIAA1915 is a metalloprotease which regulates chromatin structure by removing ubiquitin from histone H2A [127]. CSN5 is another member of the JAMM family [128].

#### 1.4.4 UCH Domain DUBs

Ubiquitin COOH-terminal hydrolases (UCHs) (EC 3.4.19.12) were reported as DUB in the 1980s [129]. The UCHs class contains a small number of structurally related DUBs that have functional roles in cleaving short ubiquitinated peptides and have important roles in recycling free ubiquitin. UCH-L1 (223 amino acids), a member of the UCH family, is an abundant multifunctional neuronal protein [129], associated with the pathogenesis of many diseases, including neurodegenerative disorders, cancer, and diabetes. In addition to its DUB activity, UCH-L1 also associates and co-localizes with monoubiquitin and elongates ubiquitin half-life (i.e. stability) in neurons [130]. UCH-L1 also has dimerization-dependent, ubiquitin ligase activity [131]. UCH-L3 is expressed only in neurons and ovary/testis, but UCH-L3 isoenzymes are expressed in all tissues. The role of UCH-L1 is controversial, as it is unregulated in many tumors and cancer cell lines. UCH-L1 has been described as an oncogene as an important regulator of tumor formation and maturation or as a tumor suppressor [132]. UCHL3 (which also acts on Ub) has a role in the processing of the C-terminus of Nedd8 [46].

#### 1.4.5 Josephin Domain DUBs

The Josephins is the smallest family of DUBs distributed in metazoans, plants, and protozoans. This broad conservation hints at important roles for the Josephin proteins, but their biological functions remain unclear in most cases [133]. Josephins are also

referred to as machado josephin domain proteases (MJDs); (EC 3.4.22.-). All Josephin proteins share a common cysteine protease domain of ~180 amino acids, known as the Josephin domain. Two of the human Josephin proteins, ataxin-3 and the ataxin-3-like protein (ATXN3L), each contains a single Josephin domain at their N-terminus plus a flexible C-terminal domain of comparable length [134][135]. Josephin-1 and Josephin-2, on the other hand, are each composed solely of a single Josephin domain. Ataxin-3 is the best characterized among the Josephin domain-containing proteins. It first attracted attention as a polyglutamine (pQ) repeat protein [136]; in its expanded glutamine repeat variant it causes spinal cerebellar ataxia type 3 (SCA3, also known as Machado-Joseph disease).

Ataxia is a medical condition in which patients initially experience problems with coordination and balance. The *Atn3* gene encodes an enzyme called ataxin-3, which is found in cells throughout the body. Ataxin-3 is classified as DUB, cleaves polyubiquitin chains of four or more Ub subunits. Ataxin-3 preferentially cleaves K<sup>63</sup>-linked chain *in-vitro* [137].

## 1.5 Catalytic mechanism

The ubiquitin module in a di-ubiquitin molecule or the ubiquitin module in a ubiquitin chain, when attached through its terminal glycine residue, is called distal ubiquitin, while the ubiquitin module attached to it is designated the proximal ubiquitin. DUBs deconjugate the distal ubiquitin from the proximal ubiquitin or directly from a substrate protein. Ubiquitin chains bury a hydrophobic patch, centered around isoleucine 44. During isopeptide bond cleavage, DUBs bind to the distal ubiquitin around the hydrophobic patch, and make little contact with the proximal ubiquitin [14].

Cysteine proteases have a conserved amino acid catalytic triad, like the classical cysteine protease papain. The catalytic triad consists of histidine (H) lowering the pKa of the catalytic cysteine (C) thus facilitating a nucleophilic attack while a third residue (asparagine; N or aspartic acid; D) is required for alignment and polarization of this H residue. During catalysis, an acyl-intermediate is formed by covalent linkage between cysteine and the carboxyl group, which will be generated after cleavage. While all cysteine protease DUBs have diverse catalytic domain structure, once bound to the ubiquitin COOH terminus, the catalytic residue superposes with little deviation.

In metalloproteases, invariant H, D, and serine (S) residues coordinate the catalytic zinc. The catalytic mechanism is same as in metalloproteases like thermolysin. The catalytic zinc ion is coordinated by two hydrogens, an Asp, and an H<sub>2</sub>O molecule. An adjacent glutamic acid (E) accepts a H<sup>+</sup> from the H<sub>2</sub>O molecule leaving an OH<sup>-</sup> ion which attacks the substrate bound at the carbonyl carbon. The transient tetrahedral intermediate, which is formed, collapses resulting in scission, with the OH group from water replacing the leaving group at the COOH terminus of the distal ubiquitin.

## 1.6 DUBomics

Apart from the catalytic domain, DUBs also contain additional domains and short structural motifs, which may regulate their activity and support protein-protein interactions. DUBs are arranged according to the sequence homology of their catalytic domains. Some human DUBs also consist of characteristic distinct domains [45][110]. A total of 16 human DUBs common also in other species (zebrafish, drosophila, *C. elegans*, yeast) are routinely used in experimental biology and are listed in **Table-2**. The copy number of individual DUBs varies in magnitude; based on quantitative proteomic analyses the most highly expressed DUBs (>300,000 copies/cell) include components of the 26S proteasome and the COP9 signalosome, UCHL1 and UCHL3, which suppress the formation of ubiquitin adducts, and OTUB1, being the most abundant active DUB in the Swiss 3T3 model cell line. For simplification only those DUBs which are localized to specific cytoplasmic organelles, the plasma membrane or nucleus are adopted from reference [138] and are listed in **Table-3**. DUBs range in size from the 358 amino acid long USP41 to USP51 containing 3546 amino acids.

## 1.7 Deubiquitinase specificity

The five DUB families share no sequence similarity and have distinct structural folds. Most DUBs hydrolyze ubiquitin chains into monomers, usually by binding to the two adjacent ubiquitin moieties, placing the isopeptide bond to be cleaved across their active site. In this mechanism of hydrolysis, the 'distal' ubiquitin monomer presents its

C-terminal Gly to the catalytic center, while the ‘proximal’ ubiquitin monomer is bound through its modified lysine. Most of the DUBs studied so far have shown binding to ubiquitin through a significant distal binding site, while the proximal ubiquitin binding site is less common. Like E3 ligases, DUBs also have some degree of substrate specificity, which is linked to three characteristics. First, DUBs have protein-protein interaction domains in addition to the catalytic domain, helping them to bind specific target proteins. Second, DUBs are ubiquitin-linkage specific, showing a preference for specific ubiquitin branches, such as K<sup>48</sup>- or K<sup>63</sup>-linked chains [43]. Third, the subcellular localization and the mode of expression of DUBs vary a lot which contributes to the diversity in their *in-vivo* function.

**Table 2. Common human DUBs used in experimental research** (adopted from reference [138])

Family	DUBs
USP	USP5, USP7, USP8, USP14, USP39, USP46 and USP52
UCH	UCLH3, UCLH5
OTU family	OTUD6B
Proteasome	Poh1, PSMD7
Others	COPS5, PRPF8, EIF3F and EIF3H

**Table 3. Subcellular localization of some human DUBs** (adopted from reference [110])

Localization	DUBs
Plasma membrane	USP6
ER	USP19, USP33
Nucleus	USP1, USP3, USP7, USP11, USP22, USP26, USP29, USP42, USP44, USP49, USPL1, BAP1, MYSM1
Microtubule	USP21, USP33, USP21, CYLD1
Golgi	USP33v3, USP32
Nucleolus	USP36, USP39
Multivesicular body	USP8, USP2a, AMSA, AMSH-LP

### Cleavage by DUBs (Ub versus ULMs)

FAT10 and ISG15 resemble di-ubiquitin. The shape and size of SUMO, Nedd8, and Atg12 are approximately the same as ubiquitin. Although, many ULMs (i.e. SUMO,

Nedd8, ISG15, FAT10, and ATG12) modify proteins using a similar mechanism compared to ubiquitin [18], DUBs can still distinguish between ubiquitin and ULMs. The C-terminal four residues preceding the Gly-Gly motif (<sup>71</sup>LRLRGG<sup>76</sup> in Ub) is one of the selectivity providers for DUBs recognizing Ub or a ULM. Nedd8 and ISG15 share sequence similarity with this residue but FAT10, SUMO, and Atg12 do not. Thus, it's not surprising, that Nedd and ISG15 can be hydrolyzed by the same cross-reactive DUBs [139][111][110].

### **Cleavage of Isopeptide versus Peptide bond**

Ubiquitin chains are linked to the epsilon-amino group of lysine residues via an isopeptide bond, but ubiquitin chains when linked to an alpha-amino group of the N-terminus of ubiquitin form linear ubiquitin chains [140]. Linear ubiquitin chains have a non-proteolytic role in NF-κB signaling [141] and linear chains also occur when linear polygenes for ubiquitin are translated in cells, which are then cleaved by DUBs into monoubiquitin [111].

### **Cleavage of Specific Linkages (K<sup>63</sup>, K<sup>48</sup>, linear) in Ubiquitin Chains**

Different DUBs cleave different linkages of Ub, this is known as DUB linkage specificity and we know that different ubiquitin linkages have different cellular roles. Therefore, to appreciate the role of DUBs in disease, it is important to know their linkage specificity. Knowing which DUBs cleaves which linkage can help to delineate which pathways are regulated by them. In a different approach, if the pathway regulated by deubiquitinases is known, the DUBs linkage specificity will tell us, what chain type was formed with the help of E2 and E3 enzymes. Some DUBs like JAMMs and OTUs can cleave only certain chain type, but USPs possess a broader cleavage specificity [14]. The USP family members do not exhibit linkage-specificity but rather are substrate specific [142][143]. The OTU class of DUBs shows specificity towards ubiquitin chain linkage and are responsible for editing and recycling of specifically linked chain [144]. **TRABID**, a human ovarian tumor (OTU) domain deubiquitinase specifically hydrolyzes both Lys<sup>29</sup> and Lys<sup>33</sup>-linked di-ubiquitin [145].

### **Cleavage within Ubiquitin Chain (Endo versus Exo)**

DUBs are 'endo' or 'exo' specific meaning that they prefer to cleave in the middle or from the end of ubiquitin chains, respectively. Some DUBs only remove ubiquitin from small peptides.

### **Ubiquitin Chain Editing**

Ubiquitin chain editing is the switch from one chain type (e.g., a 'signaling' Lys<sup>63</sup>-linked chain) to another type (e.g., a 'degradation mediating' Lys<sup>48</sup>-linked chain). Ubiquitination is a two-step process, consisting independent of chain initiation and chain elongation. Additionally, there is a clear-cut difference in the surrounding of the first ubiquitin on a substrate lysine and the one of ubiquitin for elongating the chain. Often, DUBs may only target ubiquitin-ubiquitin linkages, and their action might not remove proximal ubiquitin, leaving the substrate mono-ubiquitinated. Substrates not fully deubiquitinated end up as mono-ubiquitinated end product which would provide a platform for ubiquitin chain editing [111].

### **Sequence specific Cleavage**

DUBs that act on a monoubiquitinated target are formally not reported until now. For example, USP7 acts and removes monoubiquitin from the FOXO4 protein [146]. But DUBs are predicted to either specifically recognize the sequence ambience in the target protein and hence hydrolyze monoubiquitin, or entire ubiquitin chains at the same time (en bloc). A great level of specificity could be achieved by this. However, USP DUBs are nonspecific and can completely deubiquitinate substrates indicating they probably accommodate a wide range of sequences in their proximal binding site.

### **Substrate specificity, recognition**

For restricting their function within a particular cellular pathway, DUBs must be selective for their substrate proteins. So DUBs have additional protein-interaction domains to facilitate direct substrate interaction, moreover, there are indirect means, e.g., by localizing DUBs to specific places in the cell which may also aid in specificity. Different DUBs have been found to target the same substrate, providing an example of multifacet regulation. For example, the deconjugating enzyme USP7 deubiquitinates Mdm2 (an E3 ubiquitin ligase for p53) which leads to destabilization of the tumor suppressor protein p53 [147]. USP7 also deubiquitinate the E3 target (p53). In mammalian cells, the loss of USP5 (Isopeptidase T) causes p53 stabilization, whereas it has mild or no effect on the Mdm2 stability [148]. The UCH act on the C-terminus of ubiquitin and remove disordered sequence, whereas USP5 with its zinc-finger USP-domain (Zn-UBP) recognize the C-terminus of ubiquitin in unanchored ubiquitin chains and recycle free ubiquitin [45].

## 1.8. Regulation of DUB activity

The impact of DUBs on numerous biological and pathological processes has now been widely accepted. Often, the function of DUBs is tightly regulated by many ways, for example; substrate-induced conformational changes, binding to adapter proteins, proteolytic cleavage, incorporation into large complexes, co-factor association, sub-cellular localization and through PTMs. PTMs such as monoubiquitination, SUMOylation, acetylation, phosphorylation, and O-glycosylation can be characteristic and putative regulatory factors controlling DUB function [149]. Within the cell DUBs are expressed as catalytically active enzymes, rather than as inactive precursors. However, some DUBs achieve an active conformation when they bind to ubiquitin, this regulates their uncontrolled proteolytic activity within the cell. OTUB1, UCH-L1, UCH-L3, USP7, USP14, and yeast YUH1 are DUBs binding to ubiquitin, leading to their active conformation with induced catalytic activity. CYLD removes Lys<sup>63</sup>-linked ubiquitin chain from TRAF2. CYLD is phosphorylated in response to NF- $\kappa$ B inducing factors such as LPS or TNF- $\alpha$ , whereas phosphorylation at Ser<sup>418</sup> in its TRAF-2-binding site decreases CYLD catalytic activity leading to down-regulation of the NF- $\kappa$ B pathway [150][149]. Phosphorylation at serine residue is important for USP1, as dephosphorylation leads to a loss of its activity [151]. Many DUBs are found in pairs with E3 ligases and phosphatases. In some cases, these DUBs like USP7 have been shown to deubiquitinate both the E3 (Mdm2) and the E3 targets (p53). DUBs can bind to large complexes to attain an active state, as in the case of USP14 where binding to the proteasome improves its catalytic activity [152]. In isolation, the USP14 active site is blocked by two surface loops in its catalytic domain. With its UBL domain, USP14 binds to the proteasome. This proteasomal binding causes a conformational change in USP14 leading to loop displacement and allosteric activation of USP14 [153]. The Poh1/Rpn11/Psmd14 subunit in 19S the regulatory particle in proteasome contains a highly conserved JAMM metallo-isopeptidase motif having a role in ubiquitin deconjugation [120]. Protein-protein interaction like in the case of UCH-37 binding to the chromatin-remodeling complex can inhibit DUB activity [154]. Mostly DUBs are regulated through reversible-oxidation of the catalytic residue [155][156] while the activity of OTUD5 is regulated by its phosphorylation status [157].

## 1.9 DUBs as a component of protein degradation machinery

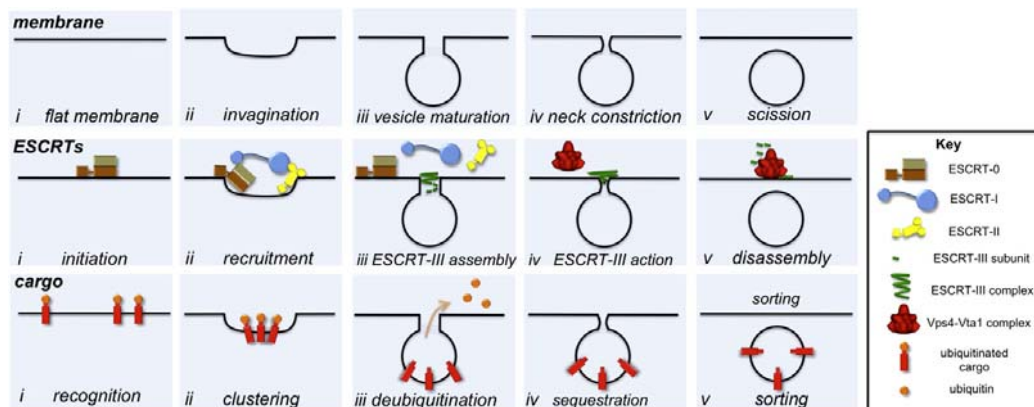
DUBs are sometimes localized to specific places in the cell which aid their selectivity and specificity to their substrate. DUBs can be organelle-specific or cellular pathways specific.

### 1.9.1 Proteasome-associated DUBs

For restricting their function within a particular cellular pathway, DUBs must be selective for their substrate proteins. In mammalian cells most intracellular cytosolic protein is degraded by the 26S proteasome. The 26S proteasome is a ~2400 kDa, 60-subunit complex which selectively degrades proteins which are covalently tagged by ubiquitin chains [6]. The 26S proteasome consists of a core 20S particle, within which proteins are digested, capped by one or two 19S regulator complexes (RP) that bind, unfold and translocate ubiquitinated substrates into the 20S catalytic chamber [6]. The RP consist of two ubiquitin receptors (Rpn13, Rpn10) and three distinct DUBs (Poh1, UCH37/UCHL5, and USP14/Ubp6). The metalloprotease, Poh1 (Psm14/Rpn11) is the constitutive component and needed for RP assembly while the other two are reversibly associated [110].

### 1.9.2 ESCRT DUBs

The ESCRT (endosomal sorting complex required for transport) pathway facilitates the trafficking of ubiquitinated proteins from endosome to lysosome via multivesicular bodies (MVBs). This is a major degradative pathway for most plasma membrane channels, pumps, and receptors. ESCRT machinery is made up of five distinct cytosolic protein complexes (ESCRTs -0, -I, -II, and -III, and vps4) which are believed to act in a sequential fashion. MVBs are formed when ubiquitin-tagged proteins enter the endosome via formation of vesicles. MVBs then deliver cargo to lysosome by direct fusion. MVB formation is important to destroy misfolded and damaged proteins in cells. Without the ESCRT pathway, these proteins can accumulate leading to neurodegenerative diseases [158][159][160]. Two DUBs, AMSH and USP8 (UBPY), interacts with different components of ESCRT-0 and ESCRT-III (**Fig. 5**). Notably, both 26S proteasome and ESCRT complexes carry a JAMM family of DUB (Poh1 or AMSH, respectively) specific for Lys<sup>63</sup>-linked chain [110].



**Figure 5. Deubiquitination in ESCRT-Mediated Vesicle Budding**

**top;** view indicated five distinct stages in intraluminal vesicle budding. A flat membrane results in scission leading to complete vesicle budding. **middle;** the view is from the ESCRT protein perspective, in which ESCRT-0 initiates the pathway by engaging ubiquitinated cargo (i), until it is sequestered and sorted by ESCRT-III (iv and v). ESCRT-I and ESCRT-II complexes bind cargo and each other to create an ESCRT-cargo-enriched zone (ii). ESCRT-II nucleates ESCRT-III assembly (iii), which drives vesicle budding (iv) and is disassembled by the Vps4-Vta1 complex (v). **bottom;** view from the cargo perspective, in which ubiquitinated cargo (protein) is first recognized by the ESCRT-0 ubiquitin-binding regions, clustered by the ESCRT-0, -I, and -II complexes, recruitment of DUB by ESCRT-III, cargo packing and sorting. The image is taken from [158].

### 1.9.3 Cdc48/p97/VCP associated DUB

Under the stressed condition, damaged proteins are not immediately degraded but are stored in aggresome. After a cell has finished essential tasks aggresomes are resolubilized and eliminated when the condition is more favorable. Both processes, aggresome formation, and aggresome elimination, are Cdc48 dependent [161]. YOD1 is an ovarian tumor (otubain) family of DUB, with yet unknown function in mammalian cells. The N-terminal UBX domain in YOD1 interacts with p97 [162]. At the surface of a damaged lysosome p62, LC3, K<sup>48</sup>-linked and K<sup>63</sup>-linked chains exist. K<sup>48</sup>-linked ubiquitin chains in damaged lysosome possess a role in degradation, whereas YOD1 interacts with p97 and cleaves K<sup>48</sup>-linked ubiquitin chains. The protein p62 binds to K<sup>63</sup>-linked chains.

## 1.10 DUBs as a player in cellular processes

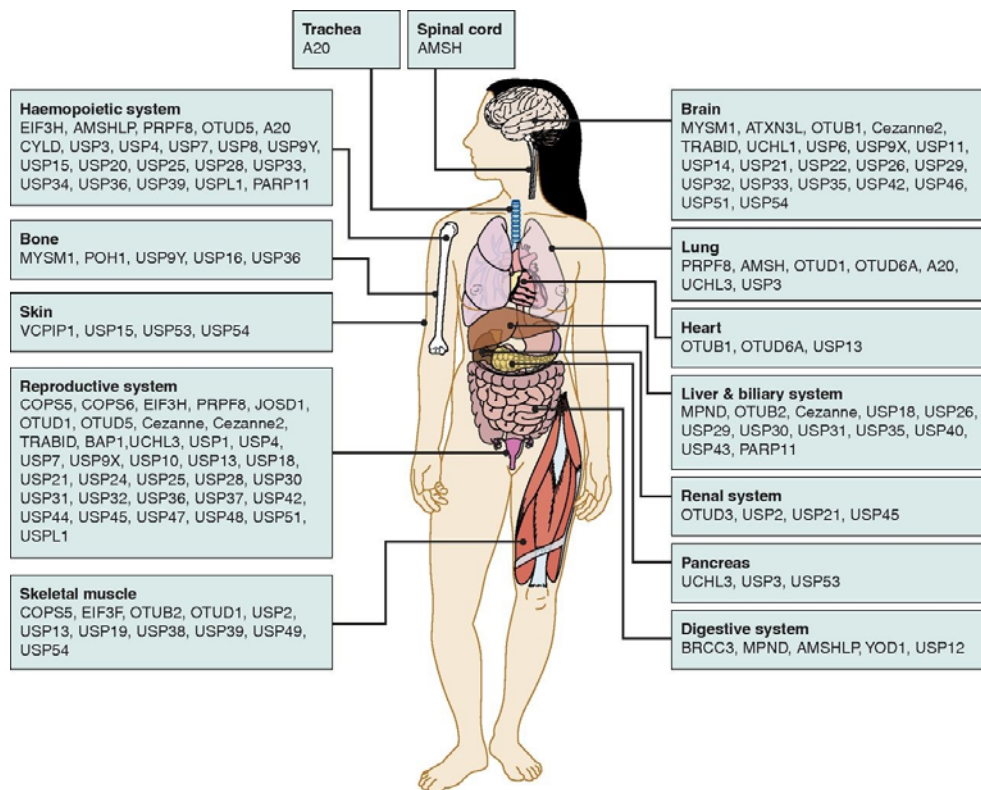
A number of DUBs regulate the process of programmed cell death (apoptosis). USP7, USP9x, USP28, and CYLD are among 14 DUBs mentioned playing a role in promoting apoptosis, whereas only three of them were regarded as negative regulators of apoptosis (A20, USP18, and UCHL3) [163]. Hepatocyte growth factor/ scatter factor (HGF/SC) promotes the proliferation, differentiation, motility, and invasion of epithelial cells by binding to its cell surface receptor, the Met tyrosine kinase. The scattering response of epithelial cell is a complex process which includes loss of cell-cell adhesion and cell mobility induction. Using siRNA library screening protocols in A549 cells (lung cancer cell line), 12 DUBs required for HGF-dependent scattering were identified (USP3, USP33, USP30, USP47, and ATXN3L) [164][165].

## 1.11 Tissue and subcellular distribution of DUBs

DUBs are organelle-specific and are distributed in various organelles of a cell with the evolution. Some human DUBs, according to their subcellular localization reported till now are enlisted in **Table-3**. Depending upon transcription profile, some DUBs are comparatively overexpressed in various organs. An overview of tissue hot spots for some of the human DUBs is depicted in **Fig. 6**.

## 1.12 Immunoregulatory DUBs

Not much is known about the role of DUBs in the immune system, but recently some immunoregulatory DUBs have been reported [166]. As phosphorylation is a reversible process, ubiquitination can also be reversed by DUBs. Deubiquitination by DUBs, CYLD and A20 is an important regulatory process for both innate and adaptive immune responses. Like ubiquitin, NF- $\kappa$ B also takes central stage in biology as both of them control processes, ranging from birth and death of a cell, or neuronal behavior of an organism [19][116]. Many stimuli (i.e. stress) converge on NF- $\kappa$ B activation, which in turn umpires diverse transcriptional programs [167].



**Figure 6. DUB expression in human tissue**

Tissue show the highest expression of some DUBs based on transcript data collected from the EMBL gene atlas. The image is taken from [110].

Transcription factor NF- $\kappa$ B binds at the  $\kappa$ B-sites in promoters and enhancers of a number of genes and induces or repress their transcription. There are five members of NF- $\kappa$ B dimers in mammalian cells. RelA (p65), RelB, c-Rel; the precursor proteins NF- $\kappa$ B1 (p105) and NF- $\kappa$ B2 (p100), which are processed into p50 and p52. NF- $\kappa$ B activation pathways can be of two types: broadly speaking the canonical pathway which is predominant in nature and the non-canonical pathway. Stimulation of TNF receptors, IL-1 receptors, Toll-like receptors by their ligands, activated TRAF proteins and subsequently TAK1, which phosphorylates and activates IKK $\beta$ . The two types basically differ in whether activation involves I $\kappa$ B-degradation or p100 processing. In the canonical pathway, stimulation of cells with TNF $\alpha$  or IL-1 $\beta$  activates the IKK-complex. The IKK-complex is composed of a regulatory subunit-NEMO and two catalytic kinase subunits, IKK $\alpha$  & IKK $\beta$ . IKK $\beta$  phosphorylates another protein called inhibitor of NF- $\kappa$ B (I $\kappa$ B). The signal induced phosphorylation allows I $\kappa$ B to be

polyubiquitinated followed by degradation by the proteasome, which releases NF- $\kappa$ B. In the non-canonical pathway which basically takes place in B-cells in response to a stimulus. Stimulus leads to the activation of the protein kinase NIK, which in turn activates IKK $\alpha$ . IKK $\alpha$  mediates phosphorylation of p100, phosphorylation at the C-terminal serine leads to selective degradation of the I $\kappa$ B like domain by the 26S relieving the mature p52 subunit. Mature p52 with REL-B translocate to the nucleus to control gene expression [19][167].

Lys<sup>63</sup>-linked polyubiquitin chains usually serve as docking sites for other proteins [166]. The Lys<sup>63</sup>-linked ubiquitin chain attached to TRAF2 is removed by a DUB of the USP family called, CYLD. TRAF2 deubiquitination cause inhibition of IKK-complex (NEMO-IKK $\alpha$ -IKK $\beta$ ), stabilizing the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  and leads to retention of the NF- $\kappa$ B heterodimer p65/p50 in the cytoplasm. Thus, mutation of the *CYLD* gene is believed to result in hyper-activation of NF- $\kappa$ B leading to tumor cell survival and cylindroma formation [168]. As, CYLD targets multiple signaling molecules such as TRAF (TNF receptor-associated factor), IKK (inhibitor of NF $\kappa$ B (I $\kappa$ B) kinase), regulatory subunit NEMO, SRC protein tyrosine kinase LCK, NF $\kappa$ B-co-activator protein BCL-3, TAK1 (TGF $\beta$  activated kinase 1), receptor interacting proteins 1 (RIP1) and transient receptor potential channel A1 (TRPA1). *Cyld*-knockdown mice deficient in CYLD promoted NF- $\kappa$ B activation by increased ubiquitination and nuclear translocation of BCL-3. CYLD regulates diverse biological functions including immune cell development, activation, and inflammation, cell survival, migration, proliferation and tumorigenesis, mitotic assembly, calcium-channel function, spermatogenesis, host defense against infection etc. It is recently reported CYLD can also cleave K<sup>48</sup>-linked ubiquitin chain [169].

A20 is an OTU class of deconjugating enzyme having both a DUB and an E3 ligase function [115]. The amino-terminal region of A20 has DUB activity and catalyzes deubiquitination of many factors in NF- $\kappa$ B signaling i.e. TRAF6, RIP1, RIP2 and NEMO (IKK $\gamma$ ). A20 catalyze both Lys<sup>48</sup> and Lys<sup>63</sup>-linked chain removal from RIP1. A20 interacts with its target via adapter proteins. The adapter proteins AB1N1 and TAX1BP1 have ubiquitin-binding domains, which helps their interaction with ubiquitinated TRAF6 and RIP1. The dual DUB and ligase activity in A20 allow it to deconjugate Lys<sup>63</sup>-chains (via its OTU domain) and replace them with Lys<sup>48</sup>-chain (via its zinc-finger ubiquitin-binding domain); thus, A20 can edit a substrates ubiquitination status [14].

Cezanne (cellular zinc finger anti-NF- $\kappa$ B), is another deconjugating enzyme of the OTU family having a role in the immune response. Cezanne negatively regulates the activation of NF- $\kappa$ B in the TNF signaling pathway. In stimulation of TNFR signaling, the expression of Cezanne is induced and is recruited to the TNFR, where it is believed to deubiquitinate RIP1 and thus negatively regulates the activity of IKK. As a feedback control of NF- $\kappa$ B activation, both A20 and Cezanne have the same role.

DUBA (deubiquitinating enzyme A), is another DUB of the OTU-family which cleaves K<sup>63</sup>-linked ubiquitin chains. Unlike A20 and Cezanne, DUBA (OTUD5) do not play a role in negative regulation of NF- $\kappa$ B but it regulates IFN expression by regulating a transcription factor that controls IFN expression i.e. transcription factors IRF3 and IRF7. Otubain-1 is a member of the OTU-family of DUBs, whose alternatively spliced isoform ARF-1, has a C-terminal domain but lacks the catalytic domain of Otubain-1. Otubain-1 was found to be interacting with an E3 ligase, GRAIL (gene related to anergy in lymphocytes) in a yeast two-hybrid screen. Otubain-1 instead of inhibiting promotes K<sup>48</sup>-linked ubiquitination and proteolysis of GRAIL.

USP15 regulates NF- $\kappa$ B activation by negatively regulating K<sup>48</sup>-linked ubiquitination of I $\kappa$ B $\alpha$ , where normally I $\kappa$ B $\alpha$  proteolysis leads to activation of NF- $\kappa$ B and its translocation to the nucleus. USP15 is a negative regulator of T cells. Mdm2 ubiquitinates NFATc2, which leads to IL-2 and IFN- $\gamma$  production; Mdm2 is itself stabilized by USP7. Knockdown to USP7 leads to more Mdm2 ubiquitination, resulting in less Mdm2 thus less NFATc2 ubiquitination and hyperproduction of IL-2 and IFN- $\gamma$  [170].

### 1.13 Cytokine-inducible DUBs

Cytokines such as IL-2, IL-3, IL-4, IL-5, and GM-CSF induces some DUBs which are important in growth and survival of lymphocytes. Among them, DUB1 (USP36), DUB1A, DUB2, and DUB2A are mouse, while DUB3 and USP17 are human deconjugating enzymes. DUB2 negatively regulates the ubiquitination of cytokine-receptor-gamma-chain ( $\gamma_c$ ) by the E3 ligase CBL. Thus, stabilization of  $\gamma_c$  leads to sustained IL-2 production which induces activation of JAK-STAT signaling leading to lymphocyte survival [116].

## 1.14 DUBs in human health and disease

Somatic or germline defects in genes responsible for coding deubiquitinases may lead to human disease. Selected DUBs are discussed below; the selection was based on the number of publications, number of citations in PubMed, and disease relevance. A20, ATXN3, BAP1, CYLD, USP7, and UCHL1 are cancer-related DUBs. The SENP1, USP11, DUB3, VCIP135, and USP7 were found to be co-immunoprecipitated with FAT10 [81]. USP30 regulates mitophagy [171]. Mutations in genes encoding DUBs are associated with various diseases like cancer and neurodegenerative disease [172][173]. Mdm2 stabilization by USP7 leads to p53 destabilization [147]. Additionally, the loss of USP5 in mammalian cells causes p53 stabilization by ubiquitin tree formation [148]. USP7 is associated with prostate cancer and solid malignancies [174]. USP7 deubiquitylates Hdm2 that is an E3 ligase for tumor suppressor p53 [174][175], thus preventing Hdm2 degradation by the 26S proteasome. Hdm2 stabilization leads to more p53 ubiquitination and degradation resulting in less cellular p53 content; this, in turn, will protect damaged cells from apoptotic death.

### 1.14.1 USP7

The ubiquitin-specific protease 7 (USP7) is a deconjugating enzyme from the ubiquitin-specific protease family. Firstly, USP7 was identified as a protein that binds to ICP0, a herpes simplex virus protein, and hence was named herpesvirus-associated USP (HAUSP) [176]. USP7 consist of five C-terminal ubiquitin-like domains, a catalytic domain, and an N-terminal TRAF-like domain. Initially, it was thought that p53 is the primary substrate of USP7 [177]. Later, the E3 ligase Mdm2 was shown to be stabilized by USP7 leading to p53 destabilization [147]. Some USP7 interacting proteins bind to the USP7 C-terminal domain including the ICP0 protein of HSV-1, GMP synthase and UHRF1 [178][179][180]. Many interacting partners binding to USP7 N-terminal domain are p53, Hdm2, Hdmx, Ube2E1, MCM-BP, EBV protein (EBNA1) and Kaposi's sarcoma-associated herpes virus protein vIRF4 [181][182][183][184]. USP7 deubiquitinates the monoubiquitinated H2B [185]. The transcription factor FOXO4 is also deubiquitinated by USP7 [146]. Functional Tregs require a stable Foxp3 transcription factor and USP7 deubiquitinates and stabilize Foxp3 [186].

### 1.14.2 DUB3

DUB3 is also known as USP17, having 12 genes in mammals (EC 3.1.2.15)[111]. The ubiquitin hydrolase DUB3 removes ubiquitin chains from Cdc25A (a phosphatase) and stabilizes it. Cdc25A activates cyclin-dependent kinases (Cdks) to promote cell cycle progress and, in addition, has oncogenic potential. The Cdc25A protein is tightly regulated, but human cancers overexpress Cdc25A [187]. DUB3 is a member of the USP subfamily of deubiquitinating enzymes. The DUB3 expression is highly down-regulated during neural conversion i.e. differentiation of embryonic stem cells to neural stem cells, which destabilizes Cdc25A. The Esrrb transcription factor regulates the Dub3 gene [188]. In DDR (DNA damage response) DUB3 phosphorylation and subsequent ubiquitination of H2AX is important, which recruit DNA repair proteins. DUB3 interacts and deubiquitinates H2AX, suggesting its role in DDR [189].

### 1.14.3 USP11

The deubiquitinase USP11 interacts with and stabilizes p53 by deconjugating it. Down regulation of USP11 leads to low p53 induction in response to DNA damage stress; suggesting a role of USP11 in regulating p53, which is needed for p53 activation in the response to DNA damage [190]. Downstream signaling by TGF $\beta$  receptors is important in regulating cell proliferation, differentiation, migration, and apoptosis. Signaling by these receptors occurs through phosphorylation and nuclear transport of SMAD2/ SMAD3. SMAD7 can recruit an E3 ligase which ubiquitinates and leads to proteasomal degradation of TGF $\beta$  receptors, thus, SMAD acts as negative regulator of the TGF $\beta$  pathway. USP11 interacts with SMAD7 and enhances TGF $\beta$  signaling by overriding the negative effect of SMAD7 [191].

### 1.14.4 USP6, and USP16

The deubiquitinating enzyme USP6 is associated with pediatric bone cancer. Earlier USP6 was known as Tre17, or Tre2. USP6 has a role in developmental regulation, perhaps through regulating BMP (bone morphogenic protein) and NF- $\kappa$ B signaling. USP16 is associated with downs syndrome. USP16 is also called Ubp-M. Recently down-syndrome phenotypes were assigned partly to elevated USP16, a DUB located on chromosome 21.

### 1.14.5 CYLD

CYLD belongs to the USP class of cysteine protease. CYLD is a tumor suppressor and was found to be mutated in an inherited cancer syndrome called familial cylindromatosis [192]. Later, CYLD was found to be a DUB [193] acting on K<sup>63</sup>-linked chains. It is the first USP family member found to regulate thymocyte development. *Cyld*-knockout mice produce early-stage thymocytes but have a decreased number of SP (single positive) thymocytes and peripheral T cells [194]. CYLD together with A20 (an OTU class of DUB) regulate the NF-κB pathway. The cylindromatosis tumor-suppressor gene (CYLD) was discovered in 2000 by the analysis of 21 families having autosomal dominant familial cylindromatosis. Cylindromatosis is also known as Brook-Spiegler syndrome. CYLD mutations occur at the C-terminal half of the protein and inactivate the USP domain either through premature truncation or missense mutation. The downregulation of CYLD increases breast cancer metastasis formation through NF-κB activation [195]. CYLD deconjugates NEMO and TRAF2 which are critical factors, thus negatively regulating (down-regulate) the NF-κB signaling pathway [196]. CYLD, like A20, cleaves K<sup>63</sup>-linked ubiquitin chain [197].

### 1.14.6 VCIP135

VCIP135 stands for valosin-containing protein p97/p47 complex interacting protein, p135 (VCIP135 or VCPIP) and belongs to the OTU family of DUBs. During mitotic golgi dis-assembly monoubiquitination acts as a regulatory signal and is required in subsequent golgi re-assembly, too. During post, mitotic golgi re-assembly, the adapter protein p47, and the AAA-ATPase p97 are proteins involved in membrane fusion. The p97/p47 complex binds to monoubiquitin through the UBA domain of p47 and this interaction is required for p97-mediated golgi membrane fusion. VCIP135 plays a role while interacting with p97/p47 [198][199].

### 1.14.7 A20 and BAP1

A20 and BAP1 belong to the class of DUBs associated with cancer predisposition and myelodysplasia. BAP1 is a BRCA1 (breast cancer early-onset 1) associated protein, which is encoded by the *BAP1* gene in the human. It is the largest member of the UCH family, discovered in 1998 just 5 years after the UCH class was identified. BAP1 contains an N-terminal UCH domain followed by a large unstructured region containing various interaction domains, a UCH-37-like-domain and a C-terminal nuclear

localization signal. A20 is associated with autoimmunity and lymphomas. It is notable from previous studies that A20 negatively regulates NF- $\kappa$ B signaling and dampens the immune response. Three independent research groups have stated that the anti-inflammatory role of A20 is contributed by its OTU-dependent DUB activity.

#### **1.14.8 ATXN3**

Ataxin-3 is classified as a DUB of the Josephin family, which cleaves polyubiquitin chains of four or more Ub subunits. Ataxin-3 preferentially cleaves K<sup>63</sup>-linked chain *in vitro* [137] and longer Ub-chains, but can remove monoubiquitin from certain proteins. ATXN3 is associated with different ubiquitin ligases such as Parkin (an E3 ligase at the mitochondrial surface) and probably functions to edit ubiquitin chains formed by E3s on other proteins. Mutations in the gene (*ATXN3*) encoding ataxin-3 lead to SCA3 (spinocerebellar ataxia type 3), also known as Machado-Joseph disease.

#### **1.15 SENPs**

The covalent conjugation of SUMO proteins to target proteins by the sequential action of E1/E2/E3 enzymes is called SUMOylation. SUMOylation can be reversed by SUMO-specific deconjugating enzymes called Ulp in yeast and SUMO-specific proteases (SENPs) in mammals [64]. *Xenopus laevis* has only one member of SENP1/ SENP2 subfamily called xSENP1 [200], which is important for mitosis [201]. Yeast has two Ulp/SENPs, Ulp1p, and Ulp2p, and is essential for mitosis as the *ulp1 $\Delta$*  strain shows mitotic arrest [65]. In mammals four Ulp1p-like Ulp/SENPs exist; SENP1, SENP2, SENP3 and SENP5 [64]; SENP1 & SENP2 are similar to each other [202] and are important for mitosis [203].

#### **1.16 DUBs and cancer**

DUBs were proposed to act both as a tumor suppressor and as tumor promotor. DUBs can have multiple substrates thus substrate nature may distinguish their oncogenic vs suppressive roles [13]. USP7, USP2a, USP20, and USP33 are oncogenic products while CYLD is a tumor suppressor [204]. DUBs that are mutated in human cancers have been characterized as oncogenes and tumor suppressors [173]. Germline

mutations in the *Cyld* gene was found in families with familial cylindromatosis and sporadic cylindromas. The gene encoding USP6 has recently been found to be causative in most aneurysmal bone cysts, but the functional mechanism by which increased USP6 transcription leads to the development of bone tumors is not well understood. USP7 stabilizes transcription factor Foxp3 and a deficiency of USP7 leads to less Foxp3, further leading to less Foxp3 positive Tregs resulting in an autoimmune disease like colitis [186]. Various SNPs in the USP7 gene were significantly correlated with several autoimmune diseases like insulin-dependent diabetes mellitus, Crohn's disease, rheumatoid arthritis etc. Thus, USP7 polymorphism is associated with multiple autoimmune diseases depicted from a genome-wide association study of 14,000 cases with seven diseases and were compared to 3,000 controls (Wellcome Trust case control consortium, 2007). In the tumor microenvironment and inside the tumor a high number of Tregs are found. These Tregs inhibit the anti-tumor specific T cells and favor the growth of tumor *in-vivo* [205]. So molecules that inhibit USP7 can award a therapeutic effect, resulting in decreased Foxp3 protein level and Tregs hence resulting in removing the anti-tumor effect through an enhanced antitumor immune response. DUBs are highly upregulated in some cancers; for example, in non-small cell lung carcinomas (NSCLCs) four DUBs were significantly overexpressed: JOSD1, COPS5 UCHL1, and USP9X [206]. Five genes (USP10, USP11, USP22, USP48, and COPS5) were found upregulated significantly, compared with benign nevi (common mole) in the initial screening of melanoma samples [206]. Thus, some DUBs can be used as diagnostic/prognostic markers (e.g., USP10, USP11 and USP22 in melanoma), or might represent therapeutic targets (e.g., upregulated DUBs in tumors, but absent in normal tissues), regardless of their exact role in tumorigenesis [206].

### 1.17 DUBs as druggable targets

Balance in protein synthesis and degradation within cells is associated with normal cellular homeostasis. The UPS is a nonlysosomal intracellular protein degradation pathway consisting of proteasome holoenzyme, ubiquitin ligases, and deubiquitinases [2]. Disturbed regulation of the UPS pathway is associated with the pathogenesis of various human diseases [207][208]. That is why inhibitors of the UPS pathway, either at the level of the 26S proteasome, ubiquitin ligases, or DUBs offer potential options

for cancer therapy such as the FDA-approved proteasome inhibitor bortezomib (Velcade) for treating multiple myelomas (a neoplasm of the bone marrow) [209]. Even if bortezomib is the first preference for multiple myelomas, still risk is associated with developing drug resistance and possible off-target toxicities [210]. The discovery and development of small molecule inhibitors of other UPS components provide an alternative approach. In contrast to inhibitors of an E1-conjugating enzyme or E3 ubiquitin ligase, DUBs are potential therapeutic target for several deadly human disease like cancer. Multiple myeloma treatments using proteasome inhibitor bortezomib and carfilzomib (PR-171) are quite toxic. DUBs regulate most cellular process whose malfunction results in so-called “hallmarks of cancer”. DUBs are highly expressed in a disease like cancers thus, it is quite convincing that targeting DUBs could reduce tumor fitness, and selectively eradicate them. Recently a small molecule inhibitor P5091 against USP7 provides an alternative therapy to improve patient outcome in multiple myelomas [211]. Small-molecule DUB inhibitors against various members of USP and UCHL families have been discovered. These include USP1, USP7, USP9x, USP14, UCHL1, and UCHL5. But these inhibitors often target more than one DUB [14]. Recently, FDA has approved Auranofin (Aur) a gold-containing compound for Phase II clinical trials to treat cancers. It is also used to treat rheumatic arthritis, that can inhibit UCHL5 and USP14 which are proteasome-associated DUBs [212]. USP5 is linked to two cancers e.g., alterations of USP5 isoforms in glioblastoma tumorigenesis and USP5 linked-suppression of p53 in melanoma [213][214]. Targeting USP9x and USP5 with deubiquitinase inhibitor EOAI3402143 has been shown to suppress melanoma growth [214]. Thus, DUBs are found to be important targets for disease therapy [215].

### 1.18 DUB inhibitors

Use of kinase inhibitors have aided our understanding of growth factor signaling, similarly, DUB inhibitors could probably accelerate our understanding of DUB function, especially if selective inhibitors can be discovered. Inhibitors of DUB activity is a natural phenomenon, for example, prostaglandin (PG) inhibits the cellular DUB activity [216]. Below DUBs inhibitors are discussed which can be purchased for study purposes. **TCID** is a potent and selective inhibitor of ubiquitin C-terminal hydrolase-L3

(UCH-L3). TCID can diminish glycine transporter GlyT2 ubiquitination in brain stem and spinal cord primary neurons [139][140]. **PR-619** is a nonselective, reversible inhibitor of DUBs that is highly useful for preserving ubiquitinated proteins during cell lysis. **IU1** is a cell-permeable, reversible and selective inhibitor of the deubiquitinating activity of human USP14. It promotes cell survival during proteotoxic stress and selectively stimulates ubiquitin-dependent protein degradation *in-vitro*. **LDN-57444** is a cell permeable UCH-L1 inhibitor with ~28-fold high selectivity over UCH-L3. It decreases 26S protease activity and is accompanied with enhanced levels of ubiquitinated proteins. This inhibitor induces apoptosis. Treatment with LDN-57444 causes dramatic alterations in synaptic protein distribution and spine morphology *in-vivo*. **ML323** binds to the complex of USP1-UAF1 resulting in the inhibition of USP1 deubiquitinase activity. **NSC-632839** is an inhibitor of ubiquitin isopeptidase activity that displays no effect on the proteolytic activity of the proteasome. This molecule induces apoptosis. **P22077** is a cell permeable inhibitor of USP7 and USP47. **P5091** is a selective inhibitor of USP7. It induces apoptosis in multiple myeloma cells. **WP-1130** is a novel DUB inhibitor directly inhibiting USP9x, USP5, USP14, and UCH37. This molecule induces rapid accumulation of polyubiquitinated proteins and triggers tumor cell apoptosis. **1,10-phenanthroline** is a chelator with high affinity for divalent metal ions. While not specific for DUBs, it has been shown to be a potent inhibitor of JAMM-type isopeptidases through chelation of the active site Zn<sup>2+</sup> ion. The compound **b-AP15** abrogates the deubiquitinating activity of the 19S regulatory particle by inhibiting UCHL5 and USP14. b-AP15 induces tumor cell apoptosis in four different solid tumor models. HBX41, 108, HBX19818, P22077, and P050429 are USP7 specific inhibitors. HBX 90, 397 is also an identified inhibitor of USP8 [165]. Several members from the JAMM class of DUB, including Poh1, CSN5, AMSH, and BRCC36, might be potential drug targets [128]. Proteasome inhibitors could be substituted by the use of DUB inhibition for more selective approaches [217].

### 1.19 DUBs in human pathogens

Many DUBs have functional roles in interferon signaling, including OTUB5 (DUBA), OTUB1/OTUB2, USP3, USP17, and USP25. Conversely, viruses encode DUBs that counteract interferon induction as a medium of escaping innate immune responses.

Pathogens also encode deconjugating enzymes such as USP7, OTU, de-sumoylase and different papain-like enzymes. A number of human pathogens including causative agents of SARS, MERS, herpesvirus, *Salmonella*, *Plasmodium falciparum* and *Toxoplasma gondii* are reported to express proteases that target ubiquitin-like modifiers and host ubiquitin. Because some of them target multiple host UBLs and cleave pathogen-encoded proteins they are often referred to as multifunctional enzymes instead of DUBs. PLP2 (papain-like protease 2) is an OTU domain DUB, encoded by arteriviruses (+RNA virus). This dual specificity protease deubiquitinates cellular proteins involved in host immunity. A virus strain expressing an engineered PLP2 with less protease activity showed a weak ability to escape the host immune response [218]. To counteract the host immune response virus uses their potential OTUs deubiquitinating or desumoylating function. OTU domains of viral L-protein (in Crimean-Congo hemorrhagic fever virus (CCHFV), Dugbe virus), and full-length protein of CCHFV decreases ubiquitin and ISG conjugates when expressed in HEK293T cells [219].

Bacteria synthesize effectors to disturb host ubiquitination by mimicking DUBs. Bacterial DUBs commonly deubiquitinate and stabilize I $\kappa$ B $\alpha$  leading to attenuation of NF- $\kappa$ B-related inflammatory responses. TssM, a DUB from the gram negative bacteria *Burkholderia pseudomallei*, targets Lys<sup>63</sup>-linked TRAF-3 & 6 affecting ISRE and IKK activation, respectively [220][221]. SseL, a DUB from *Salmonella typhimurium* alters host lipid metabolism in infected cells by modifying ubiquitination patterns of cellular targets. As infection with the deletion strain of salmonella changed host cell lipid metabolism and excessive lipid accumulation was observed in infected cells [222]. Bioinformatic analysis shows that many medically relevant parasitic protozoa encode putative DUBs. For example, *Plasmodium falciparum* encodes PfUCHL3 and PfUCHL54 while *Toxoplasma gondii* encodes TgUCHL3. PfUCHL-54 has Nedd8 hydrolase activity, but this activity is not conserved in the human homolog of UCH-L5 [223].

## Aim of the Thesis

To date, no deconjugating enzyme has been described for FAT10. Recently, we have observed that a recombinant FAT10-GFP protein was cleaved in extracts of mouse thymus, i.e. an organ where FAT10 is by far most prominently expressed [96].

The first part of the thesis was therefore to identify the enzymes which can cleave FAT10-GFP protein or cleave FAT10 from other substrates. As another approach, we used overexpression of five DUBs, which we have found to bind to FAT10 in an immunoaffinity isolation followed by mass-spectrometry [81]. We investigated if these overexpressed DUBs reduce the amount of covalent FAT10 conjugates in HEK293 cells as compared to either the bulk of FAT10 conjugation substrates or known specific substrates of FAT10 conjugation like JunB, UBE1 or p62. In parallel, systematic overexpression of a Flag-tagged DUB library consisting of 100 cDNAs [225] will be used in HEK293 cells, followed by immunoblot analysis to determine whether FAT10 conjugates of p62, UBE1, USE1 or JunB are enhanced in these cells. Once we have succeeded to identify FAT10 specific DUBs, we will characterize and determine their role in cancer cell lines. We assume their knockdown can induce the proteasomal degradation of tumor-promoting FAT10 substrates. Moreover, expression levels of such enzymes in tissues of colon cancer, hepatocellular carcinoma and thymus will be analysed to determine if they may be significantly upregulated.

In the second part of the thesis, effect of transcription factor p53 on FAT10 was investigated. It was shown, that p53 downregulates FAT10 by repressing FAT10 promoter activity [94]. Therefore, the impact of overexpression of p53 on both endogenous FAT10 and overexpressed FAT10 was tested, using p53 negative cells and HEK293T cells.

It was shown that USP7 stabilizes Foxp3 in Tregs [186]. Moreover, in Tregs FAT10 expression is high, and its level decreases upon T-cells activation [106]. Therefore, in the third part of the thesis we wanted to investigate whether FAT10 interacts with Foxp3 and what is the cellular mechanism behind their interaction.

The fourth part of the thesis was to analyse the effect of FAT10, NUB1L and FAT10 conjugates on the 26S proteasome activity, as it was shown that binding of poly-Ub chain allosterically increases 26S proteasome activity [248]. In addition, ubiquitin binds to the regulatory subunit of the 26S proteasome and signals the 20S core for gate opening [249]. All three different peptidase activity of 26S proteasome were tested.

## 3. Results

### 3.1. Search for FAT10 Deconjugating Enzymes

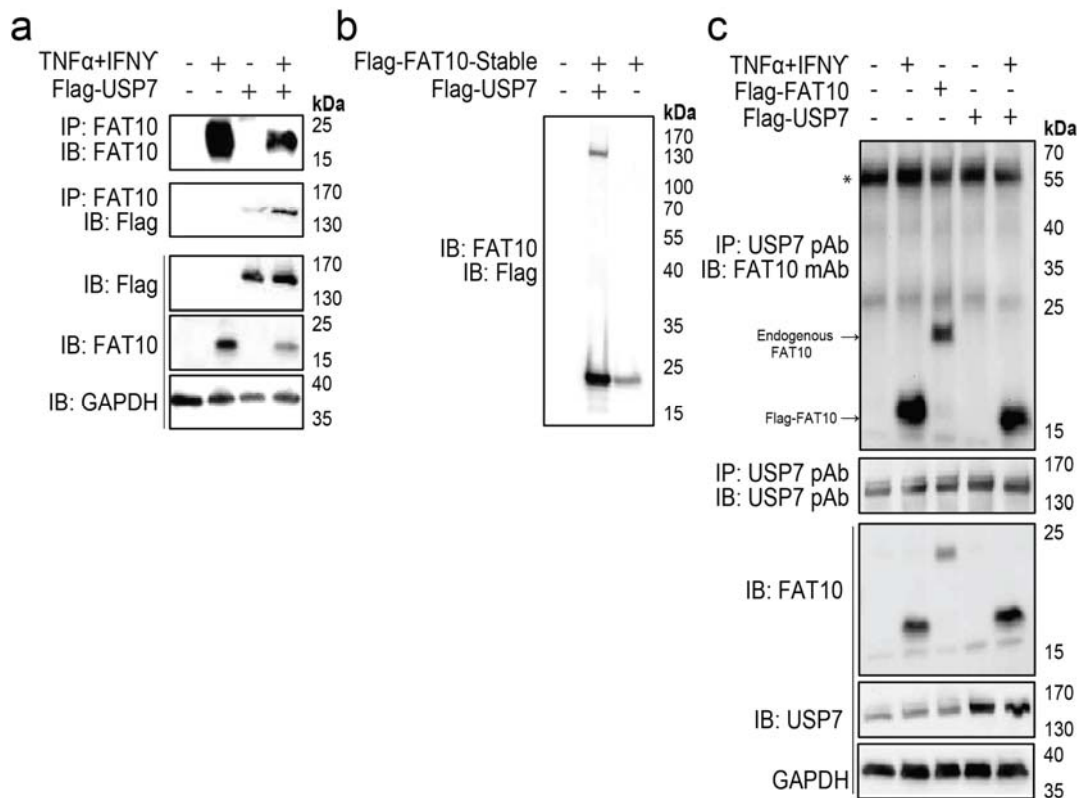
A recently published study from our laboratory identified FAT10 interacting proteins and substrates of FAT10. For this study, endogenous FAT10 was induced in HEK293T cells by proinflammatory cytokines (i.e. 200U/ml IFN $\gamma$  and 400U/ml TNF $\alpha$ ) and the proteasome was inhibited for 6 hours. Next, endogenous FAT10 was immunoprecipitated with the human reactive FAT10 monoclonal antibody 4F1 and the precipitate was subjected to mass spectrometry. Approximately, 571 proteins involved in different cellular pathways from the cell cycle, autophagy, apoptosis, and cancer were found to interact with FAT10 [83]. Among the list of proteins in mass spectrometry data, proteins showing molecular weight  $\sim$ 18 kDa (i.e. molecular weight of FAT10) higher than their predicted molecular weight were designated as putative substrates and others as putative interacting partners. Re-analysis of the identified proteins revealed that five deconjugating enzymes found to interact with FAT10, which were from different families. Three of these deconjugating enzymes e.g. USP7, USP11 and DUB3 were from the ubiquitin-specific protease family of DUB. One was VCIP135, an ovarian tumor domain-containing family of DUB. SENP1 was another DUB which has a functional role in deconjugating SUMO. In the report, USP11, SENP1 and DUB3 were designated as putative interacting partners whereas USP7 and VCIP135 as putative substrates [83].

#### **USP7 noncovalently interacts with FAT10**

The human genome encodes  $\sim$ 90 DUBs [43] specific for ubiquitin but to search for enzymes which can deconjugate FAT10 from its substrates (FAT10 specific DUBs), five DUBs which had been found during FAT10 affinity isolation [83], designated as USP7, USP11, DUB3, SENP and VCIP135 were selected for further investigations in this study. The mammalian expression plasmids encoding human DUBs were either purchased or collected from laboratories involved in DUBs research (**Table. 4**).

In our preliminary experiments, we tested the transient expression of Flag-tagged DUBs in HEK293T one by one. All DUB constructs showed detectable expression in immunoblotting, 24h post transfection (data not shown). Furthermore, in HEK293T cells, we transiently overexpressed Flag-tagged DUBs (i.e. Flag-DUB3, Flag-USP7,

Flag-USP11, Flag-SENP1 and Flag-VCIP135) one at a time and stimulated the cells with the proinflammatory cytokines TNF $\alpha$  and IFN $\gamma$  simultaneously.

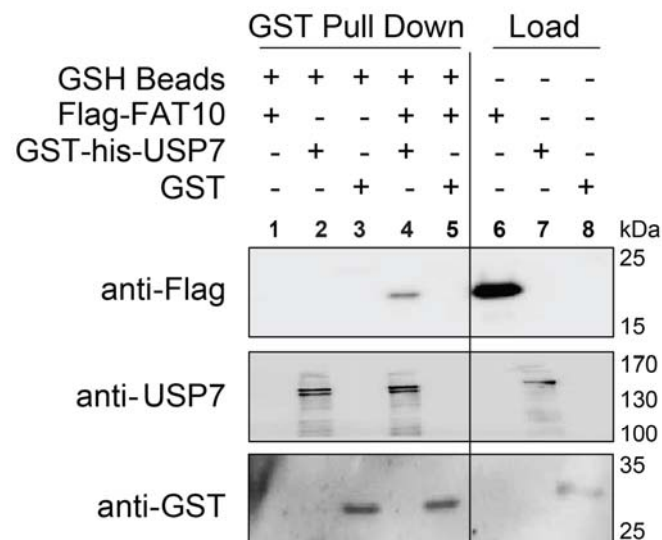


**Figure 7. FAT10 non-covalently interacts with USP7**

**[a]** HEK293T cells were stimulated with TNF $\alpha$  and IFN $\gamma$ , and Flag-USP7 was transfected simultaneously. Post 24h of stimulation/transfection total cell extracts were used to immunoprecipitate endogenous FAT10, followed by immunoblot analysis using antibodies against FAT10 and Flag. Proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels. The upper panel shows the immunoprecipitated FAT10, middle panel shows immunoprecipitated USP7 and the lower immunoblot panel shows protein expression levels in total cell lysates (load). GAPDH was used as loading control. One representative experiment out of three independent experiments with the similar outcome is shown; **[b]**. Stable HEK293 cells expressing Flag-FAT10 [226] was transfected with Flag-USP7 as described in [a]. Post 24h of transfection total cell extracts were used to immunoprecipitate FAT10 using a monoclonal antibody against FAT10, followed by immunoblot analysis using antibodies against Flag-tag. Protein was separated as in [a]. One representative experiment out of two independent experiments is shown; **[c]**. HEK293T cells were stimulated and transfected with the respective plasmids as indicated. Endogenous USP7 immunoprecipitation using polyclonal antibody (pAb), followed by immunoblot analysis using antibodies against FAT10 and USP7. One independent experiment shown. An asterisk (\*) indicates the heavy chain of the antibody used for immunoprecipitation.

24h post FAT10 induction and transfection the whole cell lysate was used to immunoprecipitate endogenous FAT10 with a monoclonal antibody specific for FAT10 (mAb; 4F1), followed by immunoblot analysis using an antibody against the Flag-tag.

Surprisingly, while analyzing immunoblots of all five DUBs we observed that the one designated as USP7 was found to coimmunoprecipitate with endogenous FAT10 (**Fig. 7a**). The experiment was repeated several times following the same protocol resulted in similar outcomes confirming that within cells there could be an interaction between FAT10 and USP7.



**Figure 8. USP7 directly interacts with FAT10 in GST pull-down assay**

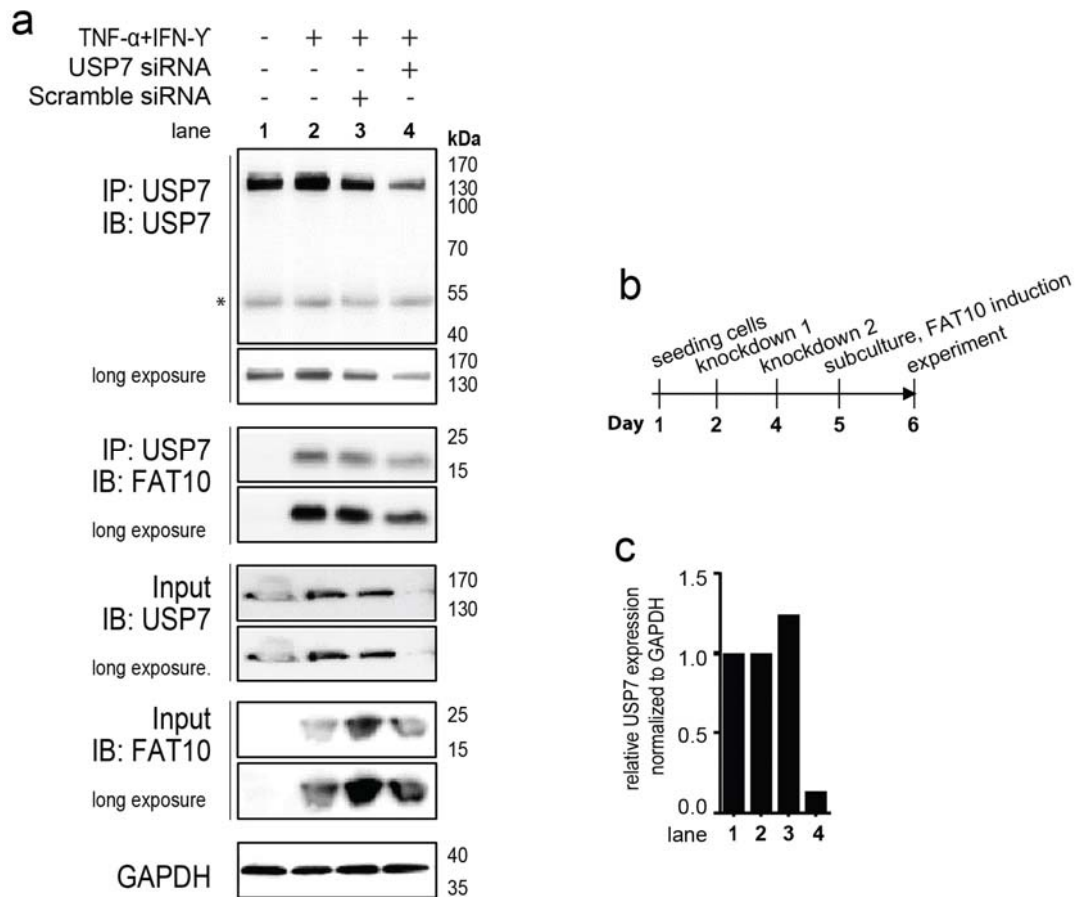
The hisGST-USP7 interacts with Flag-FAT10 in an *in-vitro* interaction assay. 0.5µg recombinant proteins as indicated were initially incubated for 2h without beads, followed by incubation with GSH-matrix for another 2h at 4°C with vertical rolling. Beads were washed twice with NET-TN and twice with NET-T buffer and proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels. The upper panel shows the immunoblot against Flag-FAT10, middle panel shows immunoblot against USP7 using a polyclonal antibody and the lower immunoblot panel shows GST protein. The quality of immunoblot against hisGST-USP7 using anti-GST antibody was not good enough to see the band. One fourth of the protein used in the reaction was taken as a loading control.

To further support this protein-protein interaction, the USP7 plasmid was transiently transfected into HEK293 cells, which express stably transfected Flag-FAT10 [226]. Whole cell lysate was subjected to immunoprecipitation using a monoclonal antibody against human FAT10, followed by immunoblot against the Flag-tag. Once again this experiment reconfirmed the interaction between FAT10 and USP7 (**Fig. 7b**). Additionally, HEK293T cells were stimulated with TNFα and IFNγ and subjected to reciprocal immunoprecipitation, in which endogenous USP7 was immunoprecipitated

using a polyclonal antibody against human USP7 followed by immunoblot against FAT10, and once again this showed the interaction of FAT10 and USP7 (**Fig. 7c**). These data suggested that USP7 interacts with FAT10, and this interaction might have some functional role within the cells. Therefore, we asked ourselves whether USP7 and FAT10 interact directly or whether an adaptor protein is involved in their indirect interaction. To answer this question, we performed GST pull-down assays in which recombinant Flag-FAT10 protein was pre-incubated with recombinant hisGST-USP7, followed by GST pull-down using GSH beads. FAT10 seemed to bind USP7 in USP7 pull-down, which shows a direct interaction between FAT10 and USP7 *in-vitro* and probably no requirement for additional factor (**Fig. 8**). FAT10 overexpression increases the population of transcriptionally active p53 [83]. As p53 was the primary substrate of USP7 [177], p53 is stabilized and deubiquitinated by USP7. But USP7 has a much higher affinity for Mdm2/Hdm2, an E3 ligase [227], which is also deubiquitinated *in-vitro* by USP7 [147]. In a parallel experiment in HEK293T cells, FAT10 interacted with transcription factor Foxp3 in overexpressed condition (**Fig. 25a**). This information enforced us to think that there could be a potential interesting pathway pursued by these two proteins.

These results and the previously reported interactions of USP7/Foxp3, p53/FAT10, USP7/Mdm2 suggest that our current finding may be of significant importance and also would be consistent with a functional role of USP7 in FAT10 biology. Keeping all these in mind, the consequence of endogenous USP7 knockdown, on endogenous FAT10 interaction was verified. HEK293 cells were subjected to two rounds of USP7 knockdown and stimulated with proinflammatory cytokines for 24h to induce the expression of endogenous FAT10 (**Fig. 9b**). Approximately, 95% knockdown of endogenous USP7 expression was achieved, which was determined by RT-PCR using primers designed for human USP7 (**Fig. 9c**) and no USP7 protein was detected in total cell lysate from cells transfected with siRNA against USP7 (**Fig. 9a**). Cells transfected with scrambled siRNA as control show normal USP7 level. Total cell lysate was subjected to immunoprecipitate endogenous USP7 followed by immunoblot analysis against endogenous FAT10. As in cells with 95% USP7 knockdown we still see some residual USP7 protein when USP7 was immunoprecipitated, (**Fig. 9a**, IP lane 4). But with immunoprecipitated USP7, a smaller amount of FAT10 coprecipitated (**Fig. 9a**, 4 versus 3). Thus, this result strengthens our finding that indeed there is an interaction between FAT10 and the deconjugating enzyme USP7 under normal

physiological conditions. This interaction raises the question if it has any functional role within cells.



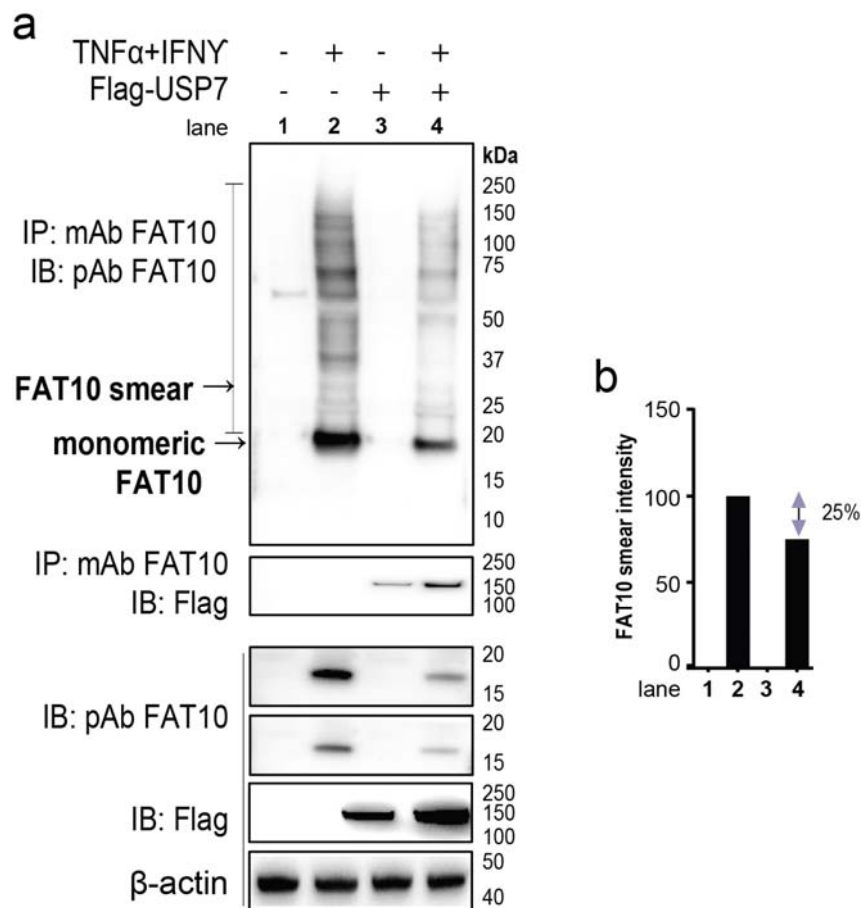
**Figure 9. USP7 knockdown HEK293T cells with less USP7 coimmunoprecipitate less FAT10**

**[a].** Endogenous FAT10 was induced with proinflammatory cytokines and only the USP7 was specifically knocked down by treating HEK293 cells with specific siRNA against human USP7. Control cells were either untreated or treated with unspecific control siRNA. An asterisk (\*) indicates the heavy chain of the antibody used for immunoprecipitation. The GAPDH staining served as a loading control. One representative experiment out of two independent experiments with the similar outcome is shown; **[b].** Experimental setup for [a] and [c]; **[c].** Knockdown of USP7 in experiment [a], confirmed by real-time PCR. 97% knockdown of USP7 transcript was achieved. Gene expression was normalized to GAPDH. One representative experiment out of two independent experiments with the similar outcome is shown.

### USP7 can reduce FAT10 conjugates in HEK293T cells

FAT10 conjugation to a spectrum of cellular protein substrates with isopeptide bond is well explored [84][81][72][78][82][74][76]. FAT10 covalently modifies p53 and high molecular weight conjugates (visible as a smear) were observed only in HEK293T cell expressing wild-type FAT10, but not in non-conjugatable FAT10 mutant (FAT10 $\Delta$ GG)

[83]. Two groups independently reported that FAT10 can be conjugated to UBA6, an E1 enzyme of ubiquitin [74][76].



**Figure 10. FAT10 conjugates can be deconjugated by USP7**

**[a].** HEK293T cells were stimulated with TNF $\alpha$  and IFN $\gamma$ , and Flag-USP7 was transfected as indicated. Post, 24h of stimulation/transfection total cell extracts were used to immunoprecipitate endogenous FAT10 using monoclonal antibody (mAb), followed by immunoblot analysis using polyclonal antibodies against FAT10 (pAb) and Flag. Proteins were separated under reducing conditions (4% 2-mercaptoethanol) on 4-12% Bis/Tris NuPAGE gels. The upper panel shows the immunoprecipitated FAT10 conjugates (smear), the middle panel shows the immunoprecipitated USP7, the lower immunoblot panel shows protein expression levels in total cell lysates (input). Beta-actin was used as loading control. One representative experiment out of three independent experiments with the similar outcome is shown; **[b].** Densitometric analysis of FAT10 conjugates visible as a smear in immunoblotting. In the presence of USP7 in lane 4, 25% less smear intensity was observed. Smear intensity was calculated as the intensity of FAT10 smear/ intensity of monomeric FAT10. Where intensity was measured as: (value intensity\*area mm<sup>2</sup>).

The E2 conjugating enzyme USE1 and the ubiquitin-binding scaffold protein p62 (sequestosome-1; SQSTM1) in autophagy is also FAT10ylated [81][80]. Recently, UBE1 the E1 activating enzyme was reported to be modified by FAT10 [84]. Htt, a

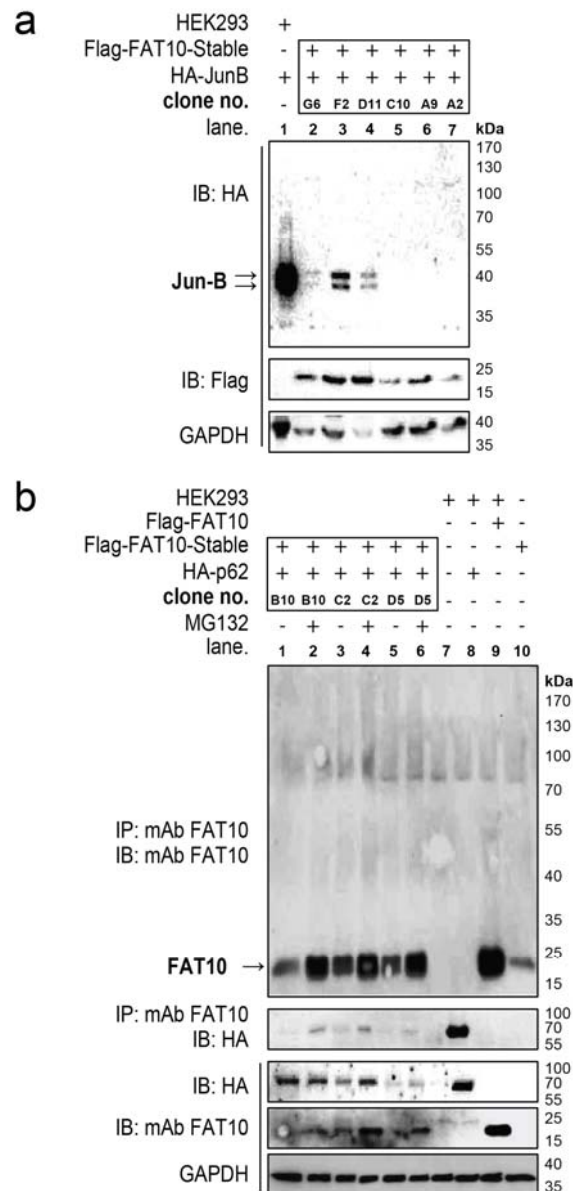
protein associated with Huntington's disease is conjugated by FAT10 [82]. Still the list of substrate proteins for FAT10 is increasing with time.

Additionally, in tumor cells deubiquitinases are highly expressed and their activated status contributes to transformed condition [228][229]. Because USP7 is a DUB which characteristically removes ubiquitin from number of its substrate proteins (e.g. FOXO4, Mdm2, p53), so an approach to investigate the effect of USP7 on conjugates of the ULM, FAT10 was undertaken. FAT10 conjugates as smear has been observed in a previous independent report from our group [81]. Thus, to visualize conjugates in immunoblot, endogenous FAT10 was induced in HEK293T cells with proinflammatory cytokines for 24h along with USP7 overexpression. Next, the whole cell extracts were subjected to immunoprecipitation for FAT10 using mAb against FAT10, followed by SDS-PAGE and immunoblotting using FAT10 polyclonal antibody (105-4). FAT10 immunoprecipitation followed by FAT10 staining in an immunoblot clearly depicted the conjugates of FAT10 of varying size (**Fig. 10a**, lane-2 and 4). Further, densitometric analysis of immunoblots were done to measure the quantitative difference in FAT10 smear in the presence or absence of USP7. The density of FAT10 smears in presence or absence of USP7 was normalized with monomeric FAT10, and 25% reduction in the FAT10 smear was observed in cells overexpressed with Flag-USP7 in three independent experiments (**Fig. 10b**, lane-4 vs lane-2) which could be the deconjugation effect of USP7 on FAT10 conjugates. As a remark, in loading control (whole cell lysate) we also observed reduced FAT10 in the presence of USP7.

#### **HEK293 cells stably expressing FAT10-p62, FAT10-JunB, or FAT10-UBE1 do not show sufficient FAT10 conjugates**

In another approach, we wanted to overexpress each of the known approximately 90 DUBs in HEK293 cells stably expressing FAT10-p62, FAT10-JunB, FAT10-UBE1 or FAT10-USE1 conjugates. So, by establishing stable cells coexpressing FAT10 and one of its substrates as conjugates (e.g. JunB, p62, UBE1, and USE1) we wanted to confirm FAT10 specific DUBs. As cells stably expressing wild-type FAT10 and non-conjugatable mutant has been previously used in other studies [83]. The objective of stable cell generation was to observe the detectable amount of FAT10 conjugate (i.e. FAT10 conjugated to its substrate with isopeptide bond) in immunoblot. Next, the cell line stably expressing detectable conjugates can be subjected to transient transfection with individual cDNA from a library of about 100 different human DUBs (Genentech, USA) [225].

First, for generating stable cells expressing FAT10 together with its substrate JunB, Flag-FAT10 stable cells were transfected with HA-JunB along with a puromycin selection plasmid.



**Figure 11. Stable cell clones co-expressing FAT10-JunB or FAT10-p62**

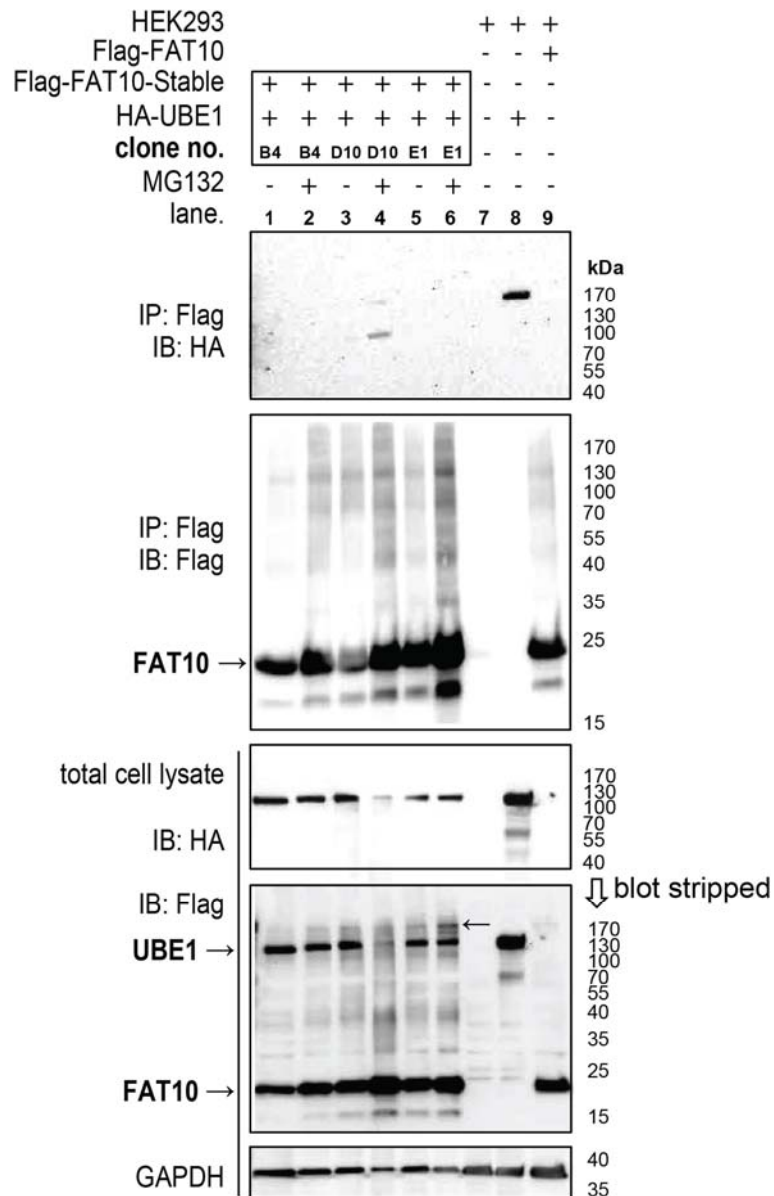
**[a]** Flag-FAT10/HA-JunB coexpressing stable clones G6, F2, D11, C10, A9, and A2. The upper panel and middle panel shows immunoblot against HA and Flag respectively for protein expression levels in total cell lysates (input); **[b]** Flag-FAT10/HA-p62 coexpressing stable clones B10, C2, and D5. The protein in total cell extracts was used to immunoprecipitate FAT10 using mAb, followed by immunoblot analysis using mAb against FAT10 and HA. Cells were treated with MG132 for 6 hours before harvesting. The upper panel shows the immunoprecipitated FAT10, the middle panel the immunoprecipitated p62, the lower immunoblot panel shows protein expression levels in total cell lysates (input); GAPDH was used as loading control. One representative experiment out of three independent experiments with the similar outcome is shown. For immunoblot analysis proteins were separated under reducing conditions on 12% Laemmli SDS-PAGE gels.

Puromycin-selected stable cell clones screened positive for both FAT10 and JunB in immunoblot were maintained in antibiotic for several passages. After months of subculture, clones found coexpressing HA-tagged substrate protein (JunB) along with Flag-FAT10, which were then used to check the conjugates in immunoblot after cell expansion. Six different clones G6, F2, D11, C10, A9, and A2 screened positively for both HA-JunB and Flag-FAT10 and were expanded and tested for visible conjugates in immunoblotting. Each clone was cultured until 90% confluency in a 10cm dish in media supplemented with G418 (i.e. for the Flag-FAT10 stable cells) plus puromycin selection antibiotics followed by SDS-PAGE and immunoblotting. Unexpectedly, we did not observe a conjugate of a size of ~60-70 kDa (predicted size of JunB ~36 kDa plus FAT10 ~18 kDa) in any of the five clones (**Fig. 11a**). We reasoned that probably, as FAT10 conjugated to JunB might accelerate JunB degradation, so for a further experimental analysis of conjugates in stable cells were considered in the presence of MG132.

Further, we took an attempt to make a stable cell line expressing another substrate of FAT10 along with FAT10. Recently p62/SQSTM1, an autophagy adapter protein has been shown to form a covalent conjugate with FAT10 [81]. Thus, p62 was selected as another substrate protein for generating stable cells. Stable clones expressing p62 and FAT10 were expanded in a 10cm dish in the presence of proteasome inhibition by MG132 followed by SDS-PAGE and immunoblotting against Flag-FAT10 and HA-p62 (**Fig. 11b**). All the clones B10, C2 and D5 express p62 and FAT10 together, unfortunately, we could not detect conjugate of size ~80-100 kDa (predicted size of p62 ~70 kDa plus FAT10 ~18 kDa) in any one of the three clones positive for both proteins.

Generation of a stable cell expressing UBE1, a FAT10 substrate along with FAT10 was done in parallel. After months of subculture clones expressing HA-UBE1 along with Flag-FAT10 were expanded in a 10cm dish to check the conjugates in the presence or absence of proteasome inhibition by MG132 for 4h. Three different clones B4, D10, and E1 were positively expressing both UBE1 and FAT10. Unexpectedly, conjugates were undetectable even in the presence of proteasome inhibition by MG132. In the presence of proteasomal inhibition, a distinct band is visible around ~170 kDa (**Fig. 12**, lane 6, input), which could be UBE1-FAT10 conjugate but repeating the experiment did not show consistent result. So all stable clones expressing FAT10 along with either JunB/p62/UBE1 or USE1 were frozen and stored for future studies. This data demonstrated stable cell lines for FAT10/p62,

FAT10/UBE1 and FAT10/JunB have been successfully made, but the levels of conjugate expression are too low for detection by plane immunoblotting.

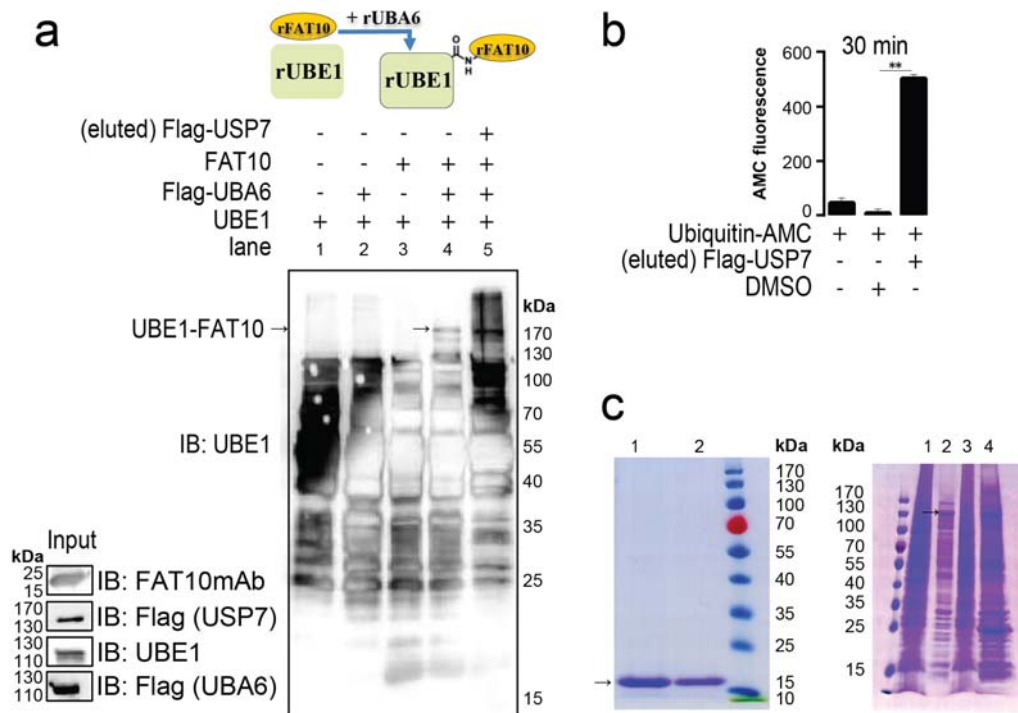


**Figure 12. Characterization of HEK293 stable transfectants expressing HA-UBE1 and Flag-FAT10**

Stable cell clones B4, D10, and E1 were expanded in 10 cm culture dishes. When cells reached ~80% confluency total cell extracts were used to immunoprecipitate FAT10 using anti-Flag beads, followed by immunoblot analysis using anti-Flag and anti-HA antibodies. Cells were treated with proteasome inhibitor MG132 for 6 hours before harvesting. Proteins were separated same as for experiment in Fig.11. The upper panel shows the immunoprecipitated UBE1, the middle panel the immunoprecipitated FAT10, the lower panel shows protein expression levels in total cell lysates (input). GAPDH was used as loading control. One representative experiment out of three independent experiments with the similar outcome is shown. After the anti-HA staining membrane was stripped using blot stripping buffer (prod# 21059, Thermo scientific) and further stained with anti-Flag.

### USP7 effect on *in-vitro* generated isopeptide bond between FAT10 conjugates

Another approach for fishing FAT10 specific DUBs was to identify an enzyme that can cleave the FAT10-GFP protein (a linear isopeptide bond) or cleave FAT10 from other substrates.

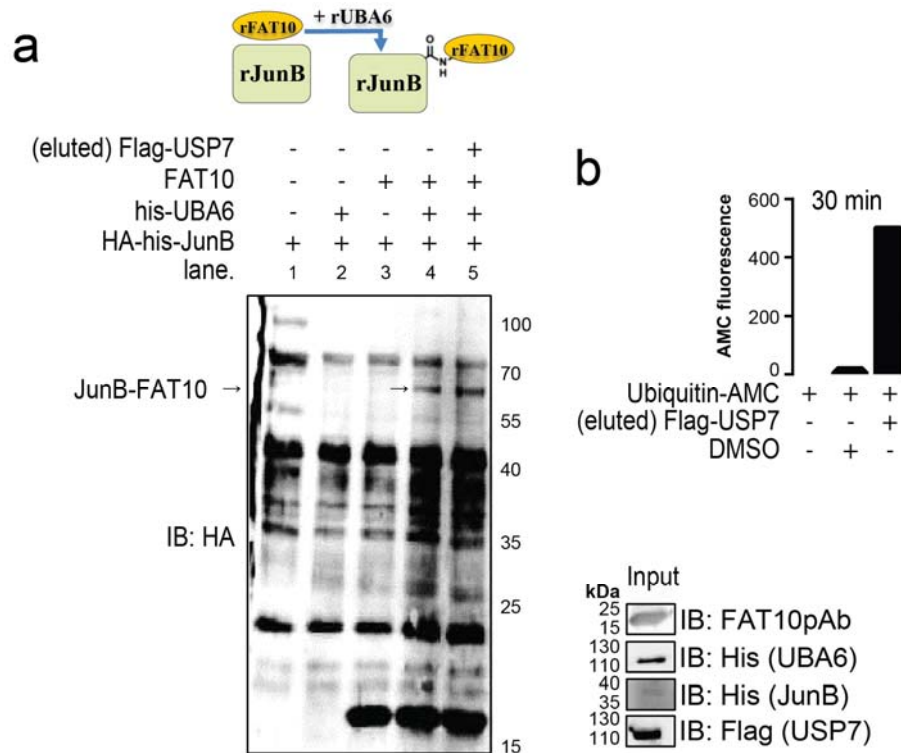


**Figure 13. FAT10ylation of UBE1 and test for a potential de-FAT10ylation by USP7**

**[a]** Incubation of recombinant proteins Flag-UBA6 together with UBE1 and FAT10 for 2 h at 30°C with vigorous shaking leads to conjugation of FAT10 to UBE1 (FAT10ylation) *in-vitro* (cartoon above [a]). Next, USP7 was added to check the deconjugation of FAT10-UBE by further incubating the reaction mixture for 2h at 30°C with vigorous shaking as indicated. Proteins were separated under reducing conditions (4% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels, followed by immunoblot analysis using anti-UBE1. One-fourth of the amount of protein for the *in-vitro* reaction was used as input control followed by immunoblot analysis using a monoclonal antibody against FAT10, anti-Flag antibody and anti-UBE1 antibody. The upper panel shows the UBE1-FAT10 conjugate (→) in lane 4 and lane 5, the lower left panels shows the input control. One representative experiment out of two independent experiments is shown; **[b]**. To confirm the catalytically active status of USP7 eluted as Flag-USP7 using flag-peptide, 20µl of eluate was incubated with Ubiquitin-AMC substrate for 30 min at 37°C; AMC fluorescence was measured using an ELISA reader at Emission-360nm/Excitation-465nm. DMSO was used as a control. Each bar represents the mean ± SD of the two independent experiments (n=2); \*\*p=0.0012. One representative experiment out of two independent experiments is shown; **[c]**. 12% SDS-PAGE followed by coomassie staining representing recombinant FAT10 in lane 1 and lane 2 (left gel, →). Flag-peptide eluted Flag-USP7 in lane 2 (right gel, →).

Recently we have found in our laboratory, that recombinant FAT10-GFP protein was cleaved in extracts from mouse thymus (Schmidtke G; unpublished data) i.e. an organ

where FAT10 expression is very high in a specialized cell type called mTECs [96]. However, so far no such enzymes have been described for FAT10.



**Figure 14. FAT10ylation of JunB and test of deconjugation of FAT10 by USP7**

**[a].** Incubation of recombinant proteins Flag-UBA6 together with JunB and FAT10 for 2 h at 30°C with vigorous shaking leads to conjugation of FAT10 to JunB (FAT10ylation) *in-vitro* (cartoon above [a]). Next, USP7 was added to check the deconjugation of FAT10-JunB by further incubating the reaction mixture for 2 h at 30°C with vigorous shaking as indicated. Proteins were separated under reducing conditions (4% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels, followed by immunoblot analysis using anti-HA antibody. One-fourth of the amount of protein for the *in-vitro* reaction was used as input control followed by immunoblot analysis using a polyclonal antibody against FAT10, anti-His antibody, and anti-Flag antibody. The upper panel shows the JunB-FAT10 conjugate (→) in lane 4 and lane 5, the lower right panels shows the input control. One representative experiment out of two independent experiments is shown; **[b].** Catalytic status of eluted-Flag-USP7 used in the experiment was confirmed by incubation of 20µl of elute with Ubiquitin-AMC substrate for 30 min at 37°C; AMC fluorescence was measured using an ELISA reader at Emission-360nm/Excitation-465nm. DMSO was used as a control. One representative experiment out of five independent experiments is shown.

So, we strongly believe that a FAT10 specific isopeptidase must exist. FAT10 is transferred from UBA6 (E1) onto USE1 (E2) *in-vitro* giving an isopeptide bond [74] and UBE1 (E1) is covalently modified by FAT10. Our intention was to analyse if an isopeptide bond formed between either FAT10/JunB or UBE1/FAT10 could be

deconjugated with any of the five putative deconjugating enzymes which had been found during FAT10 affinity isolation [81].

Thus, we first decided to generate an *in-vitro* conjugate of FAT10 and UBE1 using our standard protocol. For the generation of the *in-vitro* UBE1-FAT10 conjugate, recombinant proteins were added as depicted on top of **Fig. 13a**. In the reaction UBA6 catalyzes the conjugation of FAT10 to UBE1. The UBE1-FAT10 conjugate was clearly visible (**Fig. 13** lanes 4, 5). Next, purified eluted USP7 was added to the reaction tubes where UBE1/FAT10 conjugate can form because of the presence of all the three reaction components i.e. FAT10, Flag-UBA6, and UBE1. Moreover, to avoid improper protein folding in a bacterial expression system with posttranslational modification tagged USP7 was transiently transfected into HEK293 cells, isolated and eluted using Flag-peptide and the elute was used as purified USP7 (i.e. eluted Flag-USP7).

Out of five DUBs, only USP7 was selected as isopeptidase for this experiment because, in our previous experiment, USP7 was found to interact with FAT10 (**Fig. 7, 8, and 9**). Based on that finding we tested if USP7 can cleave FAT10 from the FAT10-UBE1 conjugate. We observed isopeptide-linked UBE1-FAT10 could appear approximately at 140 kDa which is the sum of the size of both FAT10 (~18 kDa) and UBE1 (~120 kDa), which was clear while analyzing immunoblot stained with an antibody against anti-UBE1 (**Fig. 13a**). We expected the deconjugation of UBE1-FAT10 in the presence of USP7. Unexpectedly, the band for the conjugate seen at ~140 kDa (lane 5) remained unaltered. As we don't see the disappearance of ~140 kDa band in the presence of USP7 (**Fig. 13a**, lane-5), we interpreted this might happen in case eluted USP7 had become inactive.

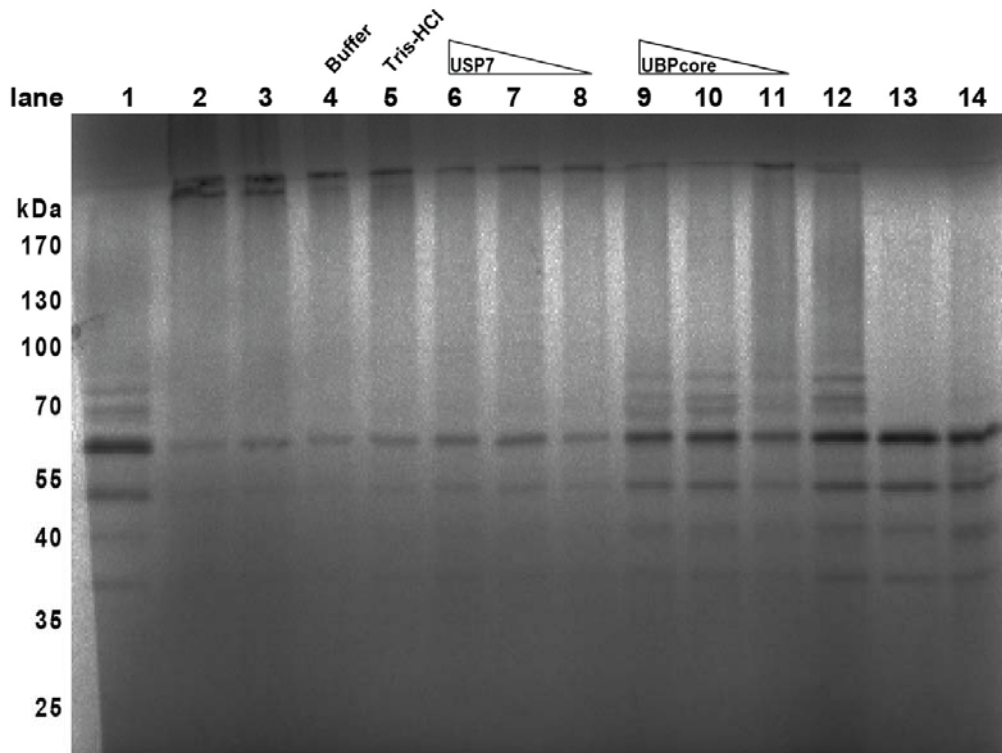
Additionally, a student in our laboratory recently, investigating FAT10 interacting partners using yeast two-hybrid screen, found Trim11 interaction with FAT10. Trim11, a member of the TRIM protein family of E3 ubiquitin ligases reported earlier to interact with several transcription factors [230][231] and transcriptional co-activators [232] and to also regulate their degradation via 26S proteasome. Trim11 is predicted to be FAT10 specific RING finger E3 ligase (A. Aichem, unpublished data). As JunB, another substrate of FAT10 was found to conjugate via isopeptide bond with either ubiquitin or FAT10 in HEK293T cells. Thus, we proceeded with the *in-vitro* generation of a conjugate between JunB/FAT10. Following our standard protocol in a reaction catalyzed by UBA6 as indicated (**Fig. 14a**; top), we were able to generate FAT10 linked by an isopeptide bond to JunB *in-vitro*. We observed isopeptide-linked JunB-FAT10 could appear approximately at ~60 kDa which is the sum of the size of both

FAT10 (~18 kDa) and JunB (~40 kDa), which was clearly visible as a distinct band while analyzing immunoblots stained with an antibody against anti-HA (**Fig. 14a**). We expected the deconjugation of JunB-FAT10 in the presence of USP7, which also did not happen in this case. As we do not see the disappearance of the ~60 kDa band in the presence of USP7 (**Fig. 14a**, lane-5), we once again assumed this might be because of catalytic inert eluted Flag-USP7 used for this experiment. Therefore, next we tested the catalytic activity of eluted Flag-USP7 using Ubiquitin-AMC substrate or using *in-vitro* ubiquitinated p53 as a substrate for USP7. We observe the full catalytic activity of eluted Flag-tagged USP7 in Ubiquitin-AMC assay (**Fig. 13b, 14b**). This was a clear indication of the functionally active USP7 used in the experiment. To further confirm the catalytic activity of eluted Flag-USP7 by another method using p53 as a substrate of USP7, deubiquitination of p53 was investigated as discussed below. These data indicated that USP7 could not cleave the *in-vitro* generated isopeptide bond between either UBE1/FAT10 or JunB/FAT10.

#### **Deubiquitination of p53 (<sup>35</sup>S-labelled) confirms USP7 catalytic activity**

Tagged USP7 isolated from transiently transfected HEK293 cells was unable to cleave the isopeptide bonds between UBE1/FAT10 or JunB/FAT10 *in-vitro*. So, p53 ubiquitination was achieved by the presence of recombinant ubiquitin, E1, E2 and E3 enzymes together. 16E6/E6AP (E3 ligase) was used, as the human papillomavirus (HPV) type 16 (HPV16) E6 protein and the ubiquitin ligase E6AP form a functional complex (16E6/E6AP) [233]. The ubiquitination reaction of p53 was performed for 30 min at 25°C followed by stopping the reaction with 20µM EDTA for another 30 min at 25°C. Furthermore, to check the effect of USP7 deconjugation on ubiquitinated p53 now Flag-USP7 (test) and UBPCore (as a positive control) [234] were added in reaction tubes and incubated for 30 min at 25°C. Finally, 10µl of 4xGSB was added to each 40µl reaction product and were subjected to SDS-PAGE followed by Phospho-imaging. The experiment was conducted, including proper controls as indicated (**Fig. 15**). We observed a clear p53 deubiquitination indicated by a decrease in smear intensity compared to lane without USP7 (lane 4, or 5) and with USP7 (lane 6, 7, 8). The decrease in smear intensity was inversely proportional to the increasing USP7 amount. As indicated in lane 6 with the lowest intensity (highest amount of USP7 loaded) compared to lane 8 (low amount of USP7 loaded). To be sure about USP7 deconjugation activity, a positive control consisting of another deubiquitinating enzyme, UBPCore was included in the reaction. With increasing UBPCore (from lane

11, 10, to 9) we clearly observed a decrease in smear intensity as an indication of p53 deubiquitination. This data tells that, as USP7 can deconjugate the  $^{35}\text{S}$ -labelled-p53 so it is functionally active.



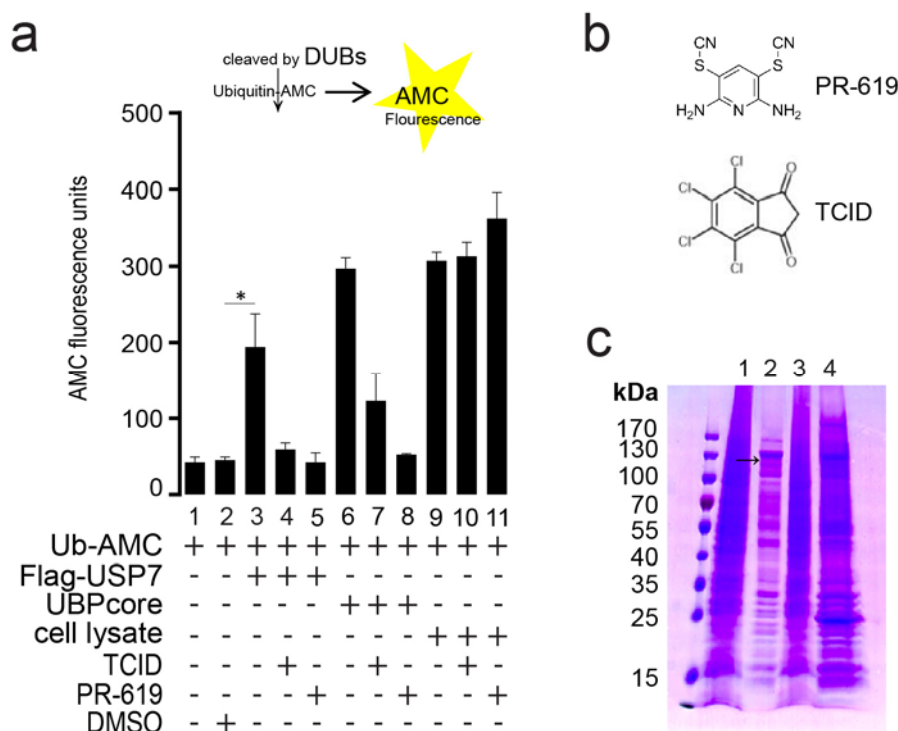
**Figure 15. Phosphoimage showing p53 deubiquitination by eluted-USP7**

The reactions consist of ubiquitination of  $^{35}\text{S}$ -labelled-p53 protein at 25°C followed by addition of USP7, UBPcore, whereas the reaction buffer and Tris-HCl only taken as negative controls. Proteins were separated under reducing conditions on 12% Laemmli gels, dried in gel drying systems (Model 583 Gel Dryer; BioRad), followed by overnight exposure to the screen. The screen scanned in a BioRad scanning system (Personal Molecular Imager™ System). All lanes represent conjugated ubiquitin as a smear. Lane 8, lane 7 and lane 6 represent increasing amount of USP7 which resulted into deconjugation of the ubiquitinated-p53 depicted as a decrease in p53 smear intensity. Lane 11, lane 10 and lane 9 represent increasing amounts of UBPcore (positive control) which also resulted into deconjugation of the p53 smear. The addition of both buffer (lane 4) and Tris-HCl (lane 5) had no influence on the p53 smear, serving as negative control. One representative experiment out of three independent experiments is shown. The catalytic activity of UBP-core and eluted-USP7 used in the experiment was first confirmed by Ubiquitin-AMC as in Fig. 13b, 14b. The reaction consisted of three steps; **[step-1]** 90 min ubiquitination at 25°C, **[step-2]** 30 min termination of ubiquitination using EDTA at 25°C, **[step-3]** DUBs & buffer addition for 2 hours 25°C. Different lanes represent different reaction samples: **1** -no ubiquitination reaction; **2** -with ubiquitination only; **3** -with ubiquitination followed by termination; **4** -with ubiquitination, termination, and reaction buffer added as control; **5** -with ubiquitination, termination and Tris-HCl added as control; **6, 7, & 8** -with ubiquitination, termination and adding decreasing volume of eluted Flag-USP7 respectively; **9, 10, & 11** -with ubiquitination, termination and adding decreasing volume of UBPcore respectively; **12** -no ubiquitination reaction (duplicate of sample 1); **13** -no E3 ligase and no ubiquitination reaction; **14** -no E3 ligase and with ubiquitination reaction.

The Ubiquitin-AMC experiment, together with  $^{35}\text{S}$ -labelled-p53-ubiquitination and deubiquitination by USP7 confirmed that although eluted Flag-USP7 was active, it was not able to cleave the isopeptide bond between both either UBE1-FAT10 or JunB-FAT10.

### DUB inhibitors did not influence bulk FAT10 conjugates

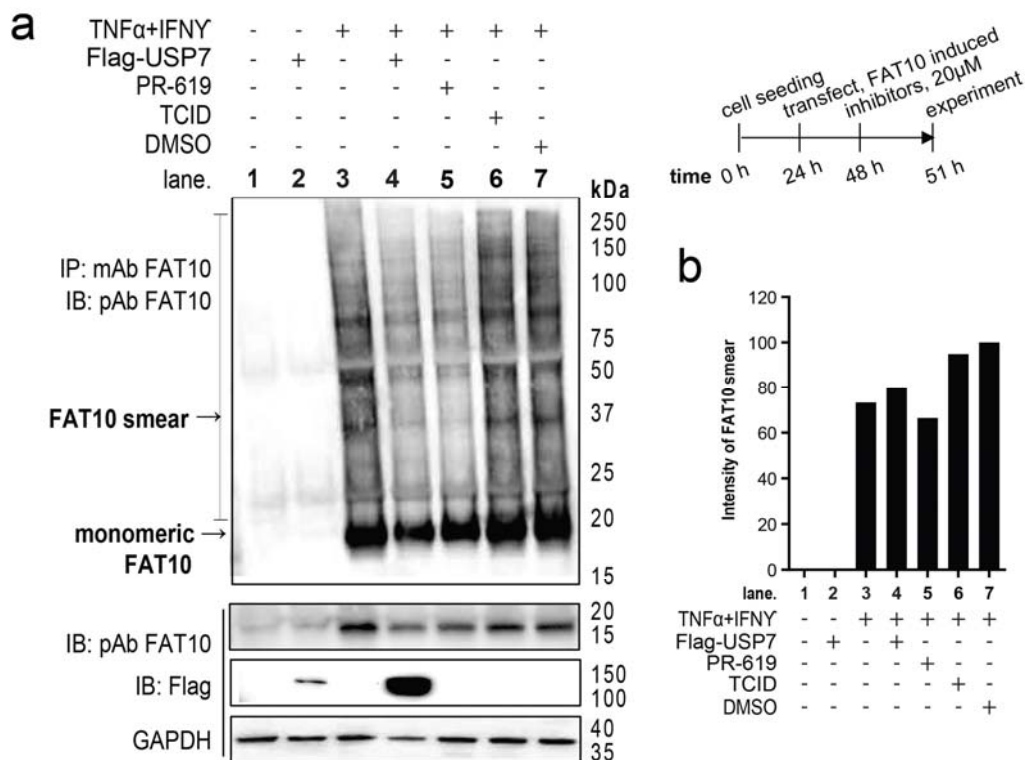
Recently, few compounds have been reported with DUB modulator activity, while DUB inhibitors have an antiviral, antitumor and antiproliferative activity which have been shown for some of the DUBs i.e. USP7[235], UCH-L1[236][237], SARS protease [238].



**Figure 16. DUB inhibitors effect on USP7**

**[a]**. DUBs or whole cell lysate were added individually to 120 $\mu\text{l}$  deubiquitinating assay buffer; inhibitors at a final concentration of 20 $\mu\text{M}$  were added and incubated for 30min at 37 $^{\circ}\text{C}$ . After adding 0.5 $\mu\text{M}$  Ubiquitin-AMC, the reaction plate was incubated at 37 $^{\circ}\text{C}$  for 15min followed by AMC fluorescence measurement using Tecan Infinite M200 pro machine with excitation and emission wavelength set at 360 and 465nm, respectively. Black flat bottom 96 wells plate (Greiner bio-one REF 655900) was used for the assay. Flag-USP7 (eluted) can cleave Ubiquitin-AMC as indicated by an increase in AMC fluorescence (lane 3 vs lane 2). The addition of either TCID inhibitor (lane 4) or PR-619 inhibitor (lane 5) can inhibit USP7 catalytic activity as indicated by a decrease in AMC fluorescence. The conserved core domain of ubiquitin-specific processing proteases (UBPs) i.e. UBPs-core, was used as positive control. UBPs-core activity was inhibited by the two inhibitors (lane 6 vs 7, 8). The global impact of the two inhibitors was tested on whole cell lysate from HEK293 cells. Inhibitors showed no effect on whole cell lysate (lane 9 vs 10, 11). Each bar represents the mean  $\pm$  SD of the two independent experiments ( $n=2$ );  $*p=0.0410$ . One out of three independent experiments is depicted; **[b]**. PR-619: Empirical Formula-C $7\text{H}5\text{N}5\text{S}2$ , Molecular Weight 223.28. TCID: Empirical Formula- C $9\text{H}2\text{Cl}4\text{O}2$ , Molecular Weight 283.92; **[c]**. 12% coomassie gel showing Flag-peptide eluted Flag-USP7 in lane 2 (indicated as,  $\rightarrow$ ).

Inhibition of major cellular DUBs (USP5, USP9x, USP14, UCH-L1, and USH37) likely induces multiple predictable cellular changes, such as (a) increased accumulation of unanchored polyubiquitin chains/polyubiquitinated proteins, (b) decrease in the monomeric ubiquitin pool, (c) slower rate of polyubiquitin disassembly, (d) an overall decrease in individual DUB activities (e) affects cellular level or activities of DUB-regulated oncoproteins [239].

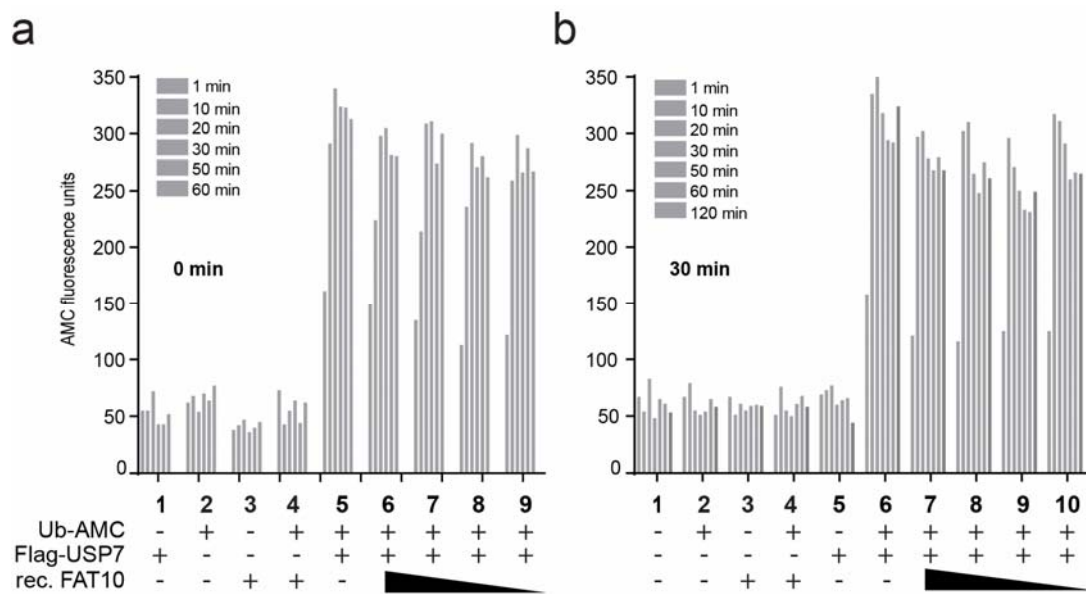


**Figure 17. Cell permeable DUB inhibitors influencing FAT10 conjugates**

**[a].** HEK293T cells were transiently transfected with expression plasmids for Flag-USP7 and stimulated with cytokines simultaneously, followed by treatment with 20 $\mu$ M of DUB inhibitors for 3 hours. As negative control cells were treated for 3 hours with 0.3% DMSO. Total cell extracts were used to immunoprecipitate endogenous FAT10 using monoclonal antibody (mAb), followed by immunoblot analysis using polyclonal antibodies against FAT10 (pAb). Proteins were separated under reducing conditions (4% 2-mercaptoethanol) on 4-12% Bis/Tris NuPAGE gels. The upper panel shows the immunoprecipitated FAT10-conjugates (smear), the lower immunoblot panel shows protein expression levels in total cell lysates (input). GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown; **[b].** Densitometric analysis of FAT10 conjugates visible as a smear in immunoblot. Smear intensity was calculated as, the intensity of FAT10 smear/ intensity of monomeric FAT10. Where intensity was measured as: (value intensity $\times$ area mm $^2$ ).

In our preliminary study where we observed, FAT10 conjugates visible as smear were reduced in intensity in the presence of USP7. It was evident that USP7 overexpression leads to the appearance of less smear intensity (**Fig. 10a**, lane 4). Predicting that this

was a USP7 effect on the conjugates, we hypothesized that inhibiting overexpressed USP7 with a DUB inhibitor could neutralize the effect. Thus, two widely used cell permeable DUB inhibitors, TCID, and PR-619 were selected to analyse the impact of inhibitors on the smear of FAT10 conjugates. PR-619 (LifeSensor) is a broad spectrum reversible DUB inhibitor, which selectively inhibits ULM-isopeptidases, without inhibiting the chymotrypsin-like activity (CT-L) of the 20S proteasome. TCID is a cell permeable DUB inhibitor selective for UCH-L3, a UCH family DUB.



**Figure 18. Influence of FAT10 on USP7 catalytic activity**

To *in-vitro* investigate if USP7 activity is influenced by the presence of the ubiquitin-like-modifier FAT10. Increasing amounts of FAT10 were incubated with a constant amount of USP7. Increasing amount of recombinant FAT10 (8.6, 4.3, 2.1, 1.3  $\mu\text{g}$  in lane 6, 7, 8, and 9 respectively) protein was added to a fixed amount of Flag-peptide eluted USP7 (20 $\mu\text{l}$ ) and incubated for **0 min** as indicated in **[a]** or 30 min as indicated in **[b]**. After adding 0.5 $\mu\text{M}$  ubiquitin-AMC, the reaction plate was incubated at 37 $^{\circ}\text{C}$  followed by measuring AMC fluorescence till 60min using the M200 Pro machine (Tecan) with excitation and emission wavelength set at 360 and 465nm, respectively. Black flat bottom 96 wells plate (Greiner bio-one REF 655900) were used for the assay. Coomassie staining for the applied recombinant FAT10 and USP7 elute is depicted in Fig.13c. One representative experiment out of three independent experiments with the similar outcome is shown.

Thus, before applying to the cells, to directly estimate the inhibitory effect on USP7, inhibitors were first tested in 96 well plates, using Ubiquitin-AMC as a substrate. AMC cleaved by USP7 was used as readout using an ELISA reader. The addition of 20 $\mu\text{M}$  of TCID could inhibit the eluted Flag-USP7 activity, indicated by a decrease in AMC

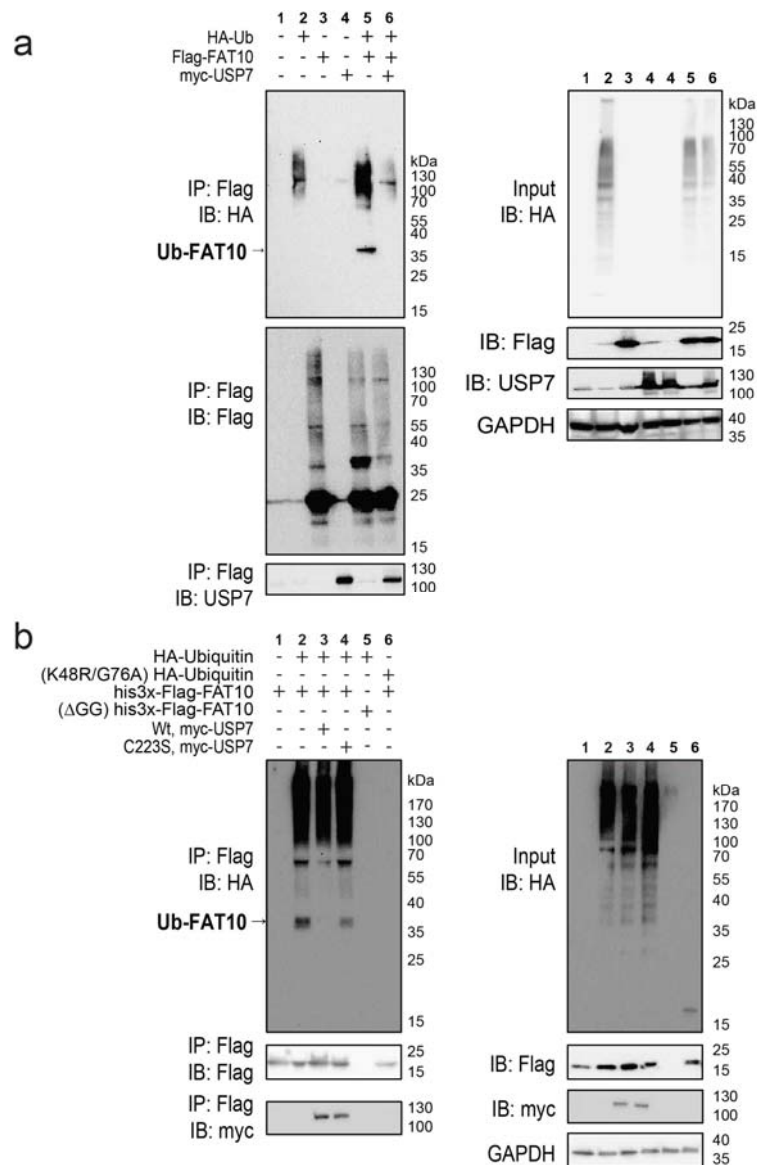
fluorescence (**Fig. 16**, lane 4). In the case of PR-619, we also observed USP7 inhibition, as indicated by a decrease in AMC fluorescence (**Fig. 16**, lane 5) but the PR-619 effect was more pronounced compared to TCID (**Fig. 16**, 5 vs 4). With this Ubiquitin-AMC assay, it was confirmed that both PR-619 and TCID individually could directly inhibit USP7 activity.

We moved one step further and wanted to determine whether inhibition of catalytic USP7 using PR-619 or TCID could impact FAT10 conjugates (smear). The treatment schedule was exactly as depicted (**Fig. 17**, top right). In HEK293 cells, FAT10 was induced and USP7 was overexpressed as indicated. After 3h of inhibitor treatment, cells were harvested and subjected to immunoblotting against FAT10 to monitor the effect of inhibitors on the FAT10 smear. The presence of USP7 diminished the high-molecular weight FAT10ylated proteins indicated by a decrease in smear intensity, which was consistent with our earlier observation (**Fig. 17**, lane 4). In contrast, PR-619 treatment did not significantly reverse the USP7 effect. TCID, in contrast, behaved as the DMSO control. These data from inhibitor experiments do not clearly tell if USP7 is really a player in FAT10 deconjugation.

Additionally, PR-957 inhibitor (ONX0914) inhibits the LMP7 active site threonine. To test if PR-957 has any off-target effect, USP7 activity was tested using Ubiquitin-AMC after adding PR-957. We found no significant effect of PR-957 on USP7 activity (data not shown).

#### **FAT10 did not affect USP7 catalytic activity *in-vitro*.**

As we observed a direct interaction between FAT10 and USP7, we thought to investigate if the presence of FAT10 could impact the catalytic activity of USP7. Thus, USP7 was co-incubated with increasing amounts of FAT10 followed by USP7 activity determination with Ubiquitin-AMC as a substrate (*in-vitro* DUB assay). However, this assay did not show any change in USP7 catalytic activity measured by AMC fluorescence (**Fig. 18a**). The coincubation of FAT10 and USP7 for either 30 min followed by AMC fluorescence reading till 2h still didn't show any significant change in USP7 catalytic activity (**Fig. 18b**). For the assay Flag-peptide eluted USP7 and recombinant FAT10 were used, coomassie staining of proteins used in the assay are depicted (**Fig.13c**). Apparently, FAT10 does not have any effect on the catalytic activity of USP7, even if they directly interact.



**Figure 19. Ubiquitination of FAT10 (FAT10-Ub) and USP7 deconjugation**

**[a].** HEK293T cells were transiently transfected with expression plasmids for HA-Ubiquitin, Flag-FAT10 or myc-USP7 as indicated. Cell lysates were subjected to immunoprecipitation with anti-Flag antibody coupled to agarose. Proteins were separated under reducing conditions (4%, 2-ME) on 12% Laemmli SDS-PAGE gels and immunoblot analysis was performed with an antibody reactive against HA, Flag or Myc. GAPDH was used as loading control. The left column shows the immunoprecipitated proteins and the right column shows the total protein expression in the whole cell lysate (input). The upper panel in the left column shows the immunoprecipitated ubiquitin-FAT10 conjugate (→) in lane 5, which disappeared in the presence of USP7 in lane 6. Middle and lower panels show immunoprecipitated FAT10 and USP7, respectively. One representative experiment out of three independent experiments with the similar outcome is shown; **[b].** HEK293T cells were transiently transfected with expression plasmids for HA-ubiquitin, for unconjugatable ubiquitin (lysine mutated to arginine at position 48; K48R, glycine mutated to alanine at position 76; G76A), Flag-FAT10, the unconjugatable Flag-FAT10 (ΔGG), myc-USP7 and the active site cysteine mutant of myc-USP7 (Myc-USP7 C232S). Protein separation, SDS-PAGE gels, and immunoblot analysis was performed as in [a]. The upper panel in the left column shows the immunoprecipitated ubiquitin-FAT10 conjugate (→) in lane 2, which disappears in the presence of USP7 in lane 3 but was unaffected in the presence of catalytically inactive USP7 in lane 4. The middle and lower panel shows immunoprecipitated FAT10 and USP7, respectively. One representative experiment out of three independent experiments with the similar outcome is shown.

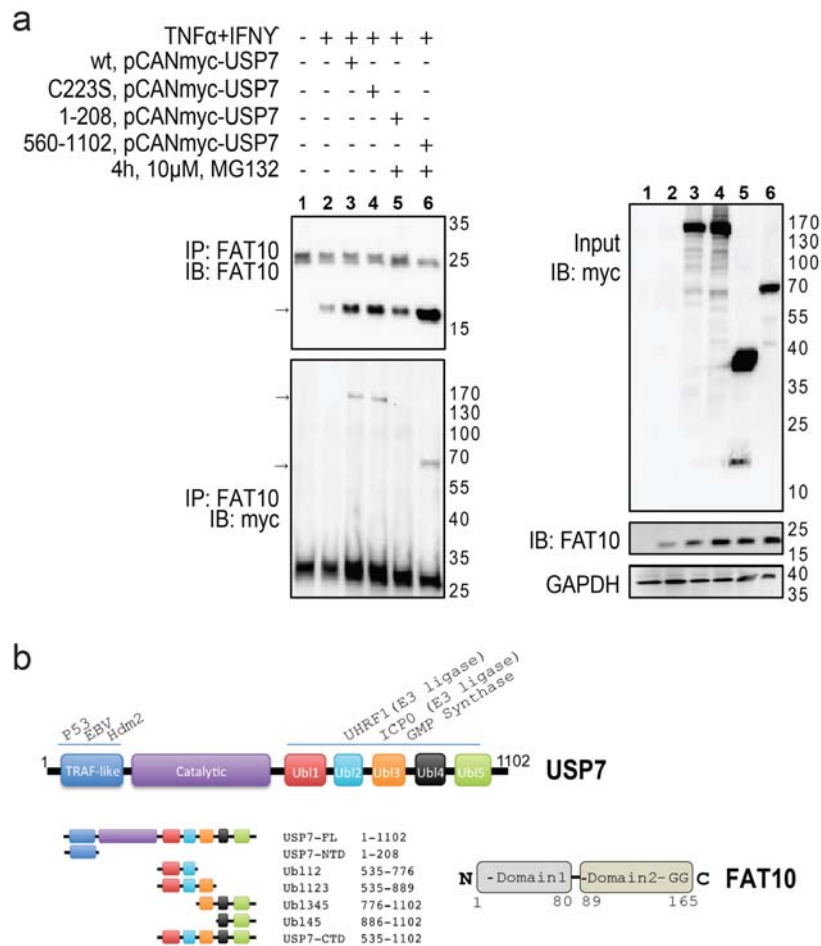
### Effect of USP7 on ubiquitination of FAT10

An earlier report by Hipp et al., 2005 [69] from our group showed that ubiquitination of FAT10 is not necessary for FAT10-mediated degradation by the proteasome. In this report, immunoprecipitation of FAT10 from HEK293T cells overexpressing FAT10 and ubiquitin together followed by immunoblotting against ubiquitin showed a clear conjugate of ubiquitin-FAT10.

Recently, it was reported that a transcription factor FOXO4 is monoubiquitinated and is deubiquitinated by USP7 [146]. Additionally, the autophagy adapter protein p62 is monoFAT10ylated but an enzyme involved in FAT10 deconjugation has remained unexplored [81]. So we set out to study the ubiquitination of FAT10 in more detail. To this end, HA-ubiquitin and Flag-FAT10 were coexpressed in HEK293T cells. Subsequently, the whole cell lysate was used to immunoprecipitate FAT10 followed by immunoblot detection using anti-HA antibody (**Fig. 19**). Several independent experiments resulted in the same pattern of ubiquitinated bands, indicating the reproducibility of this result. Notably, we observed a band at ~35-40 kDa, the molecular mass of which corresponds to the molecular mass of FAT10 plus ~8,000 Da (molecular mass of Ub), indicating that FAT10 can be monoubiquitinated at one or multiple sites. We wanted to investigate further if some DUBs can deconjugate the ubiquitin-FAT10 conjugate.

Based on the direct interaction of a DUB with FAT10, USP7 was selected to test if it can deconjugate the ubiquitin-FAT10 dimer. The rationale behind the experiment was to answer two questions: firstly, to know, whether ubiquitin can be deconjugated from FAT10 as a substrate by USP7 or whether, alternatively, FAT10 can be cleaved from ubiquitin as a substrate by USP7. To investigate whether USP7 can deconjugate FAT10, we repeated the FAT10 monoubiquitination experiment and observed that the band at ~35-40 kDa disappeared, in the presence of USP7 overexpression (**Fig. 19a**, 6 vs 5, IP lane). The experiment was reproduced three times with the same outcome, but more controls are needed to draw any firm conclusion. To this aim, catalytically inactive USP7 (USP7C223S) was included and both plasmids were simultaneously expressed to test if the deubiquitination of FAT10 relies on USP7 activity. Which holds true as the inactive USP7 was unable to cleave the band at ~35-40 kDa (**Fig. 19b**, 4 vs 3, IP lane). To validate the finding that FAT10 is monoubiquitinated. To study whether; (i) FAT10 is conjugated to ubiquitin or (ii) ubiquitin is conjugated to FAT10. We looked at the first possibility and used an HA-ubiquitin construct in which the lysine residue at position 48 in ubiquitin was mutated to arginine (K48R) together with glycine

at position 76 mutated to alanine. We realized that K48/G76A ubiquitin could not be conjugated to substrate (i.e. FAT10) because of alanine at its C-terminus at position 76 but one of its internal lysines could provide a site for FAT10 attachment.



**Figure 20. Endogenous FAT10 interacts with USP7-CTD in HEK293T cells**

**[a].** HEK293T cells were transiently transfected with expression plasmids for myc-USP7, the active site cysteine mutant of myc-USP7 (Myc-USP7 C232S), the 1-208 amino acid region of USP7 as the N-terminal domain (myc-USP7-NTD), and the 560-1102 amino acid region of USP7 as the C-terminal domain (myc-USP7-CTD). Simultaneously, cells were stimulated with IFN $\gamma$  and TNF $\alpha$  as indicated. Before harvesting, cells were treated with proteasome inhibitor MG132 for 6 hours. Cell lysates were subjected to immunoprecipitation using a monoclonal antibody against FAT10 (4F1) together with sepharose-A beads. Proteins were separated under reducing conditions (4%, 2-ME) on 12% laemmli SDS-PAGE gels and immunoblot analysis was performed with an antibody reactive against FAT10 or Myc. The left column shows the immunoprecipitated proteins and the right column shows the total protein expression in the whole cell lysate (input). GAPDH was used as loading control. The upper panel in the left column shows the immunoprecipitated FAT10 ( $\rightarrow$ ) in lane 2,3,4,5 and 6 and the lower panel shows immunoprecipitated full-length USP7 in lane 3 and lane 4, whereas lane 6 shows immunoprecipitated USP7-CTD ( $\rightarrow$ ). One representative experiment out of two independent experiments with the similar outcome is shown; **[b].** Domain architecture of FAT10 and enzyme USP7. FAT10 consist of two domains, the N-terminal-domain (NTD) or Domain-1 and the C-terminal-domain (CTD) or Domain-2 and ends with two Gly-Gly residues. USP7 has a TRAF domain, a catalytic domain in the middle and five C-terminal Ubl domains. Some proteins reported to interact with USP7 domains are indicated. The image was adopted and modified from [184].

To examine the second possibility we used a Flag-FAT10 construct in which the C-terminal glycine-glycine (GG) motif was deleted (Flag-FAT10 $\Delta$ GG). The  $\Delta$ GG FAT10 cannot be conjugated to ubiquitin but the free internal lysines in FAT10 could provide a site where still ubiquitin can conjugate. Experiments were nicely reproduced with two other independent researchers in our group, but we could not confirm if the band at 35-40 kDa is a Ubiquitin-FAT10 heterodimer or a blotting artifact. Notably, in this experiment, anti-Flag M2 beads blocked with 1x roti block (Roth) was used for immunoprecipitating Flag-FAT10. It was striking to observe when overnight blocked beads with 5% BSA were used to immunoprecipitate FAT10 the band was not detected.

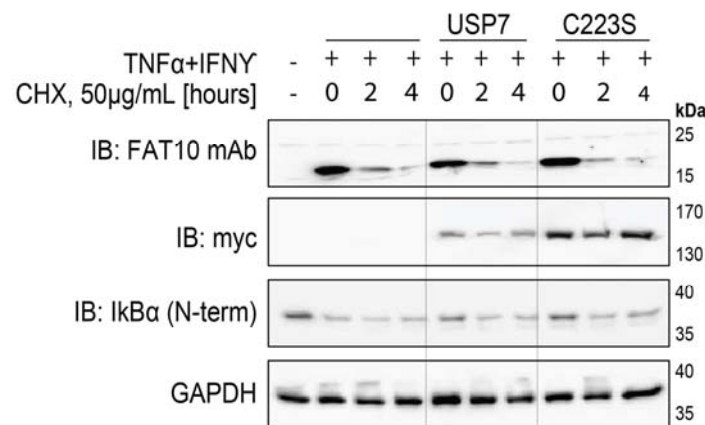
### **Mapping the FAT10 binding domains in USP7**

In order to determine whether the N-terminal or C-terminal part of USP7 is interacting with FAT10, full length catalytically active USP7 (FL-USP7wt), full length catalytically inactive USP7 (FL-USP7C223S), the USP7 C-terminal domain (USP7-CTD), and the USP7 N-terminal domain (USP7-NTD) were overexpressed and endogenous FAT10 was induced simultaneously in HEK293T cells. In total cell lysate subjected to immunoprecipitation of endogenous FAT10 followed by immunoblot against USP7 variants, we found that only the USP7-CTD could coimmunoprecipitate with endogenous FAT10. Even though the expression of USP7-NTD was higher compared to USP7-CTD in the presence of proteasome inhibition, it did not coimmunoprecipitate with endogenous FAT10. This data confirmed that the C-terminal domain of USP7 interacted with full-length FAT10 (**Fig. 20a**). Further, to analyze which of the purified Ubl domains of USP7 at C-terminus is interacting with recombinant full-length human FAT10 in GST pull-down assay. The FL-USP7wt, catalytic inactive FL-USP7C223S and all the five ubiquitin-like domains in USP7, i.e. Ubl12, Ubl123, Ubl345, and Ubl45 will be used as prey in the assay with GST-FAT10. FL-USP7 and USP7-CTD will serve as positive control while GST alone and USP7-NTD will serve as negative controls for the experiment.

### **USP7 overexpression did not influence NF- $\kappa$ B signaling in FAT10 induced cells**

Induction by proinflammatory cytokines leads to upregulation of various genes including FAT10. This is achieved by NF- $\kappa$ B signaling; NF- $\kappa$ B binds to the FAT10 promoter leading to its transcriptional induction [102]. NEMO ubiquitination is important for NF- $\kappa$ B activation and USP7 has been reported to deubiquitinate NEMO

and terminate NF- $\kappa$ B activation [240]. As from our research team A. Aichem found that siRNA-mediated knockdown of USP7 leads to decreased FAT10 levels in HEK293T cells (unpublished data). This we hypothesized, as FAT10 ubiquitination was enhanced in USP7 knockout cells, which is probably leading FAT10 to 26S proteasomal degradation, so less FAT10 is observed by immunoblot in a cycloheximide chase experiment. We tested in turn whether overexpression of USP7 could cause an accumulation of the FAT10 protein. Overexpression of USP7 and even its catalytically inactive mutant USP7C223S had no influence on the degradation of FAT10 in a cycloheximide chase experiment (**Fig. 21**). Moreover, no effect of USP7 overexpression on I $\kappa$ B degradation was observed. Taken together, these data show that, overexpression of either catalytically active USP7 or the USP7 catalytic inactive mutant do not influence the I $\kappa$ B and FAT10 level.



**Figure 21. USP7 overexpression did not downregulate endogenous FAT10**

HEK293T cells were transiently transfected with expression plasmids for myc-USP7, the active site cysteine mutant of myc-USP7 (Myc-USP7 C232S), and simultaneously treated with IFN $\gamma$  and TNF $\alpha$  as indicated. Proteins in the whole cell lysates were separated under reducing conditions (4%, 2-ME) on 12% Laemmli SDS-PAGE gels and immunoblot analysis was performed with an antibody reactive against FAT10, Myc, and I $\kappa$ B $\alpha$  (N-terminal). GAPDH was used as loading control. Cells were additionally treated for 4 hours with cycloheximide (CHX). GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown.

### 3.2. The Impact of p53 overexpression on FAT10

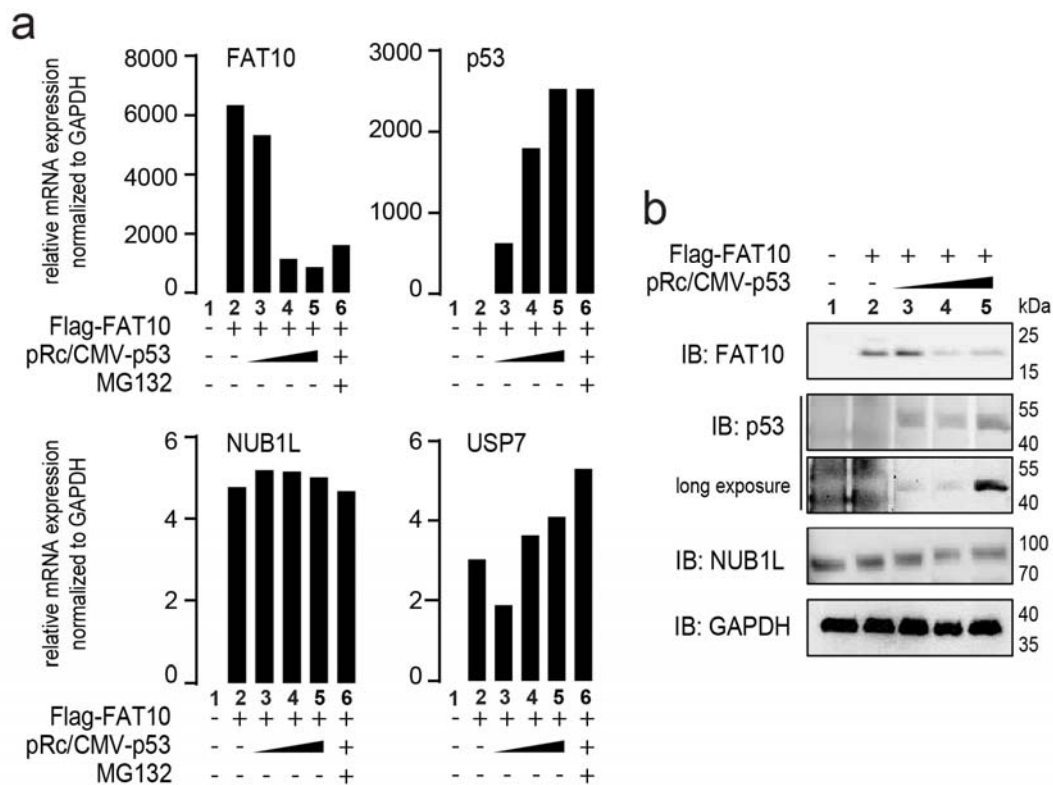
The ~53 kDa protein encoded by the *TP53* gene present in human and mice was discovered in 1979. Five independent groups found p53 to be associated with large T-antigen of SV40 in cells infected with this virus, and one group found several mouse tumor cells expressing p53. The gene for p53 was believed to be an oncogene as high levels of p53 expression were found in several cancers and overexpression of wild-type p53 transformed normal cells into cancer cells. Later in the mid-1980s, tumor suppressor function of p53 was hypothesized [241]. In a normal cell the level of p53 protein is kept low, but in response to stress, p53 is activated by post-translational modification and leads to cell cycle arrest, cell senescence or apoptosis [242]. Additionally, when USP7 was disrupted in HCT116 cells, rather than decreasing p53 level because of increasing p53 ubiquitination, the absence of USP7 resulted in p53 accumulation accompanied by reducing p53 ubiquitination. This suggested that under physiological condition Mdm2 rather than p53 is a substrate for USP7 [243]. USP7 destabilizes tumor suppressor p53 through stabilizing Mdm2 [147]. USP7 binds to p53 in cells and can deubiquitinate p53 both *in-vivo* and *in-vitro* [177]. The protein p53 is FAT10ylated while overexpression of FAT10 leads to downregulation of p53 and increases the population of transcriptionally active p53 in addition [83]. The primary substrate of USP7 is p53 [177], p53 is stabilized and deubiquitinated by USP7 [244], but USP7 has much higher affinity for Mdm2/Hdm2 (an E3 ligase for p53) [227], which is also deubiquitinated *in-vitro* by USP7 [147]. FAT10 is a target gene of STAT3 and STAT3 binding to the FAT10 promoter stabilizes NF- $\kappa$ B binding on that promoter leading to an enhanced transcriptional induction of the FAT10 gene [245]. As a transcription factor, p53 represses the FAT10 promoter activity as evident by less FAT10 transcript due to overexpressed p53 in p53 negative Hep3B cells.

Moreover, siRNA-mediated knockdown of endogenous p53 in p53-positive cell lines (KB3-1 and HEK293) significantly enhanced FAT10 promoter activity. Consequently, in hepatocellular carcinoma, FAT10 expression is high because of functionally inactive mutant p53 [94]. But, according to another report, p53 overexpression resulted in a rapid decrease of only FAT10 protein without affecting FAT10 mRNA. Also, p53 overexpression had a similar effect on the non-conjugating form of FAT10 mutant. However, the transcriptionally inactive p53 mutant failed to degrade FAT10 protein [245]. The protein NUB1 interact and suppress p53 in cooperation with Mdm2 [246]. Furthermore, NUB1 splice variant NUB1L is a cytokine inducible linker protein, which

interacts non-covalently with FAT10 through its three C-terminal ubiquitin-associated (UBA) domains and with the 26S proteasome through its N-terminal ubiquitin-like (UBL) domain. Binding of NUB1L to FAT10 accelerated the degradation of FAT10 and FAT10 linked proteins [89][90]. Taken together, these data encouraged us to investigate the consequence of p53-mediated downregulation of FAT10 and whether NUB1L may play a role in this downregulation.

### Increasing p53 decreased FAT10 level in p53 null cells

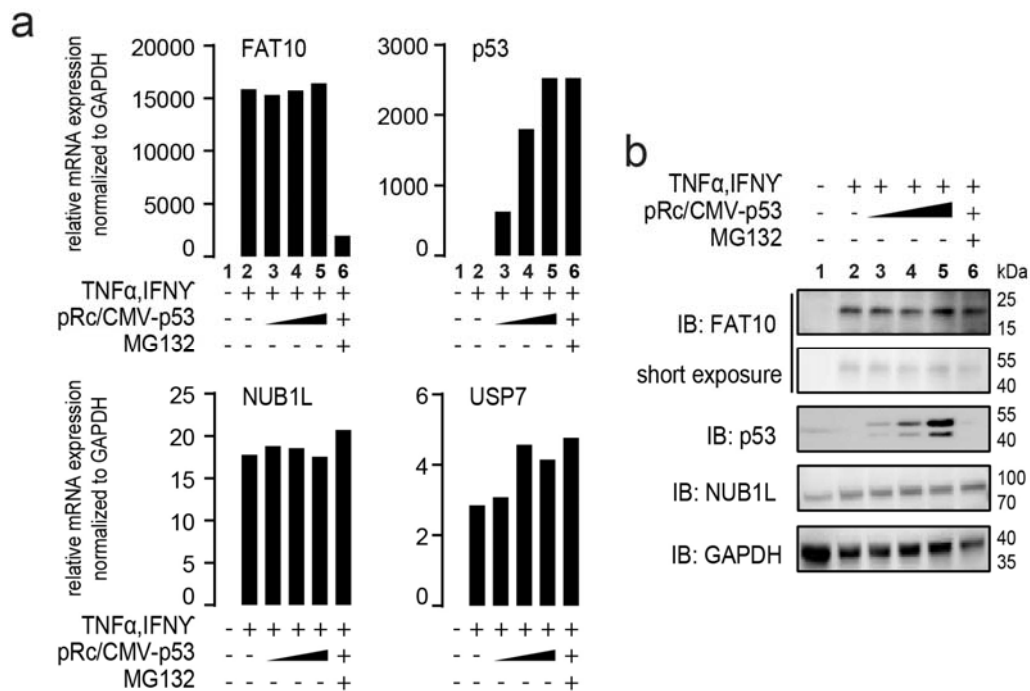
First, we wanted to reproduce a published report whether increasing p53 down regulates FAT10 at mRNA and protein level.



**Figure 22. Overexpression of p53 down regulates FAT10 in H1299 p53<sup>-/-</sup> cells**

H1299 p53 null cells were transiently transfected with expression plasmids for Flag-FAT10, untagged-p53 or Streptavidin-His-GFP. The plasmid DNA amount for transfection was always kept constant (8 $\mu$ g) for Flag-FAT10 in lane 2, 3, 4, 5 and 6 while untagged-p53 was increasing (2 $\mu$ g, 4 $\mu$ g, 8 $\mu$ g, 8 $\mu$ g in lane 3, 4, 5 and 6). The DNA amount was adjusted to 16 $\mu$ g in total using Streptavidin-His-GFP as a normalizing plasmid in lane 1, 2, 3 and 4. Before harvest, cells were treated with proteasome inhibitor MG132 for 6 hours, where indicated; **[a]**. The mRNA expression level of FAT10, p53, NUB1L and USP7 was measured by real-time PCR. The mRNA levels were normalized to GAPDH; **[b]**. H1299 p53 null cells were transiently transfected as in **[a]**, excluding lane 6. Proteins in the whole cell lysates were separated under reducing conditions on 12% SDS-PAGE gels and immunoblot analysis was performed with an antibody against FAT10, p53, and NUB1L. GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown.

We selected the human non-small cell lung carcinoma cell line H1299 which does not express functional p53. Cells were cotransfected with Flag-FAT10 and increasing amounts of p53 followed by immunoblotting and mRNA isolation after 24h of transfection. We observed a decrease in FAT10 protein with an increase in p53 protein amount in H1299 p53<sup>-/-</sup> cells.



**Figure 23. Unaffected endogenous FAT10 expression levels with increasing amounts of p53 in H1299 p53<sup>-/-</sup> cells**

H1299 p53 null cells were stimulated with TNF $\alpha$  and IFN $\gamma$ , and untagged-p53 or Streptavidin-His-GFP was transfected simultaneously. The plasmid DNA amount for transfection was kept increasing (2 $\mu$ g, 4 $\mu$ g, 8 $\mu$ g, 8 $\mu$ g in lane 3, lane 4, lane 5 and lane 6) for untagged-p53. The DNA amount was adjusted to 16 $\mu$ g in total using Streptavidin-His-GFP as a normalizing plasmid in lane 1, 2, 3 and 4. Before harvest, cells were treated with proteasome inhibitor MG132 for 6 hours, where indicated; **[a]**. The mRNA expression level of FAT10, p53, NUB1L and USP7 was measured by real-time PCR. The mRNA levels were normalized to GAPDH; **[b]**. Proteins in the whole cell lysates were separated under reducing conditions (4%, 2-ME) on 12% Laemmli SDS-PAGE gels and immunoblot analysis was performed with an antibody reactive against FAT10, p53, and NUB1L. GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown.

To be sure, if this outcome is the same at RNA level we looked at RNA expression in the same experiment using quantitative real-time PCR. With an increasing p53 level, we observed decreased FAT10 both at the protein level and at RNA level (**Fig. 22a, b**). Additionally, as NUB1L accelerates the degradation of FAT10 and FAT10 linked

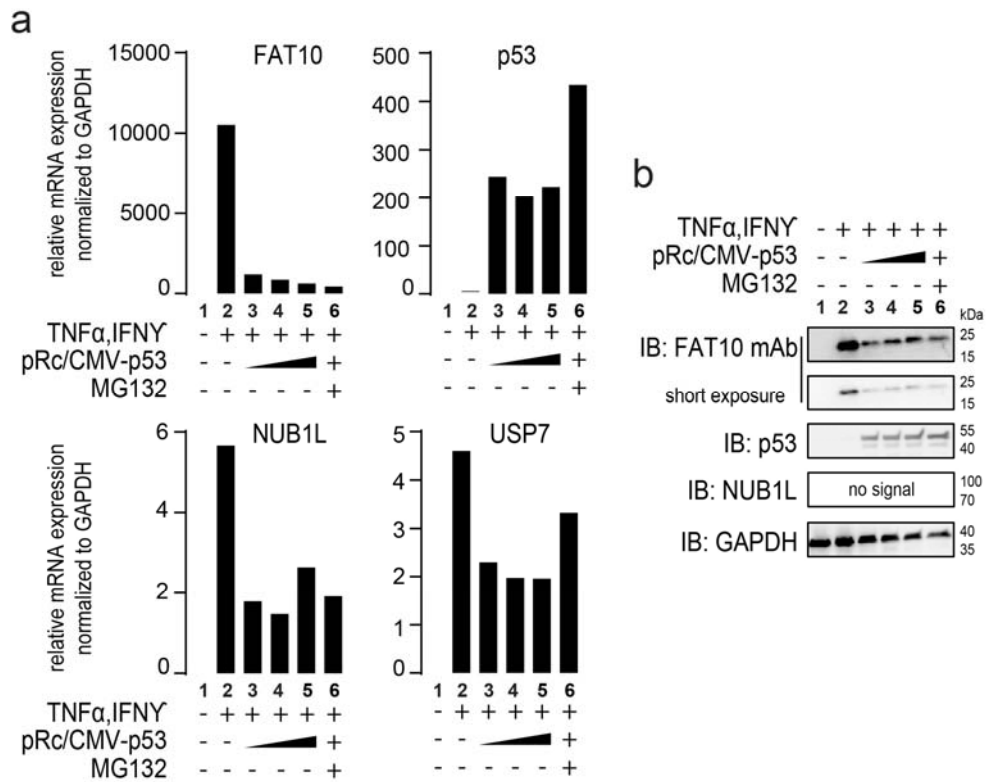
proteins [247] we investigated if increased p53 would also influence the NUB1L level. However, we found no effect of increasing p53 on NUB1L, both at protein and mRNA level (**Fig. 22a, b**).

#### **Increasing p53 does not downregulate endogenous FAT10**

FAT10 induction had not been reported in H1299 cells, so to further validate if increasing p53 overexpression could also influence endogenous FAT10 in a similar way as overexpressed FAT10. Endogenous FAT10 was induced in H1299 cells by proinflammatory cytokines TNF $\alpha$  and IFN $\gamma$  and simultaneously an increasing amount of p53 expression construct was transfected. 24h post FAT10 induction/transfection total cells were harvested and subsequently analyzed for FAT10 protein and FAT10 mRNA level. Although FAT10 was induced but with increasing p53 overexpression, we do not observe any effect on FAT10 protein level (**Fig. 23b**). Further, we looked for FAT10 mRNA and were surprised to see, that increasing p53 does not influence endogenous FAT10 mRNA as well. We also investigated the endogenous NUB1L protein and RNA level after p53 overexpression and could not find any difference (**Fig. 23a, b**).

#### **In HEK293T cells, p53 overexpression downregulated endogenous FAT10**

The HEK293T cells are derived from HEK293 cells but stably express the SV40 large T antigen which can bind to SV40 enhancers of expression vectors to increase protein expression. In addition, HEK293T are neomycin resistant. Thus, to determine if the effect of overexpressed p53 on FAT10 expression is cell specific, we repeated the experiment with the same setup but this time used HEK293T cells. With the increasing amount of p53 overexpression in HEK293T cells, we observe a decrease in endogenous FAT10 mRNA level (**Fig. 24a**). Therefore, we next examined the impact of p53 on FAT10 at the protein level. We found that p53 overexpression decreased the amount of FAT10 protein confirmed by immunoblotting (**Fig. 24b**). With p53 overexpression in HEK293T cells, we observed downregulation of NUB1L at RNA level, but as we could not detect NUB1L in immunoblotting we cannot tell if it is due to p53 or an experimental artifact.



**Figure 24. Overexpressed p53 down-regulated endogenous FAT10**

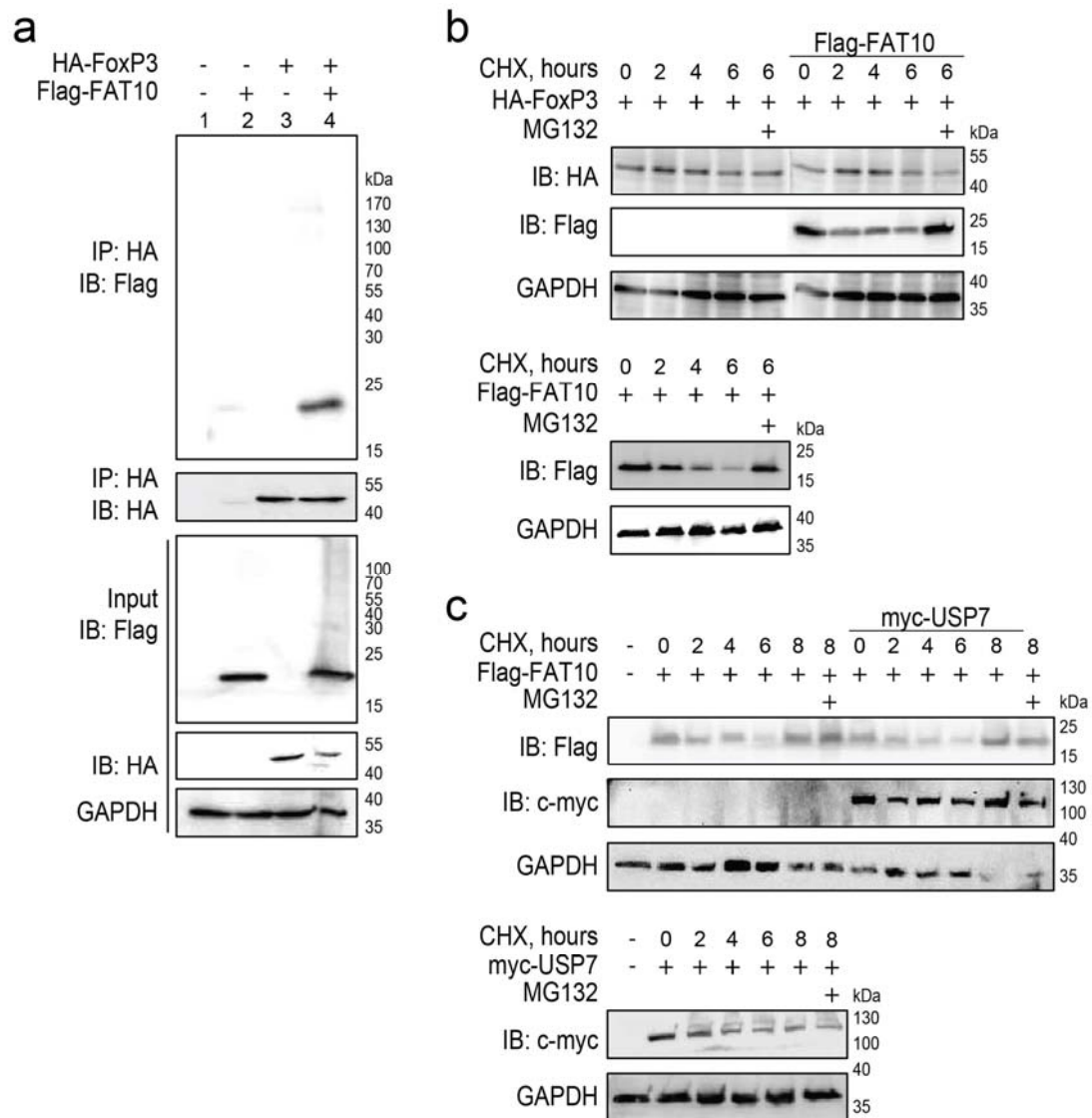
HEK293T cells were stimulated with TNF $\alpha$  and IFN $\gamma$  and transfected with increasing amount of untagged-p53 plasmid same as performed for experiments in Fig. 22 or 23. Before harvest, cells were treated with proteasome inhibitor MG132 for 6 hours, where indicated. **[a]**. The mRNA expression level of FAT10, p53, NUB1L and USP7 was measured by real-time PCR. The mRNA levels were normalized to GAPDH; **[b]**. Proteins in the whole cell lysates were separated under reducing conditions on SDS-PAGE gels and immunoblot analysis was performed with antibodies specific for FAT10, p53, and NUB1L. GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown.

### 3.3. Investigation of FAT10 and Foxp3 interaction

The transcription factor Foxp3 (forkhead box P3, scurfin), has a predicted molecular weight of ~50 kDa. Being a member of the forkhead-box protein family, Foxp3 works as a master regulator in the development and function of regulatory T cells (Tregs). USP7 stabilizes the Foxp3 transcription factor by deconjugating it and prevents its proteasomal degradation [186].

The potential role of FAT10 (ubiquitin D) in the physiology of human regulatory T cells has not been addressed in detail so far. This could be due to significant FAT10 expression in T-cells only after *in-vitro* activation [106]. As, a transcription factor, Foxp3 may not be directly involved in upregulation of FAT10 expression but FAT10 transcription may be indirectly regulated, such as by downregulating transcription repressors of FAT10. Since USP7 stabilizes Foxp3 [186] and USP7 was one of the deconjugating enzymes under investigation, therefore we thought to determine if FAT10 and Foxp3 are somehow related. Moreover, lentivirus-mediated Foxp3 overexpression in T-helper cells has been shown to induce FAT10 [106].

Initially, to test the interaction between Foxp3 and FAT10, we overexpressed both Foxp3 and FAT10 in HEK293 cells and found immunoprecipitation of Foxp3 could coprecipitate FAT10. It was striking to observe that FAT10 was able to interact with Foxp3 under overexpression conditions (**Fig. 25a**). We hypothesized that overexpression of FAT10 could downregulate the Foxp3 level, so we coexpressed FAT10 and Foxp3 in HEK293T cells and tested in cycloheximide chase experiments if FAT10 could accelerate Foxp3 degradation. However, till six hours under proteasomal inhibition, we did not observe an increase in Foxp3 degradation in the presence of FAT10 (**Fig. 25b**) while FAT10 was rapidly degraded. Although we had evidence of FAT10 being ubiquitinated (**Fig. 19b**) yet we wanted to determine the influence of USP7 on FAT10 level. But, USP7 did not show significant influence on the level of FAT10 in cycloheximide chase experiment (**Fig. 25c**).



**Figure 25. FAT10 interacts with the transcription factor FoxP3**

**[a].** HEK293T cells were transiently transfected with expression plasmids for Flag-FAT10, and pMT2-HA-Foxp3 as indicated. After 24h of transfection total cell extracts were used to immunoprecipitate Foxp3, followed by immunoblot analysis using antibodies against Flag and HA. Proteins were separated under reducing conditions (10% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels. The upper panel shows the immunoprecipitated FAT10, the middle panel shows the immunoprecipitated Foxp3, the lower immunoblot panel shows protein expression levels in total cell lysates (input). GAPDH was used as loading control. One representative experiment out of two independent experiments with the similar outcome is shown; **[b].** Post 24 hours of transfection with expression plasmids for HA-Foxp3 or Flag-FAT10, HEK293T cells were additionally treated with 50 $\mu$ g/ml cycloheximide (CHX) for 0, 2, 4, and 6 hours as indicated. Before harvest, cells were treated with MG132 for 6 hours. Proteins in the whole cell lysates were separated and immunoblotted as in [a]. The upper panel shows the immunoblot with whole cell lysates blotted and probed with anti-HA, anti-Flag. GAPDH was used as loading control. One representative experiment is shown; **[c].** 24 hours after transfection with expression plasmids Flag-FAT10, or myc-USP7, HEK293T cells were additionally treated with 50 $\mu$ g/ml cycloheximide (CHX) for 0, 2, 4, 6, and 8 hours. Proteins in the whole cell lysates were separated and immunoblotted as in [a]. All panels show the respective proteins in the whole cell lysates immunoblotted against anti-Myc and anti-Flag. GAPDH was used as loading control. One representative out of two independent experiments is shown.

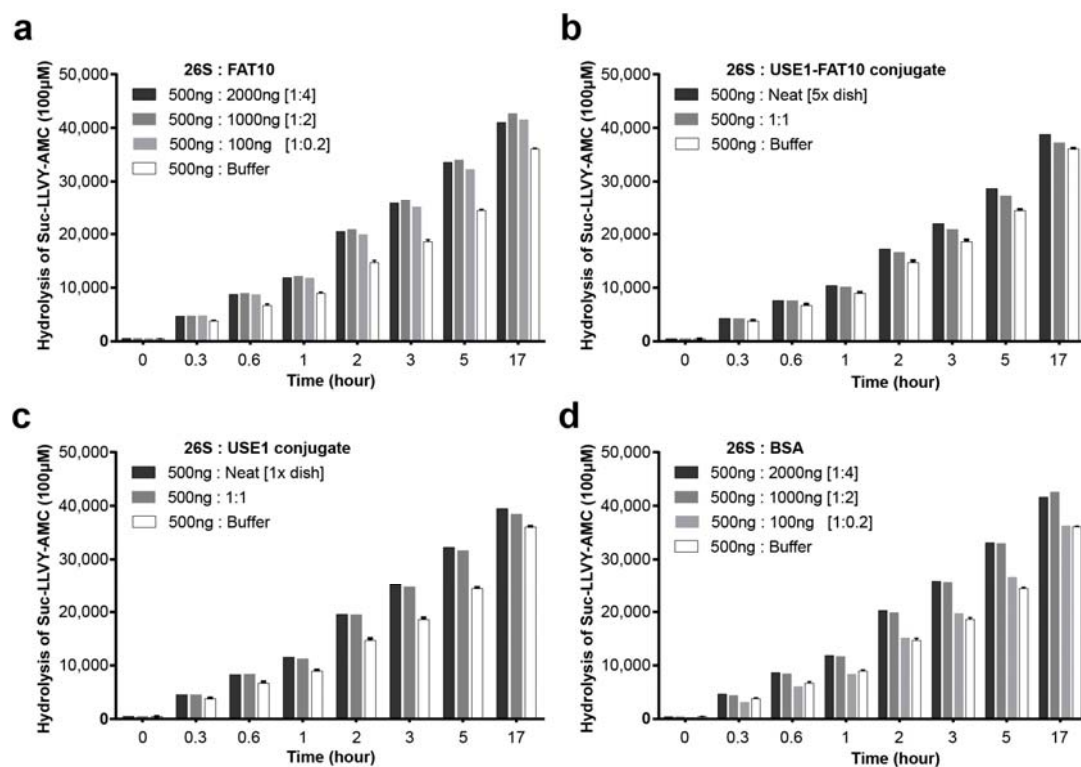
### **3.4. Investigation if FAT10 (diubiquitin) or FAT10-conjugates stimulate 26S proteasome activity**

So far abnormal, denatured or, in general, damaged proteins, as well as regulated degradation of short-lived proteins is assigned to the proteasome within mammalian cells. Maintained protein degradation is important for cellular homeostasis which influences the rate of ageing. Ageing can be reversed by reactivating the impaired mitochondria by chemical or genetic means [251]. Transfection of  $\beta 5i$  subunit into HeLa and in lymphoblasts cells increased the trypsin-like (T-L) activity, chymotrypsin-like (ChT-L) activity of the proteasome, first time shown by Goldberg and coworkers [252][253]. Like ubiquitin, FAT10 also targets protein for degradation by the proteasome. FAT10 interacts with the Rpn10 subunit whereas NUB1L interacts with Rpn10 and Rpn1 subunits of the proteasome for substrate degradation. FAT10 and NUB1L both interact with human Rpn10 via the VWA domain [247]. NUB1L binds to the 26S and to FAT10 and accelerates the degradation of FAT10 and FAT10-linked substrates [90]. Casein protein, which quickly degrades without ubiquitination does not affect the hydrolytic activity of the proteasome. Binding of the polyubiquitinated substrate increases the peptidase activity of the 20S proteasome by two-fold [248]. As, ubiquitination of proteins leads to degradation of proteins in eukaryotic cells, incubation of the 26S proteasome with polyubiquitinated proteins enhances the capacity of the proteasome to hydrolyze proteins and peptides become 2 to 7 fold faster [249].

Similarly, poly-monoubiquitinated proteins, as well as a free unanchored tetra-Ub chain with K<sup>63</sup> or K<sup>48</sup>-linkages, did not stimulate peptide hydrolysis. We studied the activation status of the human 26S proteasome in the presence of FAT10 alone, FAT10ylated substrate, or NUB1L alone and investigated the activation status of the human 26S proteasome. The substrate Suc-LLVY-AMC by itself can activate the gate opening in the 20S proteasome [254] but we still used it for our study. To test if all three peptidase activities of the 26S proteasome are stimulated by FAT10, FAT10 conjugate, or substrate of FAT10 we used specific fluorogenic substrates for its trypsin-like (BZ-VGR-AMC), chymotrypsin-like (Suc-LLVY-AMC), and caspase-like (AC-YVAD-AMC) activity. Additionally, Five E3 ligases are associated with the mammalian 26S proteasome, and many subunits of the proteasome are ubiquitinated [250].

### FAT10 stimulates the activity of the 26S proteasome

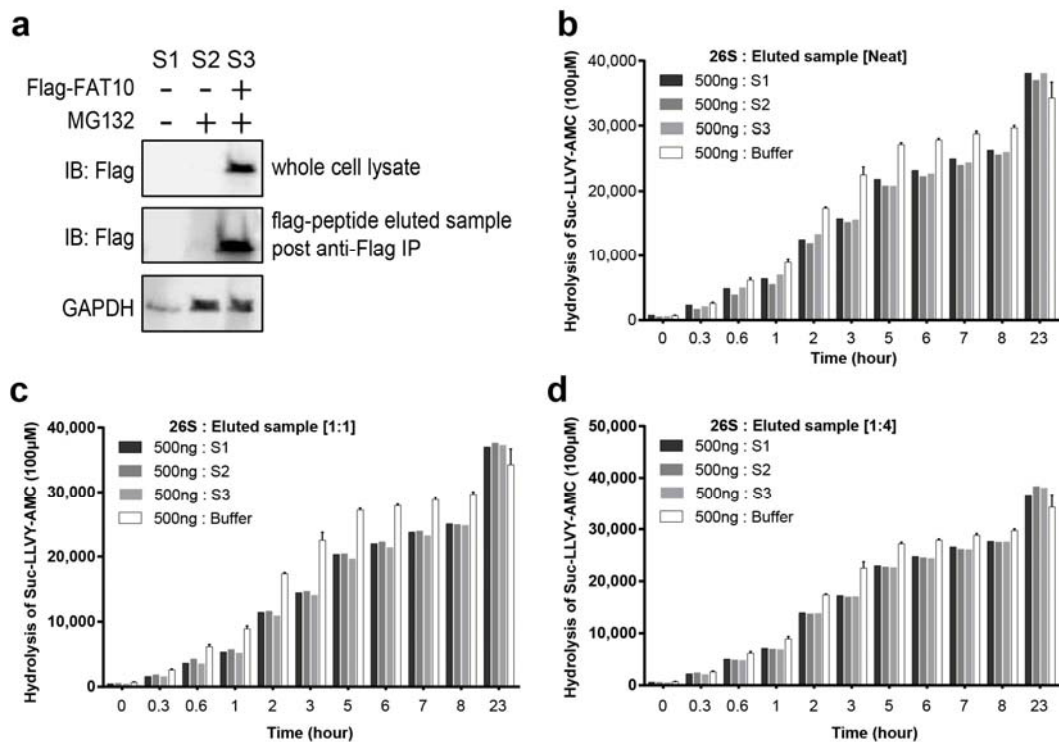
FAT10 accelerates the degradation of proteins in a ubiquitin-independent manner. So far, no information is available about whether binding of FAT10 to the 19S regulator of the 26S proteasomes influences the conformation of the enzyme complex. Thus, we analyzed the effect of FAT10 binding on the proteolytic activities of the 26S proteasome. To characterize the activation process, we incubated a constant amount of 26S proteasome with increasing concentrations of FAT10 to determine the concentrations that maximally stimulate peptide hydrolysis.



**Figure 26. Recombinant FAT10 stimulate the activity of the 26S proteasome**

Purified human 26S proteasome (500ng) was pre-incubated with or without proteins (i.e. FAT10, USE1-FAT10 conjugate, USE1 or BSA as control at different ratios of 26S proteasome to proteins as indicated for 15 min before addition of fluorogenic substrate. The ChT-like activity of the 26S proteasome was measured using Suc-LLVY (100µM) fluorogenic substrate after 15 min incubation at 37°C. The units for the y-axis are the AMC fluorescence in arbitrary units (Tecan); **[a]**. Recombinant FAT10 was pre-incubated with the 26S proteasome; **[b]**. HEK293T cells were transiently cotransfected with expression plasmids for HA-USE1 and Flag-FAT10. After 24h of transfection total cell extracts were used to immunoprecipitate HA-USE1/FAT10 conjugate using anti-HA agarose beads (Sigma). Beads were washed twice with NET-TN and NET-T buffer and eluted in 150µl of reaction buffer containing 15µl HA-peptide (Sigma, I2149). Eluates obtained from five 10 cm dishes were pooled together (5x) and used for 26S stimulation; **[c]**. HEK293T cells were transiently transfected with expression plasmids for only HA-USE1. 24h after transfection, total cell extracts were used to immunoprecipitate HA-USE1 using anti-HA agarose beads (Sigma). Beads washed twice with NET-TN and NET-T buffer and eluted in 150µl of reaction buffer containing 15µl HA-peptide. Eluate obtained from one 10 cm dish was (1x) used in the experiment and served as negative control for an experiment in **[b]**; **[d]**. Bovine serum albumin (BSA, Sigma) served as negative control for experiment in **[a]**, **[b]** and **[c]**.

In a preliminary experiment, we pre-incubated the 26S proteasome with recombinant FAT10 or FAT10 conjugated proteins for 15min and subsequently monitored the chymotrypsin-like activity of the 26S proteasome using the fluorogenic peptide substrate Suc-LLVY-AMC. The ChT-L activity of the 26S proteasome was found to be increased in the presence of different amounts of recombinant FAT10 (**Fig. 26a**). We monitored the fluorescence till 17 hours after substrate addition, at this time point, AMC fluorescence seemed to have reached saturation.



**Figure 27. FAT10-conjugate (S3) did not stimulate the activity of the 26S proteasome**

Purified 26S proteasome (500ng) was pre-incubated with or without eluate from S1, S2 or S3 at different ratios of 26S proteasome to eluate as indicated for 15 min before addition of fluorogenic substrate. The ChT-like activity of the 26S proteasome was measured using Suc-LLVY-AMC (100µM) fluorogenic substrate after 15 min of incubation at 37°C; **[a]**. HEK293T cells were transiently transfected with expression plasmids for Flag-FAT10. Before harvest, cells were treated with MG132 for 6 hours. 24h after transfection total cell extracts were used to immunoprecipitate Flag-FAT10 conjugate using anti-Flag agarose beads (Sigma). Beads were washed twice with NET-TN and NET-T buffer and eluted in 150µl of reaction buffer containing 15µl Flag-peptide. Eluates were labeled as S1, S2, and S3 and used for stimulation as indicated. Eluates were separated under reducing conditions (10% 2-mercaptoethanol) on 12% Laemmli SDS-PAGE gels. The upper panel shows an immunoblot of protein expression levels in total cell lysates (input). The middle panel shows the immunoblot of eluate S1, S2, and S3. GAPDH was used as loading control. One representative immunoblot is shown; **[b]** represents the 26S stimulation test using neat eluates; **[c]**. represents 26S stimulation with 1:1 diluted eluates; while **[d]** represents the 26S stimulation test using 1:4 diluted eluates. One representative experiment is shown.

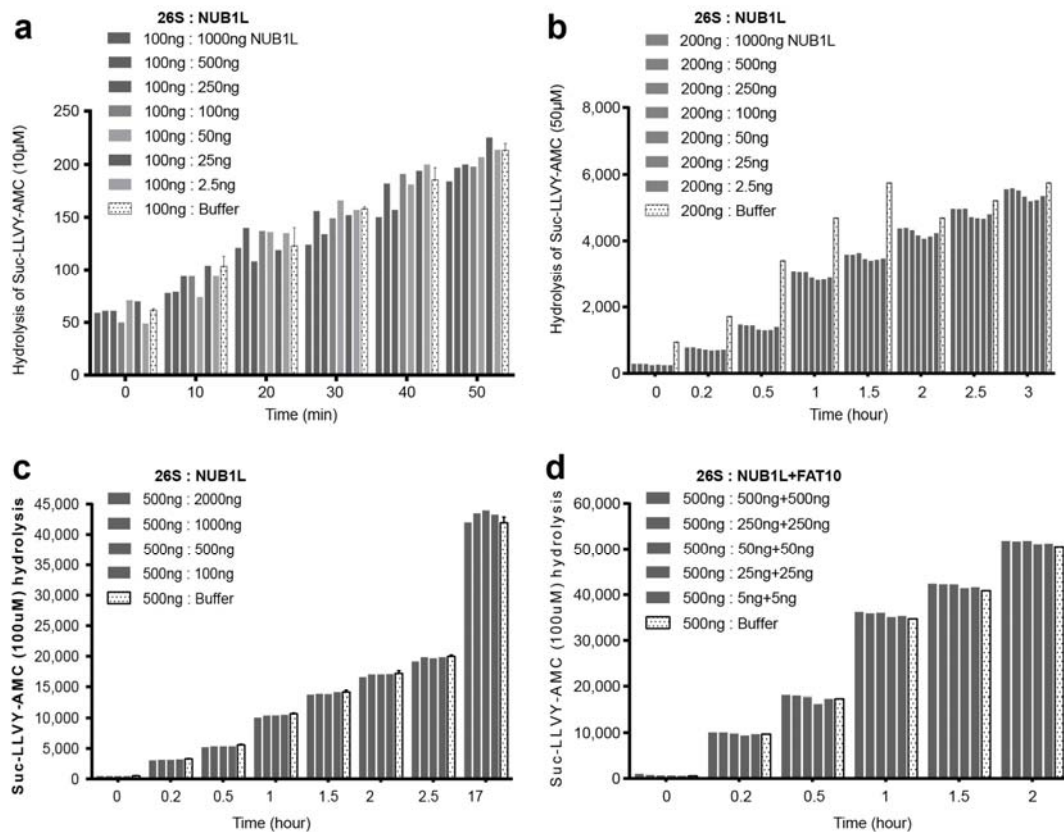
We used an excess of FAT10 compared to 26S in this experiment to test 26S stimulation, and only reaction buffer as negative control. We observed 26S stimulation by FAT10 in excess i.e. 1:4 or 1:2. It was surprising to observe even a minimal amount of FAT10 i.e. 1:0.2, could still stimulate the 26S ChT-like activity.

#### **FAT10 conjugates from HEK293 cells do not stimulate 26S proteasome activity**

Various conjugates of FAT10 have been reported up to now. USE1 is a conjugating enzyme for both FAT10 and ubiquitin, furthermore, FAT10 could form covalent conjugates with USE1 [74]. Therefore, in the present study, we tested if there is a change in 26S proteasome activity in the association of FAT10ylated protein. To obtain USE1-FAT10 conjugate, his3xFlag-FAT10 and HA-USE1 were co-overexpressed in HEK293T cells and at 24h post transfection total cell lysate was immunoprecipitated using anti-Flag beads followed by elution of tagged protein with flag peptide. We expect FAT10-USE1 conjugate in the Flag peptide eluted sample. As a negative control, cells overexpressing only HA-USE1 followed by immunoprecipitation using anti-HA beads and eluted with HA-peptide were used in the study. For the activation assay, Flag-peptide eluted USE1-FAT10 conjugate and HA-peptide eluted USE1 (negative control) proteins were incubated with 26S proteasome for 15 min. Subsequently, we determined the ChT-like activity of the 26S proteasome using the fluorogenic peptide substrate Suc-LLVY-AMC.

We observed mild stimulation with both eluates containing either USE1-FAT10 or USE1 which was surprising (**Fig. 26b, c**). As a reference control, we pre-incubated the 26S proteasome with BSA and unexpectedly found a similar pattern of stimulation as the conjugates (**Fig. 26d**). As high molecular weight conjugates have been observed in cells overexpressing wild-type conjugatable FAT10 (FAT10GG) but not its non-conjugatable (FAT10ΔGG) [72][83]. FAT10 is a degradation signal and has been claimed to be itself degraded via ubiquitination [70]. Therefore, we tested whether FAT10 conjugates alone could have any influence on the ChT-like activity of the 26S proteasome. To obtain only FAT10 conjugates we overexpressed his-3xFlag-FAT10 in HEK293 cells under proteasomal inhibition condition, followed by immunoprecipitation using anti-Flag beads. FAT10 was eluted from the anti-Flag matrix using flag-peptide and the eluate was used in the study as a FAT10 conjugate (**Fig. 27a**). We stimulated 500 nanograms of 26S proteasome with all the three eluates S1, S2 and S3 for 15 min and the ChT-like activity was monitored till 23 hours post substrate addition (**Fig. 27b**). Stimulation with either of the eluates S1, S2 or S3 diluted

in 1:1 and 1:4 using buffer did not show any stimulation (**Fig. 27c, d**). Similarly, using neat eluates showed no activation compared to the buffer control. An already published report states that NUB1L non-covalently interacts with the 26S proteasome via its N-terminus and with FAT10 via its C-terminus and this interaction accelerates the degradation of FAT10 and FAT10 linked protein [89][90].

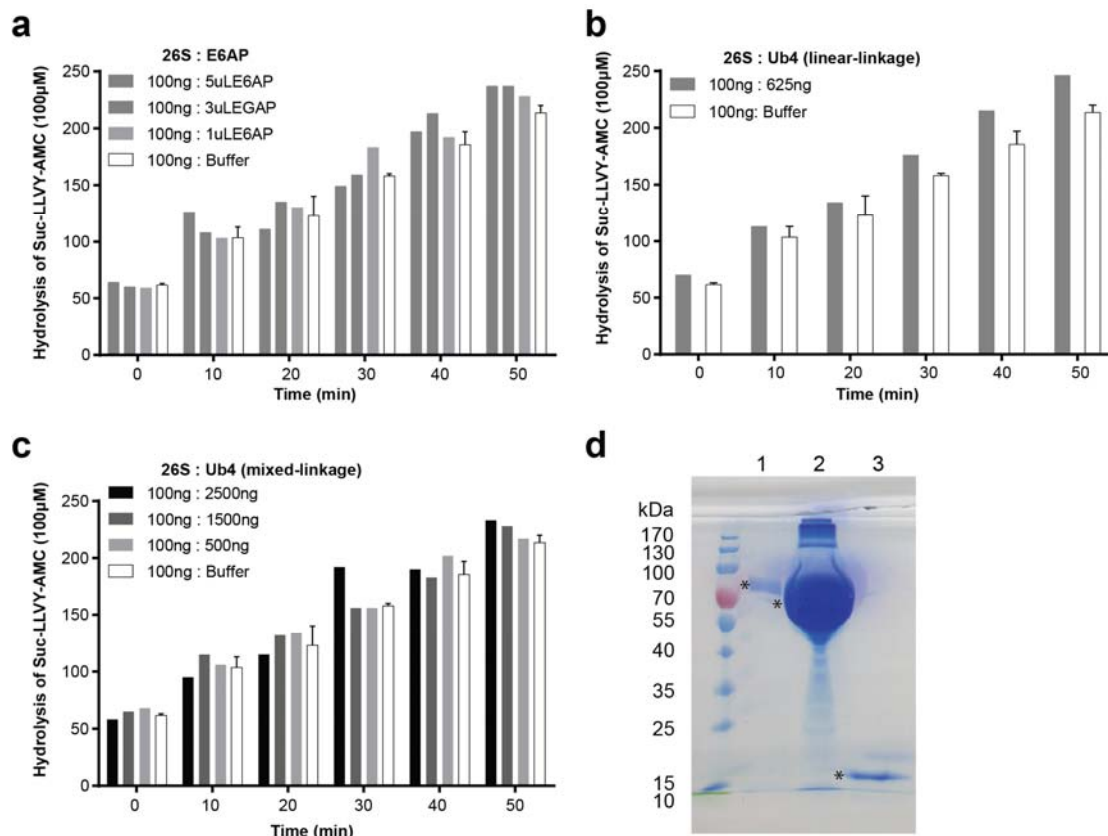


**Figure 28. NUB1L alone or with FAT10 did not influence 26S activity**

Purified 26S proteasome was pre-incubated with or without proteins (i.e. NUB1L alone or NUB1L with FAT10) at different ratios of 26S proteasome to proteins as indicated for 15 min before addition of fluorogenic substrate. The ChT-like activity of the 26S proteasome was measured using Suc-LLVY fluorogenic substrate after 15 min incubation at 37°C; **[a]** 100ng of 26S was stimulated with different amounts of NUB1L as indicated and 10 $\mu$ M of Suc-LLVY-AMC was used; **[b]** 200ng of 26S was stimulated with different amounts of NUB1L as indicated and 50 $\mu$ M of Suc-LLVY-AMC was used; **[c]** 500ng of 26S was stimulated with different amounts of NUB1L as indicated and 100 $\mu$ M of Suc-LLVY-AMC was used; **[d]** 500ng of 26S was stimulated together with different amounts of NUB1L and FAT10 as indicated and 100 $\mu$ M of Suc-LLVY-AMC was used. One representative experiment out of two independent experiments is shown.

Hence, we tested the ChT-like peptidase activity of the 26S proteasome in the presence of highly purified untagged recombinant NUB1L (**Fig. 28**). Moreover, the

ratio between the 26S proteasome/protein-substrate/fluorogenic peptide substrate was kept in a previous study at 5nM/500nM/10,000nM respectively [249], whereas in another study it was set as 30nM/300nM/100,000nM respectively [248].



**Figure 29. E6AP or Ub4 cannot stimulate 26S activity**

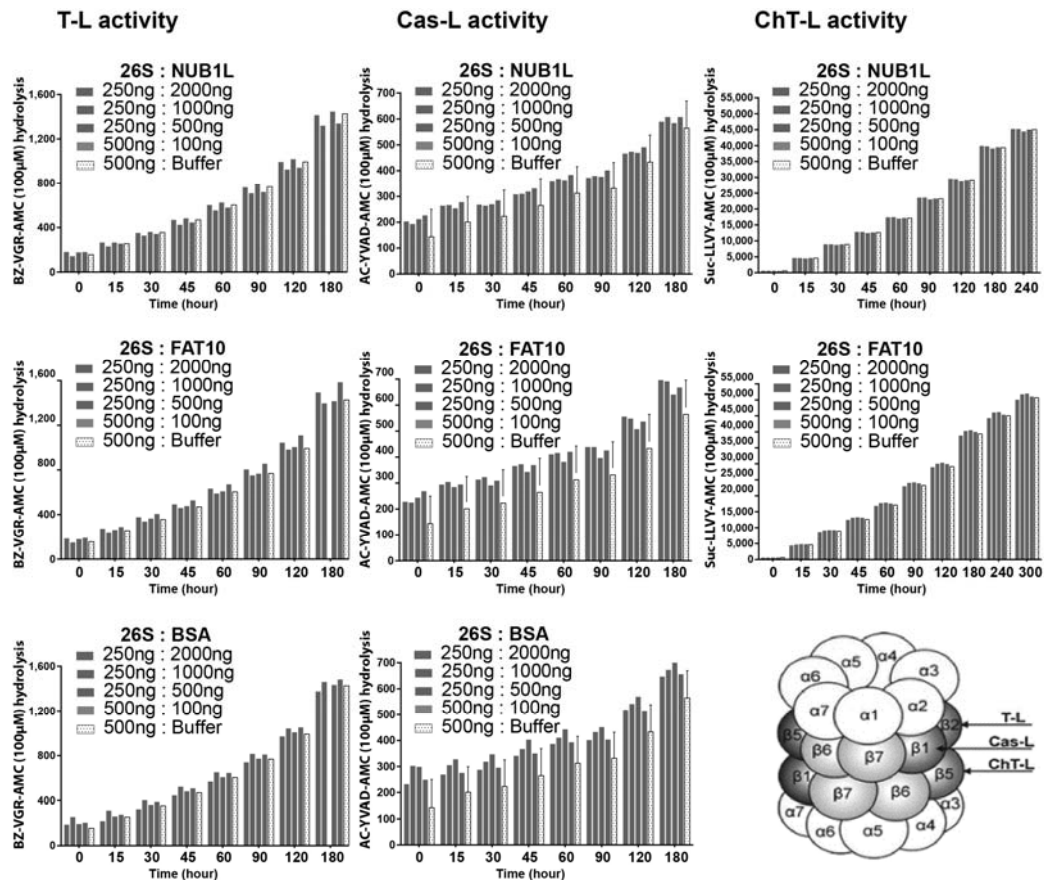
26S proteasome (100ng) was pre-incubated with or without proteins [i.e. E3 ligase E6AP, mixed-chain tetraubiquitin (Ub-K<sup>63</sup>-Ub-K<sup>48</sup>-Ub-K<sup>63</sup>-Ub), or K<sup>48</sup>-linked tetraubiquitin (Ub-K<sup>48</sup>-Ub-K<sup>48</sup>-Ub-K<sup>48</sup>-Ub)] at different ratios of 26S proteasome to proteins as indicated for 15 min before addition of fluorogenic substrate. The ChT-like activity of 26S proteasome was measured using Suc-LLVY-AMC (100µM) fluorogenic substrate after 15 min incubation at 37°C; **[a]** shows the 26S activity in the presence of different concentration of E6AP; **[b]** shows the 26S activity in presence of different concentrations of Ub4 (linear-linkage); **[c]** shows the 26S activity in presence of different concentrations of Ub4 (mixed-linkage); **[d]** indicated 12% SDS-PAGE followed by coomassie staining representing recombinant NUB1L protein in lane 1, BSA in lane 2 and recombinant FAT10 in lane 3 indicated as (\*). One representative experiment out of two independent experiments is shown.

So we tested the ChT-like activity of the human 26S proteasome in the presence of excess NUB1L protein i.e. 1000ng, 500ng, 250ng, 100ng, 50ng, 25ng, and 2,5ng, with three different concentrations of proteasome such as 100ng, 200ng or 500ng. Moreover, three different concentrations i.e. 10µM, 50µM, and 100µM of fluorogenic

peptide substrate Suc-LLVY-AMC was used, respectively. We observed no significant stimulation of the ChT-like activity by NUB1L (**Fig. 28a**). The experiment was repeated with the same setup, but this time, 50 $\mu$ M fluorogenic peptide with 200ng of 26S (**Fig. 28b**) or 100 $\mu$ M fluorogenic peptide with 500ng 26S (**Fig. 28c**) was employed, but again NUB1L did not significantly stimulate the ChT-like activity of the  $\beta$ 5-subunit.

We further tested the peptidase activity of the 26S in combination with NUB1L and FAT10 together. For the assay 500ng of 26S proteasome was pre-incubated for 17min at 37°C with an excess of NUB1L with FAT10. Post pre-incubation 100 $\mu$ M fluorogenic substrate Suc-LLVY-AMC was added and the fluorescence of liberated AMC was monitored for 2 hours. Compared to buffer control, we observed no increase in stimulatory activity of 26S proteasomes in the presence of increasing amounts of NUB1L and FAT10 in combination (**Fig. 28d**). The presence of the majority of ubiquitin ligase i.e. Ube3c, Rnf181, Ubr4, Huwe1, and Ube3a/E6AP on the proteasome is not very clear. The addition of E6AP showed no effect, but ubiquitinated E6AP showed an increase in the ChT-like activity of 26S [249]. Furthermore, the degradation process by 26S required the covalent attachment of a chain of four or more ubiquitin (Ub) molecules [6]. So we tested the ChT-like activity of the 26S proteasome in the presence of tetraubiquitin chains (Ub4) linked to lysine residues of another moiety forming mixed linkage i.e. Ub-K<sup>63</sup>-Ub-K<sup>48</sup>-Ub-K<sup>63</sup>-Ub or linear tetra-ubiquitin (Ub4). When the mammalian 26S (100ng) was incubated with different amounts of E6AP we observed a little increase in activity compared to buffer but this was no significant (**Fig. 29a**). Both linear and mixed tetra-ubiquitin (Ub4) non-significantly stimulated the ChT-L activity (**Fig. 29b, c**). In all the above experiments, we tested only the ChT-like activity ( $\beta$ 5-subunit) of the 26S proteasome in the presence of various proteins and conjugates i.e. FAT10, NUB1L, E6AP, and Ub4 as our stimulants. We also tested whether the above-observed peptidase activities were restricted to the ChT-like active site or can affect other proteasomal activity. We further tested the peptidase activities by the  $\beta$ 1-subunit and  $\beta$ 2-subunit of the 26S proteasome.

Thus, we analyzed the hydrolysis of the fluorogenic peptide AC-YVAD-AMC for the caspase-like activity (Cas-L) of the  $\beta$ 1-subunit and BZ-VGR-AMC for the trypsin ( $\beta$ 2) like activity (T-L) of the 26S proteasome. The addition of FAT10, NUB1L or BSA did not significantly stimulate the trypsin or caspase-like activity of the 26S proteasome (**Fig. 30**).



**Figure 30. None of the three activities of the human 26S proteasome are affected by FAT10 or NUB1L**

Purified 26S proteasome was pre-incubated with or without proteins (i.e. FAT10, NUB1L and BSA as control) at different ratios of 26S proteasome to proteins as indicated for 15 min before addition of fluorogenic substrate. The trypsin-like (T-L), caspase-like (Cas-L) or chymotrypsin-like (ChT-L) activity of the 26S proteasome were measured using fluorogenic substrate after 15 min incubation at 37°C as represented in the first column, second column, and the third column respectively. 100 $\mu$ M of fluorogenic substrates; Suc-LLVY-AMC (ChT-L), AC-YVAD-AMC (Cas-L), or BZ-VGR-AMC (T-L) were used. The upper row shows 26S activities in the presence of NUB1L, middle row shows different activities of 26S in the presence of FAT10, and the bottom row indicated different activities of 26S in the presence of BSA. One representative experiment is shown. The drawing at the bottom corner depicts the three different catalytic subunits in the 20S proteasome.

## 4. Discussion

The human genome encodes approximately 90 DUBs [255][43], but the biological functions and target proteins are unknown for most of them. Recently DUBs have received great attention by the pharmaceutical industry as drug targets because the inhibition of single isopeptidases allows enhancing the ubiquitin tree formation on their specific substrates. By this way, oncogenes or apoptosis inhibitors, which are often overexpressed in cancer cells, can be targeted for degradation by the proteasome. The ubiquitin-like modifier FAT10 also targets its many substrate proteins for proteasomal degradation, some of which are oncogenes and apoptosis inhibitors [247][69]. A wide class of DUBs with specificity for ubiquitin have been categorized, ubiquitin specific proteases (USPs) being the largest family, ovarian tumor proteases (OTUs), JAMM metalloproteases, ubiquitin C-terminal hydrolases (UCHs), and Josephins being the smallest family. Post-translational modification of proteins regulate the fundamental cellular processes. PTMs such as ubiquitination, FAT10ylation, neddylation, sumoylation and ISGylation have their own biological significance. The post-translation modifier ubiquitin is well understood, while other proteins which covalently modify target substrates are ULMs [256]. ULMs consist of ubiquitin-like domains and modify cellular target proteins, but in a different cascade than ubiquitin. Some of the modifiers like NEDD8, ISG15, and SUMO are well characterized, but for FAT10, the biological function is still unknown and controversial especially with respect to apoptosis induction. Additionally, DUBs have so far been found for most of the modifiers (Ub, SUMO, NEDD8, ISG15 etc.). For example, the interferon-stimulated-gene 15 was the first ULM to be discovered [257]. The UBP43/USP18 deconjugating enzyme belonging to the ubiquitin-specific protease family has ISG15 isopeptidase activity [258]. SUMOylation is reversed by SUMO-specific deconjugating enzymes called Ulp/SENPs [64][62]. USP21, UCHL1, and UCHL3 proteases have dual specificity and remove NEDD8 and ubiquitin from the cellular target protein. For FAT10, however, no such enzymes have been described. So we thought if nature has created FAT10 as an alternative or helping hand for ubiquitin (i.e. FAT10 accelerates protein degradation in a ubiquitin-independent manner), we hypothesized there must be a specific deconjugating enzyme for FAT10. Therefore, it is worthwhile and challenging to find the deconjugating enzymes specific for FAT10 in particular because FAT10 is the only ubiquitin-like modifier targeting

substrates for proteasomal degradation like ubiquitin does. The role of FAT10 has been implicated in the regulation of many cellular processes, including apoptosis, chromosomal instability, mitosis, immune system and 26S proteasome-dependent protein degradation [78]. FAT10 has been shown to be deregulated in tumor cells and ageing can be inhibited by turning off the genes encoding proteins participating in the aging process such as FAT10 [108].

### **Enzymes deconjugating FAT10 could be potential drug targets**

Accumulating evidence indicates that DUBs are important targets for pharma industries because they are like the goose that laid the golden eggs. Since attempts to cure many deadly diseases like cancer, tuberculosis etc. have failed for decades, one drug is not enough, a combination of drugs or therapies is required to combat these diseases. Thus, inhibiting DUBs could enable novel therapeutic combinations. DUBs maintain the ubiquitin pool in the cell, which they do by cleaving ubiquitin from proteins. In addition, DUBs also prevent 26S proteasomal degradation of ubiquitin-tagged proteins. So in cancers, some oncogenes and apoptotic inhibitors are up-regulated because of DUBs acting on them and remove the ubiquitin chain at the end preventing them from 26S proteasomal degradation. UPS is a coordinate system where ubiquitinating enzymes, proteasome, and the DUBs function in a cooperative manner. The presence of USP14 at the neuronal synapses function in local ubiquitin recycling by the proteasome to control the development and function of neuro muscular junction in mammals, and absence of USP14 results in neuronal dysfunction [259]. Thus, UPS dysfunction has also been implicated in the pathogenesis of various neurological diseases, like Alzheimer's, spinocerebellar ataxia, and other motor neuron diseases. Deregulated DUBs or proteins favor disease pathology by various mechanisms which can be targeted for therapeutic purpose. Overexpression of components of a multi-protein E3 ubiquitin ligase (SCF complex) has been found in a number of human cancers [260]. In addition to cancer, E3 ligases have also long been associated with neurodegenerative disorders i.e. Parkinson's, Alzheimer's, and Huntington's Disease [5]. Many DUB inhibitors are reported to date and are in various phases of clinical trials. But still is a lot is needed in this field to completely eradicate the disease havoc. Thus, this study was undertaken to search for FAT0 deconjugating enzymes, which could probably deconjugate FAT10 from substrate proteins thus preventing its FAT10 mediated degradation by the 26S proteasome. As various oncogenes and apoptosis inhibitors are substrates of FAT10, it's convincing to hypothesize that deconjugation of

FAT10 might lead to high substrate accumulation in cancer. Notably, inhibiting FAT10 specific DUBs might become a potential drug target for cancer treatment in the future.

#### **USP7 a novel FAT10 interacting partner**

According to our recent publication, showing a list of proteins interacting with FAT10 in an immune affinity isolation experiment, five DUBs belonging from two different families were interacting partners of FAT10. Two of the DUB showing ~18 kDa (mass of FAT10) more than their predicted molecular weight were reported as putative substrates (i.e. USP7, VCIP135) while the other three were classified as putative interacting partners (i.e. DUB3, USP11, SENP1)[81]. In this dissertation study, five deconjugating enzymes potentially interacting with FAT10 were selected and were investigated in detail. To date, many strategies have been applied to catch and characterize DUBs. In this study a fundamental approach of coexpression was applied, where one DUB at a time was overexpressed with FAT10 to check if it can be coimmunoprecipitated along with FAT10. In semi-endogenous condition, where the expression of FAT10 in HEK293T cells was induced by proinflammatory cytokines and Flag-tagged DUBs (USP7, SENP1, VCIP135, USP11, and DUB3) one at a time were transiently overexpressed simultaneously. Notably, following endogenous FAT10 induction with pro-inflammatory cytokines and simultaneously USP7 transfection of human embryonic kidney cells followed by immunoprecipitation of FAT10 using specific monoclonal antibody and staining for USP7 with an antibody against Flag-HRP, we observe a USP7 band at around 130 kDa (**Fig. 7a**). This indicates that USP7 coprecipitated with endogenous FAT10, in a semi-endogenous condition where endogenous FAT10 was induced and USP7 was transiently transfected. Furthermore, we could also show under transient overexpressed conditions that USP7 interacted with FAT10 (**Fig. 7b**). We even observed the positive interaction between endogenous USP7 and endogenous FAT10 in reciprocal Co-IP (**Fig. 7c**). If USP7 could have been covalently conjugated by FAT10, in that case, we should have seen a second additional band ~18 kDa above the USP7 band, but this was not the case. According to a recent report, covalent conjugation of FAT10 (FAT10ylation) to p62 (autophagy receptor) leads to its proteasomal degradation [81]. Proteins like HDAC6, NUB1L, AIPL1 interact non-covalently with FAT10. In addition, the deubiquitination by USP7 leads to Mdm2 stabilization, which further results in p53 downregulation [147]. USP7 itself is subjected to ubiquitination [261][262], NEDDylation [261], or phosphorylation [263][262] which is important for its activity. Further, for the activity of some DUBs

interaction with other proteins are important [154]. To test whether the interaction we observed between USP7 and FAT10 is direct or indirect (i.e. relying on an adaptor protein in between), purified Flag-tagged FAT10 and recombinant GST-USP7 proteins were tested for their interaction *in-vitro*. We observed that GST-USP7 could pull-down the purified recombinant FAT10 protein, which confirms a direct interaction between USP7 and FAT10 (**Fig. 8**). To further, support that the interaction between USP7 and FAT10 is not an artifact of overexpression, we performed knockdown studies in human embryonic kidney cells. Cells were subjected to two rounds of knockdown using siRNA against human USP7 and subsequently endogenous FAT10 was induced by proinflammatory cytokines. Following endogenous USP7 immunoprecipitation from USP7 knockdown cells, coimmunoprecipitated FAT10 was analyzed by immunoblotting against FAT10. Comparing the IP lanes, it was observed that with less endogenous USP7, less endogenous FAT10 was immunoprecipitated (**Fig. 9a**, 4 vs 2/3, IP lane). These results confirm that either in an endogenous or overexpression situation in human embryonic kidney cells, USP7 directly interacted with FAT10 or vice versa. Taken together, we provided evidence, that USP7 could be the deconjugating enzyme which not only interacts with FAT10 but possibly could be a deconjugating enzyme for the ubiquitin-like modifier, FAT10.

#### **USP7 and FAT10 dependence**

Many cellular proteins are conjugated with FAT10 through isopeptide bond [84][74]. The C-terminal diglycine in FAT10 is important for its conjugation to the substrates, as the non-conjugatable FAT10 mutant (FAT10 $\Delta$ GG) does not show high molecular weight FAT10 conjugates visible as smear [83]. We observed a significant reduction in FAT10 conjugates in the presence of USP7 indicated by 25% decrease in FAT10 smear intensity (**Fig. 10**). Reduced smear intensity was predicted to be an effect of USP7 on the bulk FAT10 conjugates, if this hold true in that case inhibition of USP7 could revert the smear to normal. Therefore, we overexpressed USP7 and simultaneously treated cells for 3h with cell permeable DUB inhibitors to inhibit USP7 in HEK293T cells. But, the immunoblot result does not clearly tell us if USP7 is the real player in FAT10 deconjugation (**Fig. 17**). The experiment has to be repeated with a fresh aliquot of inhibitors. Inhibitors were tested beforehand *in-vitro* and found to inhibit either eluted Flag-USP7 or recombinant hisGST-USP7 in the Ubiquitin-AMC assay (**Fig. 16**). FAT10 consist of two ubiquitin-like domains, whereas USP7 contains five C-terminal UBL domains, a catalytic domain, and an N-terminal TRAF-like domain. We

aimed to identify the binding region in USP7 interacting with FAT10. By using the USP7-NTD and USP7-CTD excluding the catalytic domain of USP7 for co-immunoprecipitation with endogenous FAT10 in HEK293T cells, we observed that the USP7-CTD interacted with FAT10 (**Fig. 20a**). Several proteins have been reported to interact with either C-terminal or the N-terminal domains of USP7. Binding of GMPS to C-terminus modulates the activity of USP7 [179][274]. So, we *in-vitro* tested the influence of recombinant FAT10 on the catalytic activity of USP7 using Ubiquitin-AMC as a substrate (**Fig. 18**). We do not observe a concentration-dependent effect of FAT10 on the catalytic activity of USP7.

In a preliminary CHX experiment, we have found that USP7 knockdown in HEK293T cells leads to a decrease of FAT10 levels (A. Aichem unpublished data). This could be because of more FAT10 ubiquitination in the absence of USP7; leading to FAT10 degradation by the proteasome hence less FAT10 level. In cancer cells high FAT10 could lead to downregulation of p53, thus less p53 in cancer cells may prevent the cancer cell from going to apoptosis and may favor cell survival. As USP7 is overexpressed in cancer cells, targeting USP7 with DUB inhibitors could lead to less FAT10 in tumor cells followed by more p53 and apoptosis of cancer cells. But, we don't know whether this downregulation of FAT10 was p53 dependent.

Additionally, FAT10 is the most upregulated gene in HCT116 cells post TNF- $\alpha$ /IFN- $\gamma$  treatment and TNF- $\alpha$ /IFN- $\gamma$  induces endogenous FAT10 through the NF- $\kappa$ B pathway [102]. The polyubiquitination of NEMO is required for NF- $\kappa$ B activation. The inhibitor HSCARG interacts with USP7 and USP7 deubiquitinates NEMO and acts as a negative regulator of NF- $\kappa$ B signaling [240]. Therefore, knockdown of USP7 could lead to more NEMO polyubiquitination, followed by activation of NF- $\kappa$ B leading to upregulation of FAT10 expression. On the other hand, we also speculated USP7 overexpression could downregulate NF- $\kappa$ B and by this means may downregulate the FAT10 level. However, we observed that the overexpression of USP7 does not have any influence on the FAT10 level in HEK293 cells. Neither wildtype USP7 nor catalytically inactive USP7 show any effect on the NF- $\kappa$ B level (**Fig. 21**).

#### **Efforts to generate stable transfectants expressing defined FAT10 conjugates for DUB overexpression screens**

Previously, it has been convincingly shown that FAT10 non-covalently interacts with MAD2 and NUB1L [87][89]. The cytokine inducible linker protein NUB1L non-covalently interacts with the 26S proteasome through its N-terminal domain and with

FAT10 through its three C-terminal domains and thus accelerate the degradation of FAT10 and FAT10-linked proteins [90]. We observed no change in USP7 level in the presence of FAT10 in whole cell lysates (**Fig. 7a, 9a, 10a**), indicating probably FAT10 is not involved in USP7 degradation. Subsequently, we investigated, if the interaction between USP7 and FAT10 has any role in deconjugating FAT10 from FAT10 conjugated substrates. So far, no chain of FAT10 has been reported, but multiple or mono-FAT10ylated substrates are well documented. As previously shown by our group, FAT10 conjugation leads to protein degradation by the 26S proteasome in a ubiquitin-independent manner [79]. Conjugates can be detected in HEK293 cells transfected with FAT10 with some of its reported substrates by immunoblotting [84][74]. Variation in transient transfection each time could affect protein expression. To avoid this caveat, we generated stable cell lines co-expressing FAT10 with one of its substrates. Stable cells expressing detectable conjugates could be subjected to transfection with cDNAs of 100 individual DUBs and change in bulk conjugates level could be observed with immunoblotting against FAT10. For DUB overexpression screens, a Flag-tagged DUB expression library consisting of 100 cDNA [225] was generated by the pharmaceutical company Genentech Inc. in South San Francisco, CA, USA, which we may be able to use.

We started our efforts with HEK293 cells stably expressing Flag-FAT10. These cells were further transiently co-transfected with puromycin and JunB plasmids in a ratio of 1:3 to generate stable cell lines co-expressing FAT10/JunB at the end. We chose JunB as one of the substrates for FAT10 because one member in our group already found that JunB can be FAT10ylated. After surviving months of puromycin pressure in 96 wells, stable clones screened both positive for FAT10 and JunB were further tested for conjugates using the immunoblotting technique. Different stable clones expressing JunB and FAT10 together were identified, but their conjugates were not observed most likely because of sensitivity limitations (**Fig. 11a**). A double band was always seen in the case of JunB which is most likely due to phosphorylation of JunB. We thought to increase the chance of seeing a conjugate by repeating the experiment in the presence or absence of proteasome inhibitor MG132. Furthermore, p62 as another substrate for FAT10 was used to generate stable cells co-expressing p62/FAT10 and screened them in the presence and absence of MG132 followed by immunoprecipitation of FAT10 (**Fig. 11b**). This time again clones expressed both proteins, but unfortunately, we could not detect any conjugate by immunoblotting. We concluded either our technique is not sensitive enough to detect the conjugate amount or it could be that

conjugate is not formed. We thought maybe if we are lucky enough, using other FAT10 substrates could show conjugates. So we selected UBE1, an E1 enzyme for FAT10. All stable clones of UBE1/FAT10 co-expressed UBE1 and FAT10, and surprisingly, we observed an intense band above UBE1 in whole cell lysate (**Fig. 12**, lane-6). The band could be UBE1-FAT10 conjugate at around 18 kDa above UBE1 (120 kDa), but to be sure, we need further validation experiments with our stable clone E1. Stable cells for USE1/FAT10 surviving the antibiotic pressure were propagated and frozen before the screening.

### **Deconjugation of isopeptide bond by USP7**

We have observed that recombinant FAT10-GFP fusion protein was cleaved in extracts of mouse thymus (Schmidtke G; unpublished data), i.e. an organ with highest FAT10 expression [96]. Keeping the central aim of this project, therefore, we tried to identify the enzymes, which can cleave the FAT10-GFP protein or cleave FAT10 from other substrates. To check if USP7 has any effect on linear peptide bonds at the C-terminus of FAT10, both FAT10-AV-GFP as non-cleavable and FAT10-GG-GFP as potentially cleavable linear peptide bond was used. We detected proper expression of the constructs by immunoblot, but deconjugation of GFP by USP7 cannot be confirmed from this experiment (data not shown). Experiments have to be repeated with proper control to answer this question.

Many studies have used *in-vitro* assay to show ubiquitination or sumoylation of proteins using their respective recombinant activating, conjugating and ligating enzymes. FAT10 was shown to be transferred onto UBE1 thus forming an isopeptide bond *in-vitro* [74]. In a parallel study in our laboratory, both UBE1 and JunB were found to be substrates of FAT10 and their FAT10ylation could be achieved *in-vitro* (unpublished). A recent report on conjugation of UBE1 with FAT10 leading to its proteasomal degradation also gives us a study model [84]. With our standard protocol using recombinant proteins, FAT10 was covalently conjugated onto UBE1 by UBA6, to form an isopeptide bond between FAT10 and UBE1. This FAT10-UBE1 conjugation *in-vitro* system was used to test the deconjugation of FAT10 using USP7, as we found its direct interaction with FAT10. Adding USP7 (Flag-peptide eluted), we did not observe any bond cleavage by USP7 (**Fig. 13a**). This raised the question whether the Flag-peptide eluted USP7 enzyme may be inactive. However, testing USP7 activity, it was found to be active in both Ubiquitin-AMC experiments (**Fig. 13b, 14b**) and in deubiquitinating poly-ubiquitinated <sup>35</sup>S-labeled p53 (**Fig. 15**). These results suggested

that USP7 cannot cleave an *in-vitro* generated isopeptide bond between FAT10 and UBE1. Similarly, using recombinant proteins, FAT10 was covalently conjugated to JunB catalyzed by UBA6. We also tested the bond deconjugation between *in-vitro* generated JunB and FAT10 by USP7, and again found no cleavage. The autophagy adapter protein p62 is covalently modified by FAT10 at several different lysines i.e. it gets multi-mono FAT10ylated [81]. Similarly, the tumor suppressor protein p53 is exported out of the nucleus after being multi-mono-ubiquitinated, suggesting a role of ubiquitination in protein localization [264]. USP10 deconjugates mono-ubiquitinated p53, which allows its re-import [265]. A report from our group stated that ubiquitination of FAT10 is not necessary for FAT10 degradation [69], but nevertheless, a Ub-FAT10 conjugate could be observed which was confirmed in the present study in three independent similar experiments (**Fig. 19**). We thought to test USP-dependent bond deconjugation by co-expressing both USP7 wild type and its catalytically inactive mutant USP7C223S. Currently, we do not have a clear result from these experiments probably because of technical reasons; still the experiment has to be repeated.

#### *Strengths and limitations*

Our study was limited to testing only five out of ~90 DUBs encoded by the human genome. As in a report, identifying substrates of endogenous FAT10 using proteomic analysis, all five DUBs were found in a list of putative FAT10 interacting proteins. Therefore, we speculated that if these DUBs coimmunoprecipitated with FAT10, they might be possibly interacting with FAT10 or could also deconjugate FAT10 or FAT10 linked substrates. Furthermore, developments of activity-based probes (ABPs) have accelerated the discovery and research on DUBs and ULM deconjugating enzymes. The ABPs contain three components [266][267], the *targeting element* gives specificity for the desired enzyme targets, and the targeting element in the case of DUB probes is ubiquitin. The second component, the *recognition* or *retrieval element* allows to immunoprecipitate or selectively retrieve and visualize the enzyme-ABP complex. Third, the *reactive group* or *warhead* reacts with the enzyme active site after the target element has bound. The epitope-tagged ubiquitin (HAUb) probe with C-terminal thiol-reactive group that acts as a suicide substrate is either alkyl halide (chloroethylamine, bromoethylamine, bromopropylamine) or michael-acceptor (glycine vinylmethylsulfone (VS), glycine vinyl methylester (VME), glycine vinylphenylsulfone (VSPH), and glycine vinylcyanide (VCN)) derived probes. In a report, where an HA-Ubiquitin derived probe using intein-based chemical ligation method was synthesized and different C-terminal

reactive groups were attached, followed by applying the probe to EL4 (mouse lymphoma cell line) cell lysates, 23 DUBs including 10 polypeptides whose enzymatic activity was not reported before were identified by mass spectrometry [267]. Therefore, our aim was to target putative FAT10 deconjugating enzymes in various tissue samples, including thymus and tumor using FAT10-VS active site probe and purify the enzyme with our FAT10 specific monoclonal antibody (4F1). We took initiative using a similar strategy to synthesize an active site probe targeting FAT10 deconjugating enzyme and teamed up with Dr. Benedikt Kessler from Oxford University and Dr. Huib Ovaa from the Netherland's Cancer Institute, two experts in this field. Long back Dr. Kessler's lab was unsuccessful in generating a soluble FAT10-thioester. Therefore, we planned to express SUMO-FAT10-intein-CBD fusion protein in *E. coli* to improve the solubility of the FAT10-thioester and received the HA-Ubiquitin-Intein-CBD and HA-FAT10-intein-CBD plasmid constructs from them. The constructs were expressed in bacteria and the cells pellets were lysed but precipitation was observed while probe synthesis after MESNa reaction. As ubiquitin contains no cysteine but FAT10 has four cysteine residues. So to improve the solubility, even after making SUMO-fusion construct together with replacing cysteine to serine in FAT10, we were unable to avoid precipitation of the probe after the MESNa reaction.

The fluorogenic substrate Ubiquitin-AMC has been used for DUBs screening [268]. Similar fluorogenic substrates SUMO-AMC, NEDD8-AMC, ISG15-AMC are available to test the activity of DUB specific for these ubiquitin-like modifiers. Synthesis of FAT10-AMC can probably provide clues for the presence of FAT10 specific deconjugating enzyme in thymus tissue or tumor cell lysate etc. Like AMC, the rhodamine derivatives such as carboxytetramethylrhodamine (TAMRA), and tetramethylrhodamine (TMR) are also used as a fluorescent agent. The reagent Ubiquitin-Lys-TAMRA (Ub-Gly-Gly- $\epsilon$ -Lys-TAMRA-Gly-OH) mimics the isopeptide bond between ubiquitin and  $\epsilon$ -Lys of a substrate, which can be used to detect DUB activity. Therefore, the synthesis of FAT10 specific TAMRA probe was initiated, in a reaction where UBA6 catalyzes the generation of FAT10-MESNa-thioester intermediate in the presence of MESNa and recombinant FAT10, followed by addition of TAMRA, which resulted, into TAMRA-Lysine-FAT10-Glycine (data not shown). The experiment was performed by one of our collaborators, Dr. Huib Ovaa, NCI, Netherland but after overnight incubation of the FAT10-TAMRA probe at 37°C, the protein precipitated. Experiments need trouble shooting and are currently being repeated with changes in buffer conditions.

**Endogenous FAT10 is not affected by p53 overexpression**

Under normal physiological conditions, the activity of tumor suppressor p53 is inhibited by RING-domain E3 ligase Mdm2. Mdm2 mediated ubiquitination leads to proteasomal degradation of p53. It is interesting to know that Mdm2 also acts as an E3 ligase in conjugating another ubiquitin-like modifier NEDD8 to p53 [276]. NUB1 regulates the balance between p53 ubiquitination and neddylation, for this NUB1 promotes p53 ubiquitination by decreasing p53 neddylation. Monoubiquitination rather than neddylation of p53 is the main signal triggering the p53 export from the nucleus to the cytoplasm, this leads to p53 inactivation. Altogether, neddylation, ubiquitylation and the activity of NUB1 cooperate to mediate this nuclear export [276]. Furthermore, binding of NUB1L to FAT10 accelerated the degradation of FAT10 and FAT10 linked proteins and one of its example is p53 [83][89][90]. Downregulation of FAT10 had been shown to be dependent on p53 [94]. Our aim was to test the relationship between p53 and FAT10 and to know whether NUB1L is also a participant or playing some role. We were able to reproduce data from Lee et al., [94] showing overexpressed p53 downregulated overexpressed FAT10 in both p53 negative cells and HEK293T cells (**Fig. 22, 24**). Additionally, we found that FAT10 can be induced in p53 null cells with proinflammatory cytokine stimulation, but with increasing p53 overexpression, we could not observe significant downregulation of endogenous FAT10 (**Fig. 23**). In all our p53 overexpression experiments in p53 negative cells, we could detect NUB1L in immunoblot but without any change in presence of p53. Also, the mRNA level of NUB1L was not affected quantified by real-time PCR.

**Foxp3 interaction with FAT10**

The stabilization of the E3 ligase (Mdm2) by USP7 leads to p53 destabilization [147]. The loss of USP5 in mammalian cells causes p53 stabilization by ubiquitin tree formation [148]. Thus, it can be inferred that different DUBs can target the same substrate. Furthermore, while roles of USP7 have been shown in several physiological processes, the role of USP7 as a deconjugating enzyme in FAT10 biology is unexplored. Similarly, Foxp3, a transcription factor is required for functional Tregs. USP7 interacts and removes ubiquitin from Foxp3 and prevents its proteasomal degradation leading to its stabilization in primary Tregs [186]. In a study conducted by Wellcome Trust Case Control Consortium in 2007, SNPs were reported in the USP7 gene among cohorts of autoimmunity patients. The effect of USP7 polymorphism on its function was not clear, but predicted to lower USP7 function, resulting in reduced

Foxp3 expression in Tregs and thus leading to autoimmunity. Likewise, FAT10 is highly expressed in mTECs [96] and found to have a functional role in human CD4<sup>+</sup> T-cells [109]. Our focus was to test whether there is any relation between FAT10 and Foxp3 and what is the cellular mechanism behind their interaction. We found that Foxp3 interacts with FAT10 when overexpressed in human embryonic kidney cells, which is quite striking (**Fig. 25a**). However, we do not observe an additional band just ~18 kDa above Foxp3 band, which might be an indication that Foxp3 is not conjugated with FAT10 (~18 kDa). Consistently, the presence of FAT10 did not significantly accelerate the degradation of Foxp3 (**Fig. 25b**). Here, we found that FAT10 not only interacts with USP7 but it also interacted with Foxp3, which cannot be ignored.

It is not only tempting to find a DUB for FAT10 but also to look for an E3 ligase, as E1 and E2 enzymes are already reported for FAT10. The number of E3 ligases is comparatively high (~600) in comparison to two E1-activating or E2-conjugating enzymes (~30) for ubiquitin. For FAT10, there is no E3 ligase reported so far. Recent reports claim that an E3 ligase (Stub1) interacts with Foxp3 [269], Foxp3 is stabilized by USP7 [186], and there are high FAT10 levels in Tregs [109]. These findings and our data on FAT10 interaction with both USP7 and Foxp3 could be an indication for the possible existence of a FAT10 specific E3 ligase. Our data and recent findings encourage us to perform future investigations on this topic.

#### **FAT10 did not enhance the peptidolytic activity of the 26S proteasome**

We observed as such no significant stimulation of the chymotrypsin-like activity of the human 26S proteasome either in the presence of recombinant FAT10 or recombinant NUB1L alone or in combination (**Fig. 25-30**). We also observed that the two additional peptide hydrolytic activities of the 26S proteasome; trypsin-like activity and caspase-like activity were not influenced by the presence or absence of either FAT10 or NUB1L. For each experiment, we have got a variation, which could be possibly due to the use of different lots of 26S proteasome, froze and thawed several times. The purified human 26S proteasome isolated from human erythrocytes obtained from Enzo Life sciences was used in this study. So for a better outcome, at least, to check the ChT-like activity in the presence of FAT10 and NUB1L together, fresh proteasome (both mice and human) could be purified with our standard protocol and screened with all possible stimulatory conditions.

Concluding, altogether we found that USP7, a deconjugating enzyme from the USP family interacts with FAT10. As the aim of this study was to search for

deconjugating enzymes for the ubiquitin-like modifier FAT10 and we were able to show that there is a direct interaction between FAT10 and the USP7 C-terminus. Notably, we have no clear evidence if USP7 can deconjugate FAT10 from its substrate but this is a novel finding in FAT10 biology as so far neither a DUB interacting with FAT10 nor any DUB which can deconjugate FAT10 from its substrate have been reported. So far, the biological function of FAT10 is not well characterized; our research study gives a new insight into the FAT10 field. Additionally, we confirmed FAT10 interaction with transcription factor Foxp3 in overexpressed condition using human embryonic kidney cells. It is known that USP7 interacts with Foxp3, and deubiquitination by USP7 leads to Foxp3 stabilization [186]. Human Tregs express FAT10 and FAT10 mRNA is induced upon Foxp3 overexpression in human CD4<sup>+</sup> T-cells [106]. Furthermore, overexpression of p53 downregulated FAT10 [94], but we thought that this could be an overexpression artifact. Thus, we tried to confirm this result in a human cell line which does not have p53 (H1299<sup>-/-</sup>). We were successful in reproducing already published data where overexpression of p53 could down-regulate overexpressed FAT10. But strikingly we found overexpressed p53 was unable to downregulate endogenous FAT10 either at the RNA or protein level. Likewise, we observed no increase in proteolytic activity of the human 26S proteasome in the presence of either FAT10 or NUB1L alone, in combination or with FAT10-conjugates from HEK293 cells. Overall, our study identifies USP7 as a new interaction partner of FAT10.

## 5. Material and Methods

### 5.1.1. *E. coli* competent cell preparation

#### LB media

Tryptone (BD)	1% (w/v)
Yeast extract (BD)	0.5% (w/v)
NaCl (Roth)	1% (w/v)

#### LB Agar

LB media	
Agar	0.5% (w/v)

#### CaCl<sub>2</sub>/Glycerol solution

100mM, CaCl <sub>2</sub> (Roth)
10% (v/v) Glycerol (VWR)
NaCl (Roth)

ampicillin 100µg/ml final conc.

kanamycin	70µg/ml final conc.
geneticin (G418)	20µg/ml final conc.
chloramphenicol	34µg/ml final conc.

Bacterial strains TOP10F', DH5α, XL1-Blue or Rosetta (ROS1) were used to prepare *E. coli* competent cells. First of all 5ml of LB broth without antibiotics were inoculated with a bacterial strain in glycerol stock and grown overnight at 37°C at 250 rpm. On the next day, 1ml of inoculum was transferred from 5ml overnight grown culture to 100ml of LB broth without antibiotics, and the culture was monitored till OD<sub>600</sub> reached 0.5. Next, cultures were subdivided into 50ml aliquots and kept on ice for 10 min, and now onward care was taken to keep culture always on ice. The 50ml aliquots were centrifuged for 10 min at 4°C, 4000 rpm. The supernatant was discarded carefully and the bacterial pellets were suspended in 50ml ice-cold 0.1M MgCl<sub>2</sub>. Bacterial suspensions were centrifuged for 10min at 4°C, 4000 rpm. Again the supernatants were discarded carefully and the bacterial pellets were re-suspended in 25ml ice-cold 0.1M CaCl<sub>2</sub>. Next, bacterial suspensions were centrifuged for 10 min at 4°C, 4000 rpm. After discarding the supernatant, the pellet was re-suspended in an 8ml ice-cold 86mM CaCl<sub>2</sub>/12.3% glycerol solution. Finally, competent cells were dispensed as 200µl aliquots into pre-cooled eppendorf tubes on ice and stored at -80°C for future experimental use.

### 5.1.2 Transformation of chemically competent bacteria

Into a pre-cooled eppendorf tubes placed on ice, 50µl of competent cells were dispensed and 2 to 5µl of the desired plasmid DNA of the respective concentration was added. The tubes were tapped few times and kept on ice for 10min. Next, heat shock was given at 42°C for 45 seconds and the tubes were incubated on ice for 2min. Afterward 500µl of pre-warmed SOC medium without antibiotics was added and kept for growth at 37°C at 250 rpm for 1 hour. After one hour 50µl, 100µl or 150µl of the transformed bacteria were plated on LB agar containing the selective antibiotics. Furthermore, LB plates were kept in a 37°C incubator overnight for bacterial colonies to appear.

### 5.1.3 Plasmid DNA purification

In 5ml of LB media containing selection antibiotics, one single colony was picked from LB culture plates and was inoculated and allowed to grow at 37°C at 250 rpm overnight. On the next day, a mini-prep was performed using a plasmid preparation kit (Macherey-Nagel) according to the procedure in the user manual.

To prepare a high amount of plasmid DNA, 200ml overnight cultures were required. For this, 1ml of inoculum from 5ml overnight culture as above was transferred to 200ml of LB broth containing selection antibiotics and further grown overnight at 37°C, 250 rpm. On the next day, the bacterial culture was harvested by centrifuging at 4000 rpm for 10min. The supernatant was discarded and the pellets were stored at -80°C or used for plasmid DNA purification using the Nucleo-Bond Plasmid Preparation Kit (Macherey-Nagel) according to the procedure in the user manual.

The 500ml cultures were allowed to grow at 37°C at 250 rpm overnight. On the next day cultures were harvested by centrifugation at 6000 rpm for 20min and pellets were used for plasmid DNA preparation. Nucleo-Bond PC500 Plasmid Preparation Kit (Macherey-Nagel) was used according to the procedure in the user manual. The DNA concentration was determined using the NanoVue Plus spectrophotometer (GE Healthcare). The blank was adjusted with MilliQ as all plasmid DNA was diluted in MilliQ water. GATC Biotech AG, Konstanz, Germany, sequenced the plasmids. In

800µl of overnight bacterial culture, 200µl of sterilized glycerol was added, vortexed and stored at -80°C as a glycerol stock.

**Table 4. Plasmid constructs used in the study**

Plasmid	Source
Flag-DUB3	[187]
Flag-HA-VCIP135	Addgene #22592
Flag-SENP1	[272], Addgene #17357
Flag-USP7C223	[270]
HA-FAT10 domain 1-GFP	PD Dr. Gunter Schmidtke, Uni Konstanz
HA-FAT10 domain 2-GG-GFP	PD Dr. Gunter Schmidtke, Uni Konstanz
HA-FAT10 $\Delta$ GG	[81]
HA-Ubiquitin	[80]
HA-Ubiquitin K48R	[69]
HA-USP11	[271]
Myc-USP7	[186]
pcDNA3.1-HA-FAT10	[1]
pcDNA3.1-his-3xFlag-FAT10	[76]
pCI-neo-Flag-USP7	Addgene #16655
pRc/CMV-p53	[273], gifted by Prof. Martin Scheffner, Uni Konstanz
Strep-his-GFP	PD Dr. Gunter Schmidtke, Uni Konstanz

### 5.1.4 Agarose gel electrophoresis

#### TAE

Tris, 40mM

Acetic acid, 20mM

EDTA, 1mM

#### 6x DNA loading dye

Glycerol 12% (w/v)

EDTA 60mM

Bromophenol blue, 0.0003% (w/v)

Agarose gel electrophoresis was performed to check the quality of purified plasmid DNA or RNA. 0.8% agarose was dissolved in TAE buffer and boiled for 1-5 min until the agarose was completely dissolved. Next, after the agarose solution had cooled down for 5min, ethidium bromide to a final concentration of approximately 0.3 - 0.5µg per 50ml gel was added and poured in a gel cassette to solidify. Samples were mixed

with the 6x loading buffer and loaded  $\leq 20\mu\text{l}$  per well, with 8 $\mu\text{l}$  of DNA marker (smart ladder 200bp to 10kb, eurorgentec) in one of the lanes for band size comparison. The gel was run at 110V for 1h and visualized using the Gel doc system (Bio-Rad).

## 5.2 Cell lines

### Freezing solution

90% (v/v) FBS

10% (v/v) DMSO

Human embryonic kidney (HEK) 293, 293T, and the human lung cancer cell line H1299wt p53<sup>-/-</sup> (a gift from Prof. Martin Scheffner, University Konstanz) were cultured in IMDM media (Gibco) supplemented with 10% FCS (Gibco) and 100U/ml penicillin and 100 $\mu\text{g}/\text{ml}$  streptomycin (Gibco). Cells were grown in 37°C incubator containing 95% relative humidity and 5% CO<sub>2</sub>. Depending upon the requirement either 75cm<sup>2</sup> flasks, 150cm<sup>2</sup> dish or 10cm dishes were used to maintain the cells and sub-cultured when the confluence reached 80-90%. The anchored cells were de-adhered from the surface of the culture flask or dish using 3 to 8ml of Trypsin/EDTA solution. Detached cells were suspended in FBS containing complete IMDM in order to inactivate the Trypsin/EDTA and the cell suspension was centrifuged for 5min at 900 rpm. The supernatant was discarded and the cell pellet washed once with IMDM medium without FBS by again centrifuging for 5 min at 900 rpm. Finally, the cell pellet was re-suspended in complete IMDM medium. Cells were counted using the Neubauer counting chamber or using Cellometer Auto 2000 Cell Viability Counter (Nexcelom Bioscience LLC., USA). Further, cells were either partially refilled into tissue culture flasks for culture maintenance or seeded for experiments. For long-term cell line storage, cells were suspended in 500 $\mu\text{l}$  to the 1ml freezing solution and dispensed in pre-cooled freezing vials. Freezing vials are then placed in a pre-cooled Nalgene freezing container, stored at -80°C for 1 week, and finally stored in liquid nitrogen stocks.

## 5.2.1 Transfection, FAT10 induction

	Cells lines	Seeding density	Growth medium
T-150 flask	HEK293T, HEK293	$4 \times 10^6$	25ml
T-150 flask	H1299	$2 \times 10^6$	25ml
T-75 flask	HEK293T, HEK293	$2 \times 10^6$	10ml
10 cm dish	HEK293T, HEK293	$2 \times 10^6$	10ml
6 well plate	HEK293T, HEK293	$0.2 \times 10^6$ per well	2ml

For transient transfection of expression plasmids or induction of endogenous FAT10 in either HEK293T, HEK293 or H1299 cells, first seeding was done as indicated. After 24h cells were transfected with expression plasmids using the TransIT®-LT1 Transfection Reagent (Mirus Bio LLC, USA). For transfection of cell in a T-75 flask, the transfection mixture was prepared in 776 $\mu$ l of IMDM media without FCS and without antibiotic, 24 $\mu$ l transfection reagent was added, vortexed and incubated at room temperature for 10min, followed by mixing 8 $\mu$ g plasmid DNA and further vortexed and incubated at room temperature for 15min before use. The transfection mixture was distributed drop by drop over the adherent cell layer. Note: the Mirus reagent to DNA ratio was kept at 3:1. For transfection of cells in a T-150 flask, 1552 $\mu$ l of IMDM, 48 $\mu$ l of transfection reagent and 16 $\mu$ g plasmid DNA was used. After 24h of incubation in a CO<sub>2</sub> incubator cells were harvested, lysed and proteins in the lysates were analyzed as per experimental requirement. For induction of endogenous FAT10, cells were stimulated with interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$ . Cells were treated with 400U/ml TNF- $\alpha$  (Pepro Tech) and 200U/ml IFN- $\gamma$  (Pepro Tech) for 24h [274]. Cells were lysed and proteins in the lysates were further analyzed as per experimental requirement.

## 5.2.2 Immunoprecipitation (IP)

The cell pellet was lysed in lysis buffer and all remaining volumes of whole cell lysate left after using 40 $\mu$ l for loading sample, was used for immunoprecipitation. Supernatants were incubated with either 30 $\mu$ l antibody-coupled beads or specific antibody together with 30 $\mu$ l protein A/ protein G beads. Immunoprecipitation was carried out at 4°C for 18h in a vertical roller followed by two washes with NET-TN and

two washes with NET-T for 2 min each at 6000 rpm. Combinations used for beads and antibodies were as follows:

#### 1% NP-40 lysis buffer

20mM Tris-HCl pH 7.6
50mM NaCl
10mM MgCl <sub>2</sub> .6H <sub>2</sub> O
1% NP-40 (IGEPAL® CA-630, Sigma, Cat. No. I-3021)
1x complete EDTA-free protease (Roche)

#### Wash buffer (NET-TN)

Tris-HCl, 50 mM, pH 8.0
NaCl, 650 mM
EDTA, 5 mM
Triton X-100, 0.5%

#### Wash buffer (NET-T)

Tris-HCl, 50 mM, pH 8.0
NaCl, 150 mM
EDTA, 5 mM
Triton X-100, 0.5%

Beads	Source	Volume [ $\mu$ l]
Red ANTI-FLAG® M2 Affinity Gel	Sigma	30 $\mu$ l
Red Anti-HA Affinity Gel	Sigma (E6779)	30 $\mu$ l
Red Protein A Affinity Gel	Sigma	30 $\mu$ l
Red Protein G Affinity Gel	Sigma	30 $\mu$ l +3 $\mu$ l, FAT10mAb (4F1; 4.5 $\mu$ g/ml)
Red Protein G Affinity Gel	Sigma	30 $\mu$ l +2 $\mu$ l USP7polyAb (1 $\mu$ g/ $\mu$ l)

Indicated beads were covered with 40 $\mu$ l of 4x GSB (containing 10%  $\beta$ -ME) and boiled for 5min at 95°C. 20 $\mu$ l of each sample was loaded per lane on 12% SDS-PAGE and analyzed by performing immunoblotting.

### 5.2.3 SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed to separate the proteins according to their molecular weight. Whole cell lysate of cell populations or the recombinant proteins were resolved by 12% or 10% SDS-PAGE. For cell lysis, the whole cell pellet was suspended in 1% NP-40 lysis buffer (1200 $\mu$ L for cells harvested from 10 cm dish, 2ml for cells harvested from a 150cm<sup>2</sup> flask) supplemented with protease inhibitor mix (Roche) and kept on ice for

30min with intermitted vortexing. Samples were centrifuged for 30min at 14,000 rpm, 4°C. The supernatant was collected in freshly labeled eppendorf tubes. Samples (input samples) were prepared by mixing 10µL of 4xGSB (10% β-mercaptoethanol) with 40µL of cell lysate supernatants and were boiled for 5min at 95°C. 20µL of boiled samples per lane or 8µL of PageRuler (10-170 kDa, Thermo scientific) in one of the lanes was loaded to compare the protein molecular weight. Gels were initially run at (60V, 60mA, 6W; 30min) followed by (110V, 112mA, 13W; 1h). Afterward, SDS gels were either subjected to coomassie staining or immunoblotting.

<b>5% Stacking Gel</b>	10ml	6.8ml H <sub>2</sub> O; 1.7ml 30% acrylamide; 1.25ml 1.0 M Tris, pH-6.8; 100µl 10% SDS; 100µl 10% APS; 10µl TEMED
<b>10% Resolving Gel</b>	30ml	11.9ml H <sub>2</sub> O; 10ml 30% acrylamide; 7.5ml 1.5 M Tris, pH-8.8; 400µl 10% SDS; 400µl 10% APS; 16µl TEMED
<b>12% Resolving Gel</b>	30ml	13.2ml H <sub>2</sub> O; 16ml 30% acrylamide; 10ml 1.5 M Tris, pH-8.8; 400µl 10% SDS; 400µl 10% APS; 16µl TEMED

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#### 4x Gel sample buffer (GSB)

40µl β-mercaptoethanol + 100µl of (\*) solution

(\*) 20%-SDS, 8ml-100% Glycerol, 6ml-1M TrisPH-6.8, 5ml/ BromophenolBlue, 40mg- H<sub>2</sub>O, 1ml

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#### 10x Tris/Glycine buffer

30.3g Tris base

144g Glycine

1000ml H<sub>2</sub>O

#### 1x Running Buffer (Towbin)

10ml 10% SDS

100ml 10x Tris/ Glycine

890ml H<sub>2</sub>O

### 5.2.4 SDS-PAGE staining

To visualize the quality of recombinant or eluted proteins used in this study, proteins after SDS-PAGE was visualized by performing coomassie blue staining. The gel was kept in coomassie stain overnight at room temperature with shaking followed by destaining. Overnight Sensitive coomassie stained gels washed with water for several hours and scanned using HP Scanjet Ilcx Scanner for the image. Sensitive coomassie stain was shaken well before use, and always stored in dark covered with aluminum foil.

**Coomassie staining solution**

0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva)
50% (v/v) Methanol
10% (v/v) Glacial acetic acid (Roth)
40% H <sub>2</sub> O

**Coomassie destaining solution**

40% (v/v) Methanol (Sigma)
10% (v/v) Glacial acetic acid
50% H <sub>2</sub> O

**Sensitive Coomassie**

5 % Coomassie G-250 in H <sub>2</sub> O (5 g in 95 ml H <sub>2</sub> O, CBB)	0.02 %	5ml
Aluminium Sulfate Hydrate (degree of hydration 14-18)	5 % (w/v)	50g
100 % Ethanol, p.a.	10 % (v/v)	100ml
85 % Phosphoric Acid (H <sub>3</sub> PO <sub>4</sub> )	2 % (v/v)	23.5ml
use ddH <sub>2</sub> O to makeup volume	-	1L

**5.2.5 Immunoblotting****1x Transfer buffer (Towbin)**

100ml 10x Tris/ Glycine
200ml Methanol (Sigma)
700ml H <sub>2</sub> O

Protein resolved by SDS-PAGE was transferred to a 0.45 $\mu$ m pore size nitrocellulose membrane (GE Healthcare) using wet electroblot transfer systems (BioRad). The wet transfer was achieved at 110V for 75 min in 1x Towbin buffer. The membrane was removed carefully, washed once in TBST, and blocked overnight in 5% milk in TBST. Next, the membrane was probed with primary antibody diluted in TBST (0.02% NaN<sub>3</sub>) for 2h at room temperature or overnight at 4°C. For secondary antibody staining blots were washed once with TBST after primary staining and probed with secondary antibody conjugated with horseradish peroxidase (HRP) diluted in TBST, followed by incubation for 1h at room temperature or overnight at 4°C. The nitrocellulose membrane was washed in TBST for 15min and protein on the blot were detected by chemiluminescence using HRP substrate (SuperSignal® West Pico Prod# 1856136 or SuperSignal® West Femto maximum sensitivity substrate Prod# 34096, Thermo Scientific) with Molecular Imager® Gel Doc™ (BioRad).

Antibodies specific for Flag, GAPDH, HA tags and directly conjugated to HRP were also diluted in TBST and either incubated 2h at room temperature or overnight at 4°C.

Cells were lysed in 1% NP-40 lysis buffer supplemented with protease inhibitor (Roche) and kept on ice for 30min with intermitted vortexing. 10µl of cell lysates were mixed with 40µl of 4xGSB containing 10% 2-ME and were boiled for 5min at 95°C. 20µl of boiled samples were loaded onto a 12% SDS-PAGE gel and subjected to immunoblot analysis.

**Table 5. List of antibodies used in the study**

Antibody	Type	Source	Dilution
2° (secondary)	polyclonal goat anti-mouse Ig/HPR	Dako Denmark	1:1000
2° (secondary)	pAb swine anti-rabbit Ig/HPR	Dako Denmark	1:3000
Flag-HRP	mAb anti-Flag, A8592	Sigma	1:1500
GAPDH-HRP	mAb G9295	Sigma-Aldrich	1:5000
GFP	MMS-118P	Bio Legend	1: 500
GST	mAb, mice, G1160	Sigma	1:1000
HA-HRP	mAb anti-HA, H6533	Sigma	1:2000
His	Penta-His mouse mAb, #34660	Qiagen	1:2000
human FAT10	mAb, mice (4F1)	EnzoLifeScience [74]	1:1000
human FAT10	pAb, rabbit (105-4), 1067, 1069	[69]	1:1000
human USP7	pAb, rabbit #A300-033A	Bethyl	1:10,000
IkBα (N-terminal)	mAb, mice	Cell signalling	1:10,000
Myc-HRP	mAb anti-c-myc, A5598	Sigma-Aldrich	1:2000
NUB1L	-	[79]	1:500
p53	pAb, rabbit #FL-393	Santa Cruz	1:500
UBE1	pAb # PW8390	Biomol or Enzo	1:2000
Ubiquitin	FK2, mAb, #BML-PW8810-0500	EnzoLifeScience	1:1000

## 5.3 Quantification of gene expression

### 5.3.1 siRNA-mediated knockdown

Knockdown of endogenous USP7 in HEK293 cells was achieved by treatment with siRNA specific for human USP7. On target plus Smart pool siRNA (Thermo Scientific Dharmacon) was purchased and used as described in the manufacturer's instructions. On the 1<sup>st</sup> day, 0.1x10<sup>6</sup> cells were seeded per well in a 12 well plate in a total of 1ml IMDM with FCS but without (w/o) antibiotics (Abx). On the 2<sup>nd</sup> day, for each well both

siRNA mix (45µl 1x siRNA buffer, 50µl IMDM w/o FCS w/o Abx, 400nM siRNA) and Dharmafect solution (195.6µl IMDM w/o FCS w/o Abx+ 4.4µl Dharmafect) were prepared, and incubated for 5 min at room temperature. Next, 100µl Dharmafect solution was mixed with prepared siRNA mix and incubated for 20min at room temperature followed by addition of 820µl media (w/o Abx, with FCS). Old media from cultured cell was aspirated and replaced with a 1ml prepared transfection solution. On the 3<sup>rd</sup> day, cells were detached using 500µl trypsin/EDTA per well and transferred to one well of a 6 well plate. On the 4<sup>th</sup> day, for the 2<sup>nd</sup> knockout reaction components were prepared (99µl 1x siRNA buffer, 110µl IMDM w/o FCS w/o Abx, 880nM siRNA) and Dharmafect solution (429µl IMDM w/o FCS w/o Abx+ 11µl Dharmafect) following incubation as above followed by aspirating old media and replacing it with freshly prepared media. On the 5<sup>th</sup> day, cells from one well were distributed into two wells of a 6 well plate, in 2ml media with FCS w/o Abx. On day 6 cells were harvested and used for experiments.

### 5.3.2 Total RNA extraction

To quantify the relative gene expression in cells or to check the efficiency of a gene knockdown using siRNA, real-time PCR was performed. Total RNA was isolated from cells with the help of the RNA isolation kit RNeasy® Plus Mini Kit (Qiagen) following the manufacturer's instructions. In the final step of the RNA isolation protocol, the RNA was eluted in 50µl of RNA-free water. The RNA concentration was determined using the NanoVue Plus spectrophotometer (GE Healthcare).

### 5.3.3 Complementary DNA synthesis

Total 1µg of RNA was transcribed into complementary DNA (cDNA) using a reverse transcription kit (Promega) and oligo-dT primer. The reaction mixture was prepared as per the reagents shown in **Table-6**. In each PCR tubes, 1µg of total RNA added first and then 19µl of the reaction mixture (without RNA) was added, capped, and spinned followed by keeping at room temperature for 10min before PCR run. Samples were placed in a PCR thermocycler (Biometra) and the cDNA synthesis program was started as in **Table-7**. Finally, reaction tubes were briefly spun and stored at -20°C.

**Table 6. Reaction mixture for cDNA synthesis**

Ingredients	Volume [ $\mu$ l] per reaction
RNA	1 $\mu$ g
25mM MgCl <sub>2</sub>	4 $\mu$ l
Reverse transcription buffer 10x	2 $\mu$ l
Oligo dT15 Primer	1 $\mu$ l
dNTP mixture	2 $\mu$ l
AMV Reverse Transcriptase	0.625 $\mu$ l
RNasin® Ribonuclease inhibitor	0.5 $\mu$ l
Nuclease-free water	8.8 $\mu$ l

**Table 7. PCR programme for cDNA synthesis**

Step	Temperature	Time [min]
1	22°C	10
2	42°C	60
3	95°C	5
4	4°C	Stop

### 5.3.4 Quantitative real-time PCR

To measure the expression levels of human genes, quantitative real-time PCR was performed with the help of LightCycler® instrument (Roche) using Light Cycler Fast Start DNA Master SYBR Green I Kit (Roche). Reaction mixtures with desired primers were prepared, whereas GAPDH primer was used as a reference gene. The amplification program as shown in **Table-9** was followed. Reaction mixture and primers for real-time PCR are listed in **Table-8** and **Table-10**, respectively.

**Table 8. Reaction mixture RT-PCR**

Components	Volume/ reaction
25mM MgCl <sub>2</sub>	2.4 $\mu$ l
50 $\mu$ M Primer forward	0.5 $\mu$ l
50 $\mu$ M Primer reverse	0.5 $\mu$ l
SYBER green	2 $\mu$ l
H <sub>2</sub> O PCR grade	12.6 $\mu$ l
Complementary DNA	2 $\mu$ l

**Table 9. RT-PCR program for USP7, DUB3, SENP1, USP11, VCIP135, FAT10 and GAPDH**

Cycle	Temperature	Time	Steps
1	95°C	10 min	Initial denaturation
	95°C	10 min	Denaturation
40	60°C	5 min	Primer annealing
	72°C	11 min	Elongation
1	65°C	15 min	Denaturation
-	4°C	∞	Cool

Note: Annealing temperatures were different for p53 (70°C), eGFP (63°C), and Hdm2 p53 (67°C).

**Table 10. List of primers used for quantitative RT-PCR**

Human gene		Sequence
USP7	forward	5'-GAGTGATGGACACAACACCG-3'
	reverse	5'-AACACGGAGGGCTAAGGAC-3'
FAT10	forward	5'- AATGACCTTTGATGCCAACC-3'
	reverse	5'-GCCGTAATCTGCCATCATCT-3'
p53	forward	5'-TGGTGCCCTATGAGCCGCCT-3'
	reverse	5'-TGGTGAGGATGGGCCTCCGG-3'
NUB1L	forward	5'-AAA GGG ATG GGC TAC TCC AC-3'
	reverse	5'-CGT CTG TTG AGG CAC TAG AGG-3'
GAPDH	forward	5'-GAAGGTGAAGGTCGGAGT-3'
	reverse	5'-GAAGATGGTGATGGGATTTTC-3'
Hdm2	forward	5'-CGGGTCTCAGATGGAAACCCAAGACAAAGAAG-3'
	reverse	5'-GCGCTCGAGTTAGGGGAAATAAGTTAGCAC-3'
eGFP	forward	5'-AGT CCG CCC TGA GCA AAG A-3'
	reverse	5'-TCC AGC AGG ACC ATG TGA TC-3'

## 5.4 GST pull-down assay

To check if there is a direct interaction between human FAT10 and human USP7, a GST (glutathione S-transferase) pull-down assay was performed. In an eppendorf tube containing 500µl of 1% NP-40 lysis buffer, 0.5µg of recombinant FAT10 ( a gift from Nicola Catone, BiTg, Switzerland), 0.5µg recombinant GST-his-USP7 (Sino biological Inc.) and 0.5µg GST was added alone or in combination as depicted in Fig. 8. Tubes were incubated for 2h in a cold room at the vertical rotation. Next, to pull-down GST

tagged USP7 100µl of lysis buffer containing 20-30µl pre-blocked GSH beads were added to each tube and further incubated for 2h as done previously. Tubes were centrifuged at 6000 rpm for 2min at 4°C and supernatants were discarded. Further, beads were washed 3 times with NET-TN buffer followed by 2 washes with NET-T buffer by centrifuging at 6000 rpm for 2min at 4°C. Washed beads were suspended in 40µl of 4xGSB containing 10% β-mercaptoethanol and were boiled for 5min at 95°C. 20µl of each sample was loaded per lane onto 12% Laemmli SDS-PAGE gels and analyzed by performing immunoblotting. Glutathione Sepharose 4B beads (GE Healthcare) used were blocked overnight in the cold room with 5% BSA in MilliQ in order to reduce unspecific binding. Blocked beads were washed twice with NP-40 lysis buffer and were used in consecutive experiments.

## 5.5 Generation of stable cells

Stable cells constitutively expressing Flag-FAT10 endogenously (a gift from S. Lukasiak) [226] were maintained in complete media supplemented with G418 (2mg/ml, Gibco) and were used to generate another HEK293 stable transfectants. For generating FAT10/UBE1 coexpressing stable transfectants the Flag-FAT10 stable cells were transfected with HA-UBE plus puromycin selection plasmid in a ratio of 3:1. After 24h of transfection, cells were harvested and seeding was done in a round bottom 96 well plate at a density of  $10^3$  cells per 100µl complete media. Two days after seeding, 100µl of complete medium containing selection antibiotic puromycin (3µg/ml, Sigma) was added to each well and cultivated until resistant clones appeared. Resistant clones, which survived the antibiotic pressure were further expanded in separate culture dishes in complete media supplemented with both G418 and puromycin together. It was expected that positive clones which survived antibiotic pressure retained the puromycin plasmid only or puromycin plasmid plus the UBE1 expression plasmid integrated into the genome. Expanded clones that were screened positive for both UBE1 and FAT10 in immunoblot were used to further check the conjugates in the presence or absence of proteasome inhibition by MG132. Clones were named according to their original wells in 96 well plate e.g. clone B10, where B indicates the row and 10 indicated the column number in a 96 wells plate.

For making FAT10/p62 coexpressing stable transfectants the Flag-FAT10 stable cells were transfected with HA-p62 plus puromycin selection plasmid in a ratio of 3:1. After 24h of transfection, cells were harvested and seeding was done in a round bottom 96 well plate at a density of  $10^3$  cells per 100 $\mu$ l media. Two days after seeding, 100 $\mu$ L of medium containing selection antibiotic (3 $\mu$ g/ml) was added to the wells and cells were cultivated until resistant clones had appeared. Resistant clones, which survived the puromycin pressure, were further expanded in separate culture dishes in media supplemented with G418 and puromycin. Next, resistant clones screened positive for both p62 and FAT10 in immunoblot were used to further check the conjugates in the presence or absence of proteasome inhibition by MG132 (Sigma).

For generating FAT10/JunB stable transfectants the Flag-FAT10 stable cells were transfected with HA-JunB plus puromycin selection plasmid in a ratio of 3:1. At 2-4 days after transfection, selection antibiotic was added to the wells and cultured until resistant clones had appeared. Resistant clones, who survived the puromycin pressure, were further expanded in separate culture dishes in media supplemented with G418 and puromycin. Next, resistant clones screened positive for both JunB and FAT10 in immunoblot were used to further check the conjugates. For long-term storage, stable cells were suspended in a 1ml freezing mixture containing 10% DMSO and 90% FBS and dispensed in pre-cooled freezing vials. Freezing vials were placed in a pre-cooled Nalgene freezing container, stored at  $-80^{\circ}\text{C}$  for 1 week, and finally stored in liquid nitrogen or  $-150^{\circ}\text{C}$  freezer.

### 5.5.1 Antibiotics killing curve

For positive selection of stable transfectants in 96 well plates, the effective concentration of puromycin to kill HEK293 cells was determined on a killing curve.  $1 \times 10^3$  cells per well were seeded in 100 $\mu$ l complete medium (CM) per well of a flat bottom 96 well plate. On the next day, cells seemed ~50-70% confluent, then 100 $\mu$ l puromycin (1mg/ml) solution (2x antibiotics in CM) was added in each well, and incubated. Plated cells were observed daily for the percentage of cell survival. Optimum effectiveness should be reached in 1-4 days with puromycin. The minimum antibiotic concentration to use is the lowest concentration that kills 100% of the cells in 1-4 days from the start of the antibiotic selection. The dilutions of puromycin used per well; 20 $\mu$ g/ml, 10 $\mu$ g/ml, 5 $\mu$ g/ml, 4 $\mu$ g/ml, 3 $\mu$ g/ml, 2 $\mu$ g/ml, 1 $\mu$ g/ml, 0.5 $\mu$ g/ml, 0 $\mu$ g/ml. The cell viability was tested by MTT assay at different time points after puromycin

addition; 20, 42, 67, 95, and 120 hours. Puromycin concentration of 3µg/ml was found optimum for killing HEK293 cells.

MTT assay: Add 20µl MTT per wells (i.e. 5mg/ml MTT in PBS). One well with only MTT (without cells) was taken as control. Next, the plate was incubated for 3.5 hours at 37°C. Without disturbing the cell layer, all media were aspirated from the wells. 150µl DMSO solvent was added to each well and covered with tinfoil and cells were agitated on an orbital shaker for 15 min. The absorbance was measured at a wavelength of 570nm (590 nm with a reference filter of 620 nm).

## 5.6 DUB activity assay

To determine the catalytic activity of a deubiquitinating enzyme (Flag peptide eluted USP7 or recombinant hisGST-USP7), an *in-vitro* assay was conducted to detect the increase in AMC-derived fluorescence upon cleavage of Ubiquitin-AMC. The assay was performed as reported previously [239] with slight modifications. DUBs were added individually to 120µl deubiquitinating assay buffer (lysis buffer) per well, after adding 0.5µM Ubiquitin-AMC (# BML-SE211-0025, Enzo Life Sciences, Germany) the reaction plate was incubated at 37°C. The fluorescence intensity was monitored using the Tecan Infinite M200 pro machine with excitation and emission wavelength set at 360 and 465nm, respectively. Black flat bottom 96 wells plates (Greiner bio-one REF 655900) were used for the assay.

### 5.6.1 Elution by Flag-peptide or HA-peptide

For elution with Flag-peptide,  $4 \times 10^6$  HEK293T cells were seeded on 150 cm<sup>2</sup> culture dishes and on the next day cells were transfected with 16µg of the Flag-USP7 plasmid. After 24h of transfection, cells were harvested and lysed in 2ml of 1% NP-40 lysis buffer for 30min on ice with intermitted vortexing. Lysates were centrifuged at 14,000 rpm for 30min at 4°C and the collected supernatant was subjected to anti-Flag immunoprecipitation using 30µl Flag-M2 beads, at 4°C overnight on a vertical roller. The input sample was prepared before immunoprecipitation by mixing 10µl of 4xGSB (β-ME) with 40µl whole cell lysate and boiled for 5min at 95°C. The beads after overnight immunoprecipitation were washed twice with NET-TN and twice with NET-

T for 2 min. Washed IP beads were suspended in 150µl buffer containing Flag-peptide [Mix 3µl of 3xFlag peptide (stock 5mg/ml, SIGMA F4799) +147µl reaction buffer]. The suspension was incubated at 30°C for 2h under agitation at 1100 rpm followed by centrifugation at 6000 rpm at 4°C for 2min. The supernatant was collected (eluate) and the presence of the Flag-USP7 protein was confirmed by SDS-PAGE (Fig. 16c) and used for *in-vitro* assays (Fig. 13a, b; 14a, b; 15; 16a, 18a, b).

For the elution of HA-tagged USE1 by HA-peptide (I2149, Sigma), the same protocol was followed as for Flag-peptide elution. The supernatant was collected (eluate) and used for the experiment in Fig. 26b, c.

### **5.6.2. *In-vitro* conjugation assay**

Conjugation of FAT10 to its substrates UBE1 or JunB was catalyzed by UBA6. The reaction buffer supplemented with Tris-HCl (pH 7.6), 50mM NaCl, 10mM MgCl<sub>2</sub>, 5U/ml inorganic pyrophosphatase, 4mM ATP, 20mM creatine phosphate, 0.1mM DTT, 4µg/ml creatine phosphokinase and 1x protease inhibitor was prepared. For the generation of UBE1-FAT10 conjugate indicated in Fig.13, reaction tubes containing recombinant proteins UBA6 (0.65µg), UBE1 (4µg) or FAT10 (10µg) in a total volume of 20µl were incubated for 2h with 1150 rpm shaking at 37°C. For the generation of JunB-FAT10 conjugate indicated in Fig.14, reaction tubes containing recombinant proteins UBA6 (0.65µg), UBE1 (4µg) or FAT10 (10µg) in a total volume of 20µl were incubated for 2 hours with 1150 rpm shaking at 37°C. Next, 20µl eluted Flag-USP7 was added to the 20µl reaction and incubated further for 1h at 37°C with agitation during shaking at 1150 rpm. The reaction was terminated by adding 15µl 4x GSB (10% 2-ME) and boiled for 5min at 95°C. 30µl of samples were loaded per lane in SDS-PAGE and were subjected to immunoblotting.

### **5.6.3. *In-vitro* transcription and translation of p53 (IVTT)**

Using rabbit reticulocyte lysate and the TNT® T7 coupled transcription/translation kit (Promega) the pRcCMVp53 plasmid was translated and transcribed *in-vitro* into <sup>35</sup>S-methionine-labelled p53 protein. Reaction components were placed into 500µl microcentrifuge tubes with a total reaction volume of 40µl and incubated at 30°C for

2h to complete the reaction. Next, the terminated reaction mixture was dispensed as 20 $\mu$ l aliquots and stored at -80°C. Aliquots were used in the study as <sup>35</sup>S-methionine-labelled p53 protein. Plasmid DNA used in IVTT was pre-cleaned before use with NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel) as per the manufacturer's instructions.

#### **5.6.4. *In-vitro* p53 ubiquitination assay**

The <sup>35</sup>S-methionine (IVTT) p53 was used in this ubiquitination reaction. For p53-ubiquitination, 1 $\mu$ l of translated <sup>35</sup>S-labeled-p53 protein (1 $\mu$ l from 20 $\mu$ l aliquot) was incubated with 40 $\mu$ l reaction mixture containing 25mM Tris (pH-7.5), NaCl 100mM, DTT 1mM, 0.5 $\mu$ g ubiquitin, 2mM ATP, 2mM MgCl<sub>2</sub>, 1 $\mu$ l E1 enzyme, 1 $\mu$ l E2 (His-UbcH5b), E3 (i.e. 2.5 $\mu$ L 16E6 and 5 $\mu$ L E6AP together). Reactions consisted of two steps; [step-1] 90 min ubiquitination at 25°C, [step-2] 30 min termination of ubiquitination using EDTA at 25°C. As ubiquitination control, in one reaction tube, the E3 ligase was omitted. The ubiquitination reaction in the tubes was stopped by adding 20mM EDTA. Post reaction termination, 10 $\mu$ l 5xGSB was added to each tube and the tube was boiled at 95°C for 5min. 20 $\mu$ l reaction samples per lane were subjected to SDS-PAGE (10%). The SDS-PAGE was vacuum dried over Whatman filter paper. Dried gels were exposed overnight to a radio-imaging plate and visualized on a radio image (BioRad).

#### **5.6.5. *In-vitro* p53 de-ubiquitination**

Eluted Flag-USP7 was added to the tubes just after the p53-ubiquitination reaction had ended, to check if USP7 is active in removing or deconjugating ubiquitin from p53. The deconjugating enzyme UBP-core [234], known to deconjugate ubiquitin from p53 was taken as positive control while only buffer and Tris-HCl were taken as negative control. 20 $\mu$ l, 10 $\mu$ l, and 5 $\mu$ l of USP7 corresponding to lane 8, 7, and 6 were applied to ubiquitinated-p53, while 5 $\mu$ l, 2.5 $\mu$ l, and 1 $\mu$ l of UBPcore corresponding to lane 11, 10, and 9 were applied to ubiquitinated p53 (Fig.15). The reaction consisted of three steps; [step-1] 90 min ubiquitination at 25°C, [step-2] 30 min termination of ubiquitination using EDTA at 25°C, [step-3] USP7/UBPcore/Tris-HCl/Buffer were added for 2 hours

at 25°C. Different lanes represent different reaction samples in Fig.15: Lane 1, contains all ingredients but terminated at 0 min by adding 4xGSB, boiling followed by freezing i.e. no ubiquitination reaction; 2, with ubiquitination only; 3 -with ubiquitination followed by termination; 4 -with ubiquitination, termination and reaction buffer added as control; 5 -with ubiquitination, termination and Tris-HCl added as control; 6, 7, & 8 -with ubiquitination, termination and adding decreasing volumes of eluted Flag-USP7 respectively; 9, 10, & 11 -with ubiquitination, termination and adding decreasing volumes of UBPcore, respectively; sample 12 -no ubiquitination reaction (duplicate of sample 1); 13 -no E3 ligase and no ubiquitination reaction; 14 -no E3 ligase and with ubiquitination reaction.

## 5.7 Peptidase activities of the 26S proteasome

To check whether the catalytic activity of mammalian 26S proteasome could be increased or stimulated in the presence of FAT10 (diubiquitin), NUB1L or FAT10 conjugate. The mammalian 26S proteasome (Enzo Life Sciences) was incubated at 37°C with or without proteins for 15min before adding the fluorogenic-substrate in the reaction buffer (reaction buffer: 20mM Tris-HCl, pH-7.6; 50mM NaCl; 10mM MgCl<sub>2</sub>.6H<sub>2</sub>O; 2mM ATP; 1mM DTT; 50U inorganic pyro-phosphatase; 10% glycerol). The peptidase activity was monitored at several time points after adding the fluorogenic substrate. List of proteins used in the study for stimulation were FAT10 (BiTg, Switzerland), NUB1L [247], Tetra-ubiquitin mixed linkage i.e. Ub-K63-Ub-K48-Ub-K63-Ub (Boston Biochem), Linear Tetra-ubiquitin i.e. Ub<sub>4</sub> (Boston Biochem), BSA (Sigma), E6AP (gifted by Prof. Martin Scheffner). To measure different peptidase activities of proteasome preparations different fluorogenic-substrate were used; SLLVY-AMC for chymotrypsin-like activity; AC-YVAD-AMC for caspase-like activity; BZ-VGR-AMC for trypsin-like activity. Fluorogenic-substrates was tested using various concentration ranges such as 10µM, 50µM or 100µM. The desired concentration of a stimulant protein in 50µl assay buffer was mixed with 26S proteasome in 50µl assay buffer to yield a total volume of 100µl per reaction well. Next, 100µl fluorogenic substrate was added to obtain a final reaction volume of 200µl. Finally, post substrate addition plates were incubated at 37°C and the fluorescence intensity was monitored

using Tecan Infinite M200 pro machine with excitation and emission wavelength set at 360 and 465nm, respectively. Black flat bottom 96 wells plate (Greiner bio-one REF 655900) were used.

### **5.8 Softwares**

Softwares used in this study include Quantity One, MS Office, Graph Pad Prism 6, Adobe illustrator CS6 (64bit), Clone Manager Professional 9, Primer3 Input (online version 0.4.0).

### **5.9 Statistical analysis**

For statistical analysis, groups from similar experiments were repeated and analyzed for significant differences as indicated in the graph. Significant difference is given as p-values in the figure legends. The p-values were calculated by unpaired t-test. All statistical analyses were performed using Prism software.

## 6. References

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## Record of contributions

I designed and performed all the experiments

the experiment presented in Fig.10a and Fig.17a was technically supervised by Dr. Annette Aichem, Biotechnology Institute Thurgau (BiTg), Switzerland.

for the experiment presented in Fig.13a and Fig.14b, I learned the technique from Johanna Bialas at BiTg, Switzerland.

for the experiment presented in Fig.15, I learned the technique in the laboratory of Prof. Dr. Martin Scheffner, Konstanz University.

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