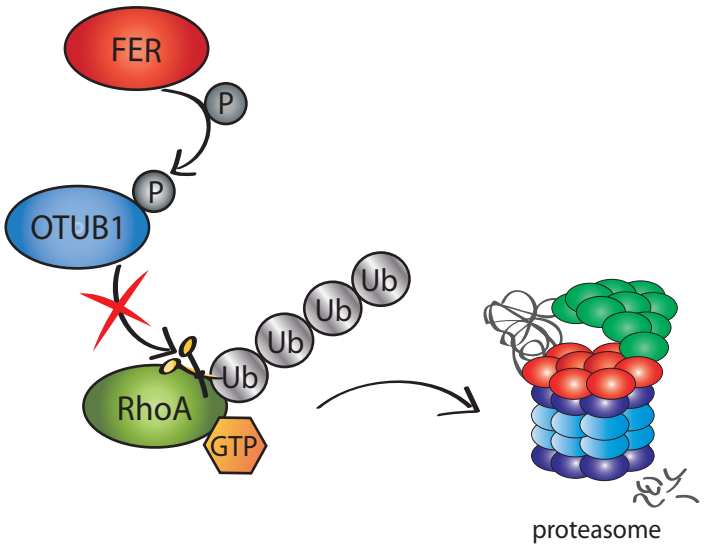
Biochemical characterization of a novel deubiquitinating enzyme otubain 1 and investigation into its role in *Yersinia* infection

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Thesis submitted for the degree of Doctor of Philosophy

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Abstract

Deubiquitinating enzymes (DUBs) constitute a diverse protein family. The specificities and functions of the majority of DUBs are unknown, although their impact on many biological and pathological processes is widely appreciated. This dissertation entails a detailed characterization of otubain 1 (OTUB1), an ovarian tumor domain-containing DUB. The presented work describes OTUB1's specificity, localization, protein interactions, importance in infection with *Yersinia*, and proposes a novel model of regulation of its enzymatic activity.

I first discuss the structural and biochemical properties of OTUB1, demonstrating its selectivity towards ubiquitin and NEDD8. Moreover, I show that OTUB1 cleaves lys^{48}- but not lys^{63}-linked polyubiquitin, emphasizing its role in ubiquitin-mediated proteasomal degradation. Mass spectrometric identification of interaction partners and localization studies suggest possible involvement of OTUB1 in RNA processing and cell morphology.

Furthermore, I demonstrate that invasion of the host cells by the enterobacteria *Yersinia* can be altered by changing OTUB1 expression. This effect is dependent on the catalytic activity of OTUB1 and its ability to stabilize RhoA-$_{GTP}$ prior to infection. YpkA and OTUB1 modulate RhoA-$_{GTP}$ stability in opposing ways, leading to cytoskeletal rearrangements that may be involved in bacterial invasion. Moreover, OTUB1 is post-translationally modified by phosphorylation that modulates its ability to stabilize RhoA-$_{GTP}$ and counteracts its effect on bacterial invasion. These findings provide a novel entry point for the manipulation of the host—pathogen interactions.

Lastly, a kinase screen revealed that FER, an oncogenic kinase with a role in cell morphology, phosphorylates OTUB1, as demonstrated by overexpression, siRNA and *in vitro* studies. The phosphorylated site was mapped to tyr^{26} and the activity-based labeling revealed that this modification interferes with the deubiquitinating activity of OTUB1.

In summary, the results presented in this thesis confirm that OTUB1 exerts properties of a "classical DUB" and uncover some of its physiological functions.

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Edelmann MJ, Kessler BM. *Ubiquitin and ubiquitin-like specific proteases targeted by infectious pathogens: Emerging patterns and molecular principles.* Biochimica and Biophysica Acta 1782(12): 809-16. 2008.

Batycka MJ[1], Inglis NF, Cook K, Adam A, Fraser-Pitt D, Smith D, Main L, Luben A, Kessler BM. *Ultra-fast tandem mass spectrometry scanning combined with monolithic column liquid chromatography increases throughput in proteomic analysis.* Rapid Communications in Mass Spectrometry 20:2074-2080. 2006.

[1] This article was published under my maiden name.

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List of abbreviations

AMC	7-amino-4-methylcoumarin
ATP	Adenosine-5'-triphosphate
Br2	2-bromoethyl
BSA	Bovine serum albumin
Cl2	2-chloroethyl
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
ECL	Electrochemiluminescence
EDTA	Ethylenediamine tetra acetic acid
ER	Endoplasmic reticulum
ERα	Estrogen receptor α
ESI	Electrospray ionization
FAT10	F-Adjacent transcript-10
FER	Fes related (proto-oncogene tyrosine-protein kinase)
FITC	Fluorescein isothiocyanate
FUS	Fusion involved in t(12;16) in malignant liposarcoma
GDP	Guanosine diphosphate
GRAIL	Gene related to anergy in lymphocytes
GST	Glutathione-S-transferase
GTP	Guanosine-5'-triphosphate
HA	Haemagglutinin

HECT	Homologous to E6-AP carboxyl terminus
HEK	Human embryonic kidney
HPLC	High pressure liquid chromatography
HRP	Horseradish peroxidase
IAA	Iodoacetamide
IEF	Isoelectric focusing
Ig	Immunoglobulin
IL	Interleukin
IPG	Isoelectric focusing protein gel
ISG15	Interferon stimulated gene 15
IVTT	In vitro transcription-translation
LC	Liquid chromatography
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MBP	Myelin basic protein
NEDD8	Neural-precursor-cell-expressed developmentally down-
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP-40	Nonidet P40
OTU	Ovarian-tumor-domain-containing protease
OTUB1	Otubain 1
OTUB2	Otubain 2
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDI	Protein disulfide isomerase
PTM	Posttranslational modification
Pup	Prokaryotic ubiquitin-like protein
PVDF	Polyvinylidene fluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RACK1	Receptor for activated kinase 1
RhoA	Ras homolog gene family member A
RING	Really interesting new gene
RNA	Ribonucleic acid
SBP	Streptavidin-binding peptide
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SENP	SUMO1/sentrin/SMT3-specific peptidase
SUMO	Small ubiquitin-related modifier
TBS	Tris-buffered saline
TEV	Tobacco etch virus
TLS	Translocation in liposarcoma
TRAF	Tumor necrosis factor receptor-associated factor
TTSS	Type three secretion system
Ub	Ubiquitin
UBA	Ubiquitin-associated domain
UBD	Ubiquitin binding domain
Ubl	Ubiquitin-like protein
UBP	Ubiquitin processing protease
UCH	Ubiquitin C-terminal hydrolase
ULP	Ubiquitin-like specific protease
UPLC	Ultra performance liquid chromatography
UPS	Ubiquitin proteasome system

USP	Ubiquitin-specific peptidase
VME	Vinyl methyl ester
YopO	*Yersinia* serine/threonine kinase
YpkA	*Yersinia* serine/threonine kinase

I refer to all amino acids by their three-letter abbreviations, and when describing mutants I use their single-letter abbreviations.

1. Introduction

1.1. Role of ubiquitin in protein targeting and function

1.1.1. Early history of ubiquitin

"*It has long been clear how proteins are built up in the cell. But the opposite, how they are broken down, was long thought to be less exciting to study*"[2]

The discovery of ubiquitin and the ubiquitin proteasome system took place in mid-1970'. Previously, it was commonly believed that most proteins are long-lived, although some aspects of protein turnover were recognized [(Schoenheimer & Clarke, 1942), (Schimke & Doyle, 1970)]. In 1978 Hershko and Ciechanover reported on the proteolytic activity of two chromatographic fractions of rabbit reticulocyte lysate, which were inactive when separated, and if combined their proteolytic activity was reconstituted. The first fraction contained a small protein that weighed around 9000 Da, at that time named **A**TP-dependent **p**roteolytic **f**actor [APF-1; (Ciehanover et al, 1978)]. One year later Hershko, Ciechanover and Rose discovered that the second fraction contained a 450 kDa, heat-labile factor called ATP-stabilized protein, as well as the third fraction that was a heat-stable component. All three fractions were necessary to degrade a substrate in an ATP-dependent fashion (Hershko et al, 1979). In 1980 it was suggested that as an intermediate process in proteolysis APF-1 forms covalent high-molecular weight

[2] *http://nobelprize.org/nobel_prizes/chemistry/laureates/2004/illpres*

conjugates attached to proteins [(Ciechanover et al, 1980), (Hershko et al, 1980)]. In 1975 Goldstein and his group reported on a 8500 molecular weight polypeptide isolated from the thymus, referred to as **ub**iquitous **i**mmunopoietic **p**olypeptide [UBIP; (Goldstein et al, 1975)], also known as **ub**iquitin [Ub; (Schlesinger & Goldstein, 1975)], as this reflected its presence in various tissues from evolutionary distant organisms (Goldstein et al, 1975). Finally, in 1980 Wilkinson, et al. showed that APF-1 was the same protein as the formerly identified ubiquitin (Wilkinson et al, 1980). In this way ubiquitin and its function as a signal molecule in protein degradation have been discovered and established. In 2004 Aaron Ciechanover, Avram Hershko and Irwin Rose were awarded the Nobel Prize in Chemistry for this indisputable breakthrough in the field of protein turnover (Ciechanover, 2009).

1.1.2. Ubiquitin conjugation

A significant aspect of the ubiquitin system is the process of how proteins become labeled with ubiquitin molecules, which is called ubiquitination or ubiquitylation. This subject was first studied between 1981 and 1983 by Hershko and his collaborators [(Ciechanover et al, 1982), (Hershko et al, 1983)]. His research group identified a cascade of three enzymes involved in the conjugation of ubiquitin to a substrate, which they termed E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). The E1 is an enzyme containing a cysteine residue that is required for catalysis and in the cascade of reactions it first binds MgATP. ATP-binding may open accessibility of the E1 to ubiquitin because prior to the ATP binding its affinity for ubiquitin is very low

(Haas et al, 1982). The E1 adenylates then the C-terminal carboxyl group of ubiquitin, forming a high-energy ubiquitin adenylate (Ub-AMP) intermediate. This enables the binding of ubiquitin to the E1 active site via formation of a thiol ester between the carboxyl group of gly^{76} of ubiquitin and a thiol group of the E1. This series of reactions activates the C terminus of ubiquitin for a nucleophilic attack.

Next, the E1 loaded with an active ubiquitin molecule transfers it to a sulfhydryl site of the E2 with which ubiquitin also forms a thiol ester (Ciechanover et al, 1982). Ubiquitin bound to the E2 is then transferred to a lysine of a target protein, forming an isopeptide bond. This is the third step of ubiquitination and it is catalyzed by the E3. The only known exception is monoubiquitination for which only the E1 and E2 might be necessary (Hochstrasser, 2007). A protein substrate is first recognized by the E3, which catalyzes the covalent transfer of ubiquitin to a lysyl side chain of the substrate (Hershko et al, 1983). In some cases the substrate is not bound to the E3 directly, but via an adaptor molecule (Hershko & Ciechanover, 1998).

There are approximately 40 E2s encoded in the human genome and all of them share a conserved core domain that consists of ~150 amino acids, and in some cases the extensions from the core domain are minimal. Despite their similarity, the biological functions of E2s vary substantially. This could be due to their association with various E3s that play distinct physiological roles [reviewed in (Hershko & Ciechanover, 1998), (Pickart, 2001)]. The specificity of E3s determines which substrates become ubiquitinated, based on recognition signals on the target proteins, such as an N-terminal residue (Hershko & Ciechanover, 1998), or post-translational modifications, for instance phosphorylation [(Yaron et al, 1997), reviewed in (Ciechanover, 1998)]. There are three categories into

which hundreds of known E3s are divided and this classification is based on the characteristics of their E2~Ub-binding domain:

(1) HECT (**h**omologous to **E**6-AP **c**arboxyl **t**erminus) domain E3s

(2) RING (**r**eally **i**nteresting **n**ew **g**ene) and RING-like domain E3s

(3) E3s with E2~Ub-binding domains not resembling any of the above

Importantly, the type of domain possessed by the E3 determines the catalytic mechanism it facilitates. The RING-domain containing E3s act as molecular scaffolds bringing the E2 and the target protein together and thus enabling ligation of ubiquitin that is still bound to the E2, whereas the HECT-domain containing E3s act as direct catalysts by forming a thiol ester with ubiquitin before transferring it to a lysine side chain of the target protein [(Wang & Pickart, 2005), (Zheng et al, 2000), (Scheffner et al, 1995)]. Identification of E3s and characterization of their mechanisms still require further studies and their large diversity presents one of the major challenges in the ubiquitin field.

1.1.3. Ubiquitin chains

Once ubiquitin is linked to the target protein it may have various effects on its fate. The effect primarily depends on the type of ubiquitin modification with which the protein was tagged. Ubiquitin is a 76-residue polypeptide containing seven lysine residues in its sequence: lys^{6}, lys^{11}, lys^{27}, lys^{29}, lys^{33}, lys^{48} and lys^{63}. All of them can participate in the formation of ubiquitin chains [(Peng et al, 2003), (Xu et al, 2009)], although linear conjugates of ubiquitin [head-to-tail; (Kirisako et al, 2006), (Rahighi et al, 2009)] and forked chains [also known as mixed, branched chains; (Kim et al, 2009), (Kim et al, 2007)] have been reported as well. Ubiquitin can also

modify proteins through single or multiple monoubiquitination, in which case ubiquitin does not form conjugates with other ubiquitin molecules (Haglund et al, 2003). The mechanism of formation of ubiquitin chains is still not entirely clear and there are several alternative models explaining this process (Hochstrasser, 2006). The oldest is the sequential addition model in which ubiquitin chains can be assembled on a substrate by the addition of ubiquitin monomers, perhaps via chain elongation factors (E4s). New data suggest that ubiquitin polymers are preassembled on E2/E3 enzymes prior to a transfer *en bloc* onto a target protein [(Chau et al, 1989), (Li et al, 2007), reviewed in (Hochstrasser, 2006)]. It is plausible that several mechanisms exist, depending on the substrate and the enzymatic cascade. Understanding this process would be useful for the manipulation of polyubiquitin chain formation on specific substrates in order to modulate their function and fate.

The most studied function of ubiquitin modification is the induction of proteasomal degradation of a modified protein, although ubiquitin plays a role in a whole range of other cellular processes, as summarized in Table 1.1. According to a non-quantitative mass spectrometry based study, lys^{48}-linked polyubiquitin is the most abundant amongst the ubiquitin conjugates (Peng et al, 2003). It is an efficient signal for proteasomal degradation, thereby controlling the lifespan of proteins, although some non-proteolytic functions of lys^{48}-linked polyubiquitin chains have been proposed as well (Li & Ye, 2008). Interestingly, a recent report suggests that polyubiquitin chains formed with all lysyl residues with the exception of lys^{63} can target substrates for proteolysis (Xu et al, 2009), making lys^{63}-polyubiquitin conjugate the only ubiquitin chain not involved in proteasomal degradation.

The questions then arise as to what is the molecular basis for the ubiquitin chain to be a signal for proteasomal degradation, and why are certain chains better signals than others? Firstly, the ubiquitin molecule must be efficiently bound to the 26S proteasome. The number of ubiquitin molecules is crucial in this process and it has been demonstrated that at least four ubiquitin molecules must form a conjugate (i.e. tetraubiquitin) for an efficient binding to the proteasome [K_d < 1μM; (Thrower et al, 2000)]. Furthermore, the spatial relationship between ubiquitin molecules and the quaternary structure of the chains are important for the efficient recognition. Different ubiquitin conjugates have distinct ubiquitin—ubiquitin binding interfaces and this affects their binding to the 26S proteasome (Varadan et al, 2004). For example, in terms of the structure, lys^{63}-linked polyubiquitin chains are closer to a linear ubiquitin and have an extended conformation in comparison to lys^{48}-linked polyubiquitin chains that form a more compact structure.

Moreover, lys^{63}-linked polyubiquitin chains lack direct contact between the hydrophobic residues leu^{8}, ile^{44} and val^{70}, which play a role in ubiquitin chain recognition by the **ub**iquitin-**a**ssociated (UBA) domains of the proteasome [(Ikeda & Dikic, 2008), (Pickart & Raasi, 2005), (Tenno et al, 2004), (Varadan et al, 2004)].

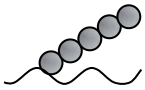
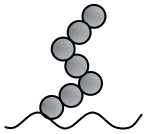
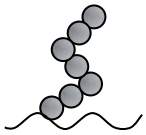
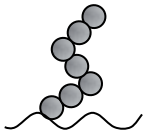
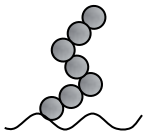
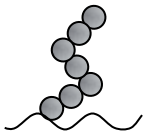
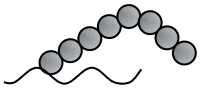
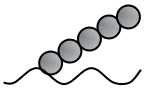
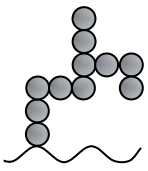
Type of Ub chains		Biological role	Reference
	monoubiquitin	trafficking, endocytosis, virus budding, nuclear export, DNA repair, gene expression	Haglund, Di Fiore et al. 2003
	linear ubiquitin	signalling	Rahighi, Ikeda et al. 2009
	K^{6}	proteasomal degradation	Baboshina and Haas 1996 Xu, Duong et al. 2009
	K^{11}	proteasomal degradation, protein stability	Baboshina and Haas 1996 Nishikawa, Ooka et al. 2004 Xu, Duong et al. 2009
	K^{27}	proteasomal degradation	Xu, Duong et al. 2009
	K^{29}	lysosomal degradation, proteasomal degradation, regulation of kinase activity, protein stability	Nishikawa, Ooka et al. 2004 Chastagner, Israel et al. 2006 Al-Hakim, Zagorska et al. 2008 Xu, Duong et al. 2009
	K^{33}	proteasomal degradation, regulation of kinase activity	Al-Hakim, Zagorska et al. 2008 Xu, Duong et al. 2009
	K^{48}	proteasomal degradation	Chau, Tobias et al. 1989
	K^{63}	trafficking, kinase activation, proteolytic degradation of misfolded proteins	Olzmann, Li et al. 2007 Tan, Wong et al. 2008 Wooten, Geetha et al. 2008
	forked chains	negative effect on proteasomal degradation	Kim, Kim et al. 2007 Kim, Kim et al. 2009

Table 1.1. ***Functions of various ubiquitin conjugates.***

Consequently, lys^{63}-polyubiquitin chains do not efficiently bind to the UBA domains and therefore are not implicated in the ubiquitin proteasome pathway. Instead, lys^{63}-polyubiquitin chains play an essential role in signal transduction events of the NF-κB pathway, endocytosis and DNA repair [(Haglund & Dikic, 2005), (Hayden & Ghosh, 2008), (Krappmann & Scheidereit, 2005)]. The biological relevance of the five remaining polyubiquitin conjugates, especially in the context of the proteasomal degradation, are less well understood and still need to be characterized further [(Al-Hakim et al, 2008), (Chastagner et al, 2006), (Wooten et al, 2008), (Xu et al, 2009)].

1.2. Degradation of ubiquitinated substrates by the 26S proteasome

Most of the ATP-dependent intracellular protein degradation is catalyzed by the 26S proteasome that represents a controlled and specific mechanism of degrading only those proteins that have been tagged for proteolysis. The 26S proteasome is a 2.4 MDa multi-protein complex consisting of two large components, the 19S **r**egulatory **p**article (RP; also known as PA700) and the 20S proteolytic **c**ore **p**article (CP; Figure 1.1). The RP is attached on one or both sides of the CP (Walz et al, 1998) and it has six AAA-ATPase subunits required for the substrate unfolding and channel opening, and four non-ATPase subunits, some of which are responsible for the deubiquitination of protein substrates.

The UBL-UBA proteins Ddi1, Dsk2 and Rad23 are bound to the proteasome through the N-terminal **ub**iquitin-**l**ike (UBL) domain and to the ubiquitin through the UBA domain. Ddi1, Dsk2, Rad23 as well as proteasomal ubiquitin receptors Rpn10 and Rpn13 contain polyubiquitin binding domains that initially recognize

and bind polyubiquitinated substrates to the proteasome [(Schreiner et al, 2008), (Sharon et al, 2006)]. Regulation of cellular concentration of UBL-UBA proteins determines whether proteasomal degradation is inhibited or activated [(Matiuhin et al, 2008), (Walters & Zhang, 2008)]. For instance, recent findings suggest that Dsk2 inhibits the degradation of ubiquitinated substrates by the proteasome thereby having a cytotoxic effect on cells, while Rpn10 interacts with Dsk2 and interferes with its effect on cell viability and with the Dsk2—proteasome association [(Matiuhin et al, 2008), (Walters & Zhang, 2008)].

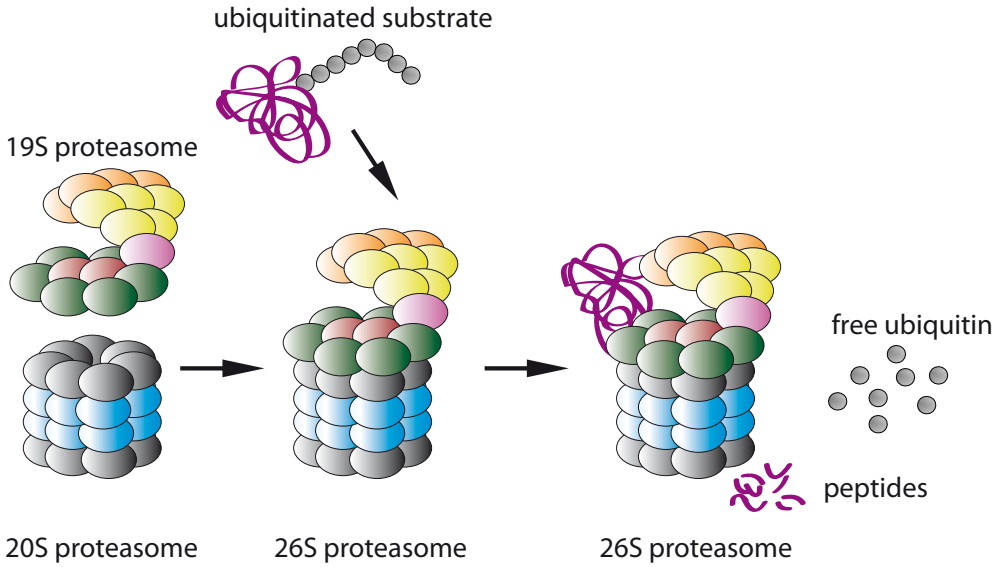


Figure 1.1. ***Proteasomal degradation***[3]

Thus, the RP controls proteolytic functions of the CP in multiple ways: the RP facilitates gate opening for substrate entry [(Kohler et al, 2001), (Smith et al, 2007)], it recognizes proteins tagged with specific ubiquitin conjugates and it

[3] The general structure of the illustration is based on, but not copied from Marteijn JA, Jansen JH, van der Reijden BA (2006) Ubiquitylation in normal and malignant hematopoiesis: novel therapeutic targets. *Leukemia* **20**(9)**:** 1511-1518; the permission was granted by the Nature Publishing Group (license number: 2365101087349).

deubiquitinates and unfolds them. Only then are they able to translocate to the CP through its gated pore, a process also facilitated by the RP [reviewed in (Glickman & Ciechanover, 2002), (Goldberg, 2007), (Voges et al, 1999)].

Having been translocated into the CP, the substrates are subjected to proteolysis. The CP has a cylindrical structure and is composed of α and ß subunits that form four stacked rings. The inner ß rings are proteolytically active and perform the proteolytic cleavage of substrates entering the CP, while the N-termini of the outer α subunits block non-specific degradation of proteins. The CP normally exists in an autoinhibited state and the binding of one or two RPs at either end of it releases the inhibition[4].

The proteolysis results from the action of six active sites located on ß subunits of the CP (ß1, ß2 and ß5). Due to the specificity of these subunits, acidic (ß1), basic (ß2) as well as hydrophobic (ß5) amino acid residues can be recognized and cleaved, thus displaying caspase-like (ß1), trypsin-like (ß2) and chymotrypsin-like (ß5) proteolytic activities [reviewed in (Finley, 2009), (Voges et al, 1999)]. The CP has an efficiently controlled proteolytic chamber that does not release degradation intermediates, but only small peptides that can vary in length from 4 to 25 residues (Voges et al, 1999). Therefore such peptides are rather short lived and do not accumulate in the cell, but are rapidly processed further, probably by downstream proteases [(Baumeister et al, 1998), (Glickman & Ciechanover, 2002)].

Proteasomal proteolysis is an essential cellular event and as such it has been linked to diverse physiological and pathological processes, including

[4] Other proteasome activators have also been identified, e.g. PA28; Smith DM, Chang SC, Park S, Finley D, Cheng Y, Goldberg AL (2007) Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. *Mol Cell* **27**(5)**:** 731-744.

infection, inflammation, cell cycle, or neurodegradation [reviewed extensively, e.g. (Petroski, 2008)]. Its homeostatic function is essential for the viability of the cell, yet in certain pathological cases inhibition of the proteasome has been proven effective, such as the therapeutic treatment of cancer patients. For instance, the treatment of multiple myeloma with the proteasome inhibitor Bortezomib [Velcade; (Curran & McKeage, 2009)] has been shown to induce apoptosis of cancer cells and inhibit cell adhesion (Blade et al, 2005). The clinical importance of the proteasome highlights the relevance of research focused on the **u**biquitin-**p**roteasome **s**ystem (UPS) as there is an increasing interest in novel proteasome inhibitors and activators [(Hoeller & Dikic, 2009), (Huang & Chen, 2009), (Tsukamoto & Yokosawa, 2009)].

1.3. Ubiquitin-like molecules

Since the discovery of the ubiquitin modification, a whole family of **ub**iquitin-**l**ike modifiers (Ubls; ubiquitin-like proteins) has been established. It is not for the sequence similarity that makes them members of the same family of proteins, but for their homology in three-dimensional structures characterized by the ubiquitin fold and their C-terminal residue, glycine. The C-terminal glycine residue is linked through an isopeptide bond with a lysine residue of the substrates it modifies. The Ubls function in a similar way as ubiquitin, modulating function, localization or the half-life of the proteins. The members of this family in the mammalian proteome currently include around ten different Ubls. Below is a brief description of some of the most well defined Ubls.

1.3.1. Interferon-stimulated gene product of 15 kDa (ISG15) – the immunological kin of ubiquitin

The **i**nterferon-**s**timulated **g**ene product of **15** kDa (ISG15; **u**biquitin **c**ross-**r**eactive **p**rotein, UCRP) was described in 1979, making it the first Ubl identified (Farrell et al, 1979). It covalently modifies other proteins in the process of ISGylation, or attachment of ISG15 to substrate proteins. ISG17, which is a precursor of ISG15, is processed by protease USP18 (UBP43), activated by **ub**iquitin-activating enzyme **E1**-**l**ike (UBE1L), and ISG15 is then able to modify other proteins [reviewed in (Herrmann et al, 2007), (Welchman et al, 2005)]. Its transfer to substrates requires the E2 UBCH8, which is the same as the E2 in the ubiquitin-conjugation pathway (Zhao et al, 2004), demonstrating the functional overlap of this E2.

Expression of ISG15 is stimulated by type I interferons, as well as by bacterial **l**ipo**p**oly**s**accharide (LPS) or double-stranded RNA in viral infection (Ritchie & Zhang, 2004). In turn, ISG15 expression stimulates IFN-γ production which results in the natural killer cell proliferation (D'Cunha et al, 1996). This defines ISG15 as the "immunological kin of ubiquitin" (Ritchie & Zhang, 2004). In addition, there seem to be crosstalk between the ISG15 and the ubiquitin processes. Specifically, overexpression of ISG15 is correlated with decrease in protein polyubiquitination, and proteasome inhibition causes increase in ISGylation [(Desai et al, 2006), (Liu et al, 2003)].

1.3.2. Small Ubiquitin-Related Modifier (SUMO)

In the human proteome there are four **s**mall **u**biquitin-related **mo**difier (SUMO) isoforms, SUMO-1, SUMO-2, SUMO-3 and SUMO-4. As SUMO-2 and -3 are almost identical, I will refer to them in this study as SUMO-2/3. SUMO-2/3 is able to form SUMO chains, while SUMO-1 can only modify proteins in its monomeric form (Tatham et al, 2001), although some conjugates of SUMO-1 have been also detected [reviewed in (Ulrich, 2008)]. SUMO molecules are transferred to substrates in the E1/E2/E3 cascade. However, contrary to the ubiquitin system, SUMO-specific conjugating enzyme (E2) is the factor determining substrate specificity [reviewed in (Ulrich, 2008)]. Physiological roles of sumoylation are diverse. SUMO isoforms regulate protein translocation to the nucleus, affect protein—protein interaction, are involved in transcription and DNA repair, and regulate NFκB signaling [(Wang et al, 2009a), reviewed in (Herrmann et al, 2007), (Welchman et al, 2005)]. Recent studies suggest a role of sumoylation in the ubiquitin proteasome system. SUMO modification can signal recruitment of E3 ubiquitin ligases to substrates, therefore promoting their ubiquitination and subsequent degradation in a proteasome-dependent manner [reviewed in (Geoffroy & Hay, 2009)].

1.3.3. Neural precursor cell-expressed developmentally downregulated-8 (NEDD8) – regulator of cullin function

Neural precursor cell-**e**xpressed **d**evelopmentally **d**ownregulated-**8** (NEDD8) is the Ubl with the highest homology to ubiquitin (59% sequence identity). It is synthesized as a precursor and is further processed by C-terminal hydrolases

deneddylase 1 and UCH-L3. Its conjugation to substrates (neddylation) requires activation by the E1 APPBP1-UBA3 and transfer by the E2 UBC12 (Xirodimas, 2008). Some E3s specific for NEDD8 have been identified, including MDM2 mediating conjugation of NEDD8, which has been correlated with inhibited p53 activity (Xirodimas et al, 2004). However, NEDD8 primarily functions in the regulation of E3 ubiquitin ligases, modifying the majority of members of the cullin family. Cullins are scaffold components of the SCF (**S**-phase kinase-associated protein 1/**c**ullin/**F**-box) ubiquitin ligases that control the proteasomal degradation of proteins involved in cell-cycle, transcriptional regulation, oxygen regulation, and signal transduction [reviewed in (Herrmann et al, 2007), (Xirodimas, 2008)]. Neddylation of cullins results in increased ubiquitination of these proteins and their subsequent proteasomal degradation.

1.3.4. F-Adjacent Transcript-10 (FAT10)

F-**a**djacent **t**ranscript-**10** (FAT10) is a Ubl encoded in the **m**ajor **h**istocompatibility **c**omplex (MHC) class I locus. Its expression is induced by IFN-γ and TNF-α (Raasi et al, 1999). FAT10 is able to directly interact with the 26S proteasome through one of its two UBL domains (Schmidtke et al, 2009). A recent study evaluated the role of FAT10 in protein degradation, establishing the ubiquitin-independence of FAT10-mediated targeting to the proteasome (Schmidtke et al, 2009). This makes FAT10 the second member of the ubiquitin-like protein family capable of a direct signaling of the substrates to the 26S proteasome.

1.3.5. Prokaryotic ubiquitin-like protein (Pup) – a bacterial relative of ubiquitin

The function of proteasomes in bacteria and *Archaea* has been relatively poorly characterized and until recently detection of ubiquitin or Ubls in *Prokaryota* had not been successful. This had lead to the conclusion that ubiquitin- or Ubl-mediated protein degradation is not present in those organisms and is performed via different pathways. However, recent findings suggest that some bacteria may use a small protein modifier as a marker for degradation after all (Pearce et al, 2008). Darwin et al. identified the **p**rokaryotic **u**biquitin-like **p**rotein (Pup; Rv2111c) in *Mycobacterium tuberculosis* and observed its covalent conjugation to several substrates (Pearce et al, 2008). Unlike ubiquitin and its relatives that contain C-terminal glycine, Pup has a di-glycine motif at the penultimate position, but it ends in a glutamine residue that becomes deamidated in the conjugated complex [reviewed in (Burns & Darwin)]. Therefore conjugation of Pup to substrates (pupylation) may involve a mechanism that is distinct from ubiquitination and the properties of Pup itself may be also unique. It has been further confirmed by structural and phylogenetic studies that Pup indeed belongs to a unique and ancient branch of Ubls [(Chen et al, 2009), (Liao et al, 2009)].

1.4. Deubiquitinating enzymes

1.4.1. Classification of deubiquitinating enzymes

As many other post-translational modification, ubiquitination is also reversible and the deconjugation of ubiquitin (deubiquitylation, deubiquitination) is accomplished by a large group of proteolytic enzymes known as **d**e**ub**iquitinating enzymes (DUBs, deubiquitylating enzymes, deubiquitinases, ubiquitin proteases),

which predominantly consists of cysteine proteases and a small number of metalloproteases. It has become evident that a vast number of proteases have acquired the ability to process ubiquitin and Ubls, probably far beyond the currently known classifications, because novel DUBs are still being discovered and new predictions made by bioinformatic analyses [(Hotson & Mudgett, 2004), (Iyer et al, 2004), (Makarova et al, 2000)]. The recent studies predict approximately 79 deubiquitinating enzymes to be encoded by the human genome (Komander et al, 2009a). I shall therefore refer to the classification of proteases into clans and families as proposed by the MEROPS database[5].

In the case of ubiquitin, five classes of enzyme families have been defined to date, including **u**biquitin **C**-terminal **h**ydrolases (UCH — C12 family), **ub**iquitin processing **p**roteases (UBP, also referred to as **u**biquitin **s**pecific **p**roteases, USP — C19 family), **j**osephin **d**omain containing proteases (JD), **o**varian **tu**mor domain containing proteases (OTU — C64, C85, C87 and C88 families) and metalloproteases [MM; Table 1.2, (Nijman et al, 2005), (Amerik & Hochstrasser, 2004), (Ha & Kim, 2008)]. The UCH class contains only three members, which were originally identified to process ubiquitin precursors and hydrolyze small conjugates at the C-terminus of ubiquitin. UBPs (USPs) form the largest enzyme family, and all its members share the conserved cysteine and histidine boxes as short motives, but otherwise can be very diverse. Another family of proteases sharing the ovarian tumor domain (OTU) as a common feature is conserved throughout evolution, and is found in approximately 100 genes derived from viruses, bacteria, plants and mammals [(Balakirev et al, 2003), (Makarova et al,

[5] See http://merops.sanger.ac.uk/index.htm for a more detailed discussion of these enzymes.

2000)], suggesting their involvement in fundamental biological processes. Deubiquitinating activity was also assigned to a small number of josephin domain and JAB metalloprotease domain containing proteases.

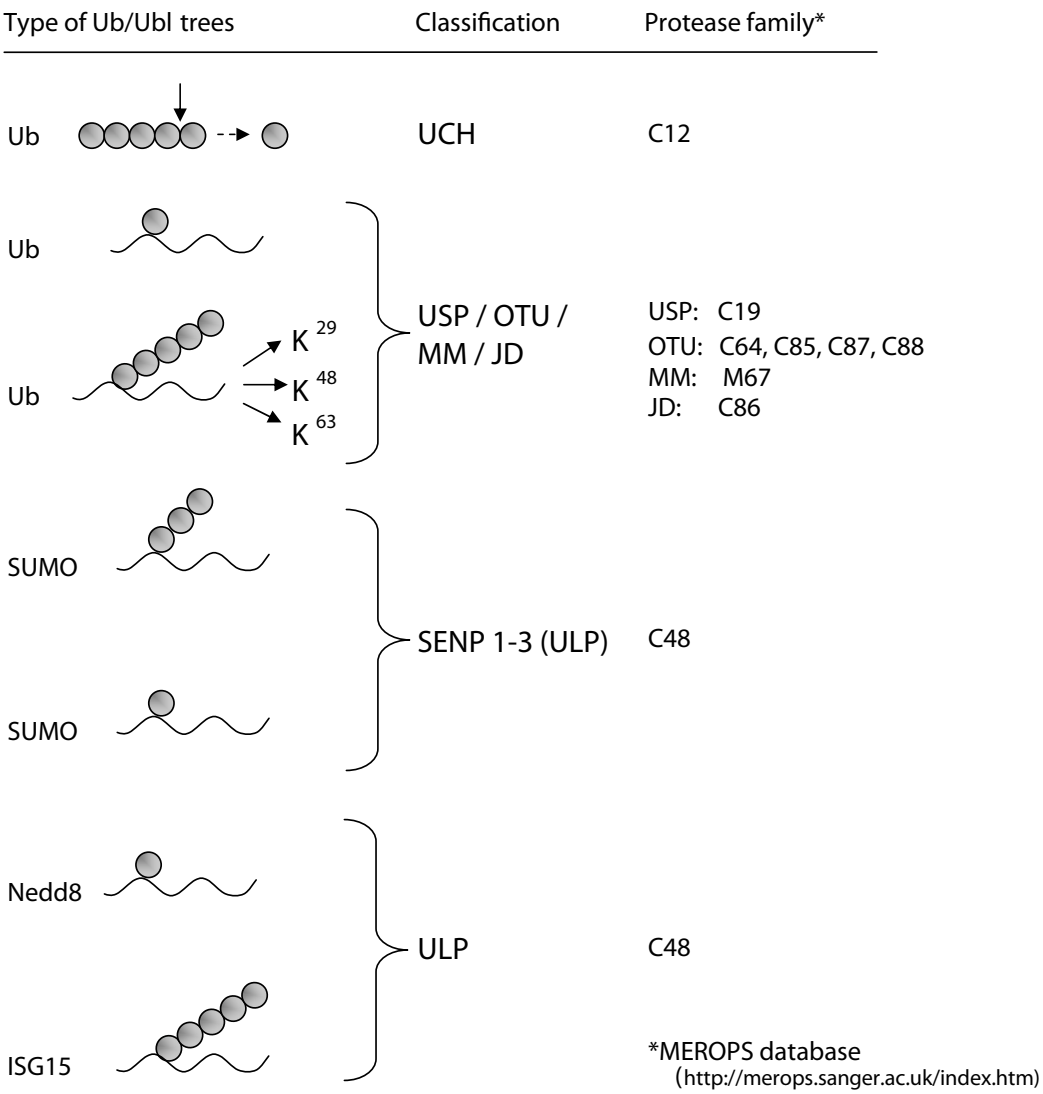


Table 1.**2.** ***Scheme of Ub/Ubl-specific proteases***. Proteases with specificity to **ub**iquitin (Ub) or **ub**iquitin-**l**ike proteins (Ubls) are currently classified into the following protein families: UCHs: **u**biquitin **C**-terminal **h**ydrolases; USPs: **u**biquitin **s**pecific **p**roteases (also referred to as UBPs: **ub**iquitin processing **p**roteases); OTUs: **o**varian **tu**mor domain containing proteases; MMs: metalloproteases; JDs: **j**osephin **d**omain containing proteases; SENPs: small ubiquitin-like modifier specific proteases; ULPs: **u**biquitin-**l**ike protein specific **p**roteases.

It is anticipated that more enzymes may be added to this list since bioinformatics-based predictions suggest larger families of cysteine proteases to be potential DUB candidates, including **p**ermuted **p**apain fold **p**eptidases of **D**sRNA viruses and **e**ukaryotes (PPPDE) and **W**ss1p-**l**ike **m**etalloproteases (WLM) domains (Iyer et al, 2004).

Proteolytic enzymes with specificities towards Ubls are classified as **u**biquitin-**l**ike specific **p**roteases (ULPs) and **S**UMO/s**en**trin/SMT3-specific **p**eptidases (SENPs), both of which belong to the cysteine protease superfamily [reviewed in (Ha & Kim, 2008), (Mukhopadhyay & Dasso, 2007)]. In addition, experimental evidence using activity-based profiling strategies based on Ub/Ubl scaffolds and tandem mass spectrometry retrieved a number of novel gene products (including pathogen-derived ones) with Ub/Ubl-specific protease activities, which previously escaped to be predicted by any sequence or structure-based alignment algorithms. Such active-site probes mainly detect the ability of proteins to interact with Ub/Ubls with sufficient affinity for the occurrence of the suicide inhibition of the catalytic site by covalent attachment, thereby providing a list of DUB candidates. A number of these were subsequently confirmed to exert Ub/Ubl-specific processing activities [reviewed in (Kessler, 2006), (Love et al, 2007), (Ovaa, 2007)]. Clearly, these findings provide the framework for biochemical and functional characterization of novel proteolytic enzymes with the Ub/Ubl-specific processing activity.

1.4.2. Otubain 1 (OTUB1) – a member of OTU-domain containing proteases

Members of the OTU family of proteases are found in viruses, bacteria, plants and mammals [(Balakirev et al, 2003), (Makarova et al, 2000)], emphasizing the importance of their function in biological processes. OTUs are putative cysteine proteases homologous to the OTU protein in *Drosophila melanogaster*. The otu gene in *Drosophila* functions as a regulator of ovary development (Steinhauer et al, 1989) and is involved in sex determination of the germ cells (Pauli et al, 1993). Phenotypes associated with mutations in specific alleles of the otu gene in *Drosophila* involve ovarian cancer and other ovarian pathologies (King & Storto, 1988).

Amongst the mammalian OTUs, **otub**ain **1** and **otub**ain **2** (OTUB1 and OTUB2) were the first ones for which a deubiquitinating activity was demonstrated *in vitro* (Balakirev et al, 2003). At the present, OTUs are a relatively uncharacterized group of DUBs and very few mammalian OTUs have been linked with functional pathways, as summarized in Table 1.3.

A20 is an OTU with a well-defined function in pro-inflammatory events. It down-regulates activation of the transcription factor NF-κB and therefore plays an important role in inflammation. Interestingly, next to the OTU domain involved in cleavage of lys^{63}-linked polyubiquitin chains from TRAFs and RIPs, it also contains the C-terminal zinc finger domain that acts as a ubiquitin ligase and is responsible for building lys^{48}-linked polyubiquitin conjugates on RIPs, thus targeting them to the proteasome [(Balakirev & Wilkinson, 2008), (Heyninck & Beyaert, 2005)]. Therefore, A20 has dual, or editing functions on its substrates, removing one type of polyubiquitin chain and attaching another.

Gene	Ubiquitin Chain Specificity	Substrates	Pathway	References
A20	lys^{63}	RIPs, TRAFs	NF-κB signaling	(Wertz et al, 2004)
Cezanne	lys^{48}, lys^{63}	Unknown	NF-κB signaling	(Enesa et al, 2008)
DUBA	lys^{63}	TRAF3	IFN-I production	(Kayagaki et al, 2007)
OTUB1	lys^{48}	Erα, TRAF3, TRAF6	Transcription mediated by Erα, T cell anergy, virus-triggered IFN-I production	(Soares et al, 2004), (Edelmann et al, 2009), (Stanisic et al, 2009), (Li et al, 2009c), this study
OTUB2	lys^{63}, low activity towards lys^{48}	TRAF3, TRAF6	Virus-triggered IFN-I production	(Edelmann et al, 2009), (Li et al, 2009c), this study
Trabid	lys^{63}	APC tumor suppressor protein	Wnt pathway	(Tran et al, 2008)
VCIP135	Unknown	Unknown	ER and Golgi assembly	(Wang et al, 2004)
YOD1	lys^{48}, lys^{63}	Unknown	Unknown	(Ernst et al, 2009)

Table 1.3. ***Characterized mammalian OTUs, their specificity towards polyubiquitin linkages, substrates, and functional pathways they are involved in.***

Cezanne, another OTU member, is able to cleave linear and branched ubiquitin *in vitro* (Evans et al, 2003) without a clear preference for lys^{48}- or lys^{63}-polyconjugates (Enesa et al, 2008). Cezanne is induced by TNFα in cultured cells and it has an inhibitory effect on NF-κB signaling, where its deubiquitinating activity is involved. This activity is also essential in controlling the polyubiquitination of RIP1 (Enesa et al, 2008). This together represents a negative

feedback loop in pro-inflammatory signaling, although more evidence is required to demonstrate that RIP1 is a bona fide substrate of Cezanne.

DUBA is an OTU involved in the immune system, cleaving lys^{63}-linked, but not lys^{48}-linked polyubiquitin. It has been identified as a positive regulator of **i**nte**r**fero**n** type **I** (IFN-I) production and shown to deubiquitinate TRAF3, the E3 that plays an important role in the IFN-I response (Kayagaki et al, 2007).

Trabid is a DUB with a preference for lys^{63}–linked polyubiquitin due to the presence of the OTU and zinc finger domains. Depletion of Trabid from cells is associated with the reduced activation of the Wnt (Wingless/Integrated) signaling pathway, where both domains of Trabid, the zinc finger and OTU, are required (Tran et al, 2008). Moreover, Trabid deubiquitinates the **a**denomatous **p**olyposis **c**oli (APC) tumor suppressor protein that constitutes a negative regulator of the Wnt-mediated transcription (Tran et al, 2008).

Specificity of **V**CP(p97)/p47 **c**omplex–**i**nteracting **p**rotein of **135** kDa (VCIP135) for polyubiquitin chains has not been yet established. This OTU is known to bind to the p97 ATPase complex, where it functions as a necessary factor for formation of the Golgi vesicles [(Uchiyama et al, 2002), (Wang et al, 2004)].

YOD1 is a recently described OTU with an unknown physiological role. It cleaves lys^{48}- and lys^{63}-linked polyubiquitin chains with similar efficiency. Similarly to VCIP135, it was found to be in a multi-protein complex with p97, placing it in the context of ER (**e**ndoplasmic **r**eticulum) function. The substrates for this DUB have not yet been identified, but its deubiquitinating activity has been linked with the stabilization of several ER-dislocation substrates (Ernst et al, 2009).

In 2003 Balakirev et al. identified OTUB1 and OTUB2 proteins as binding to ubiquitin aldehyde (Balakirev et al, 2003) that suggested their DUB activity. They also demonstrated that both enzymes can process ubiquitin, although at that time they proposed that in case of OTUB2, five C-terminal amino acids had to be removed to obtain an enzymatically active DUB ($OTUB2\Delta_{1-229}$). OTUB1 and $OTUB2\Delta_{1-229}$ processed tetra-ubiquitin chains, but neither OTUB1, OTUB2, nor $OTUB2\Delta_{1-229}$ were able to cleave a peptide substrate LRGG-7-amido-4-methylcoumarin, or **ub**iquitin fused to **g**reen **f**luorescent **p**rotein (Ub-GFP). Further mass spectrometry evidence indicated that the proteolysis by OTUB1 occurred at the isopeptide bond of ubiquitin, identifying OTUB1 as a specific isopeptidase (Balakirev et al, 2003). The structural studies on OTUB2 (Nanao et al, 2004) revealed that its active site is sterically restricted by a novel loop conformation, leading to a hypothesis that the OTUB2 structure represents a self-inhibited state. Geometrically, the active site of OTUB2 is in a catalytically productive formation, even though in the absence of ubiquitin. OTUB2 structure could represent a novel mechanism of auto-inhibition, as suggested by the authors. Moreover, OTUB2 has been shown to have the highest levels of expression in brain tissue (Balakirev et al, 2003), but its physiological role in this context has not been yet revealed. Recent studies have found both OTUB1 and OTUB2 involved in negative regulation of virus-triggered type I IFN induction and therefore in cellular antiviral responses by affecting the ubiquitination status of E3 ubiquitin ligases TRAF3 and TRAF6 (Li et al, 2009c).

In contrast, OTUB1, a close relative of OTUB2, has been much better characterized in terms of its function. In human tissue it is a widely distributed DUB, and in leukocytes it appears in two forms that differ in their molecular

weight (Balakirev et al, 2003). The subcellular localization of OTUB1 has been recently investigated, concurrently with this study, and apart from the cytoplasm, OTUB1 has been shown to be present in the nucleus [(Shan et al, 2009), (Stanisic et al, 2009)], what is not surprising since OTUB1 contains a nuclear localization signal (Balakirev et al, 2003).

Intriguingly, transcriptional profiling of T cells (thymus cells, lymphocytes) revealed that protein expression of OTUB1 is increased in CD4+ and CD8+, but decreased in CD3+ T cells, placing OTUB1 in the communication network between CD4+ and CD8+ T cells (Wang et al, 2008). Moreover, OTUB1 has been shown to regulate T cell anergy via its interaction with ubiquitin E3 ligase GRAIL [**g**ene **r**elated to **a**nergy **i**n **l**ymphocytes; (Soares et al, 2004)]. GRAIL, a crucial regulator of T cell anergy, binds to two isoforms of OTUB1. Expression of the OTUB1 **a**lternative **r**eading **f**rame **1** (OTUB1 ARF-1) is associated with an increase in levels of endogenous GRAIL and with functionally anergic T cells, effects reversed by OTUB1 expression. Both isoforms of OTUB1 serve therefore as epistatic regulators of GRAIL. They function as such through controlling proteasomal degradation of GRAIL that depends on its auto-ubiquitination status. It has been further shown that GRAIL may be deubiquitinated by USP8, thereby preventing its degradation in the proteasome. OTUB1 ARF-1 binds to USP8, inducing its deubiquitinating activity towards the GRAIL substrate, while OTUB1 bound to USP8 reduces this effect, allowing degradation of the polyubiquitinated GRAIL (Soares et al, 2004). Further studies demonstrated that OTUB1 expression is induced during activation of CD4+ T cells, a process positively correlated with a decrease of GRAIL expression level (Lin et al, 2009).

Moreover, similarly to OTUB2, OTUB1 also deubiquitinates TRAF3 and TRAF6, thus negatively regulating the virus-triggered IFN-I induction and cellular antiviral responses (Li et al, 2009c).

Apart from its role in immunology, OTUB1 has been identified as a binder and a DUB of the **e**strogen **r**eceptor **α** [ERα; (Stanisic et al, 2009)] that mediates the effects of the steroid hormone 17β-estradiol and is a transcription factor regulating gene expression implicated in several cancer types. OTUB1 regulates ERα levels through its DUB activity as well as via regulation of the ERα transcription. The effect of OTUB1 on ERα transcriptional activity is negative due to the OTUB1-mediated stabilization of chromatin-bound ERα (Stanisic et al, 2009).

Finally, using a chemical genetics approach, OTUB1 has been shown to interact with YpkA, a *Yersinia* protein kinase that disrupts the actin cytoskeleton of the host cells. OTUB1 bound to YpkA also interacts with the actin cytoskeleton. Interestingly, OTUB1 interacting with YpkA has an approximate molecular weight of 37 kDa that is different from its expected molecular weight of 31 kDa. The authors further demonstrated that YpkA phosphorylates OTUB1 *in vitro* and hypothesized that OTUB1 function in T cell anergy (Soares et al, 2004) may be imperative in *Yersinia* survival within the host cell (Juris et al, 2006).

Clearly, OTUB1 is an enzyme with diverse physiological functions, such as T cell activation, antiviral response, transcription, and some aspects of bacterial infection.

1.4.3. General functions of deubiquitinating enzymes

Functions of DUBs can be classified into three major categories.

(1) Firstly, DUBs process linear polyubiquitin precursor proteins into single ubiquitin molecules. Ubiquitin is not expressed in a monomeric form but it is encoded either as a polyubiquitin gene or as ribosomal fusion genes. Subsequently DUB activity is required to cleave off monomeric ubiquitin from these adducts [(Baker et al, 1992), reviewed in (Wing, 2003)].

(2) Secondly, DUBs recycle ubiquitin by processing polyubiquitin chains to generate free ubiquitin that can subsequently enter the ubiquitin pool for the next ubiquitin conjugation events. It is a critical process since free polyubiquitin chains can competitively inhibit the binding of polyubiquitinated substrates to the 26S proteasome (Piotrowski et al, 1997). An example of such a DUB is **Iso**peptidase **T** (IsoT) that disassembles lys^{29}-, lys^{48}-, and lys^{63}-polyubiquitin conjugates [(Raasi et al, 2005), (Wilkinson et al, 1995)].

(3) Finally, DUBs remove ubiquitin from ubiquitinated substrates, antagonizing function of ubiquitin ligases and thus inhibiting various functions of the ubiquitin protein modification [(D'Andrea & Pellman, 1998), (Komander et al, 2009a)].

Deubiquitination is an important proteasome function and while DUBs can control proteasomal degradation of protein substrates by reversing the action of ubiquitin ligases, three DUBs have been identified to associate with the 26S proteasome itself, including USP14 (Borodovsky et al, 2001), UCH37 (Yao et al, 2006) and Rpn11 [POH1; (Koulich et al, 2008)]. USP14 and UCH37 cleave ubiquitin at the distal end of the ubiquitin chain (Koulich et al, 2008), thereby editing the polyubiquitin chains by trimming them. Knockdown of these DUBs is

associated with accelerated protein degradation but not with altered activity of the proteasome itself (Koulich et al, 2008). In contrast, knockdown of POH1 influences proteasomal activity by disrupting the assembly of the 26S proteasome and therefore impairs proteasomal degradation (Koulich et al, 2008). Unlike USP14 or UCH37, POH1 cleaves ubiquitin near or at the proximal end (Yao & Cohen, 2002). Interestingly, all above DUBs are inactive or less active if not associated with 26S proteasome, clearly demonstrating modulation of their enzymatic activity by the proteasome [(Borodovsky et al, 2001), (Verma et al, 2002), reviewed in (Reyes-Turcu et al, 2009)].

1.4.4. Specificity of deubiquitinating enzymes

The vast number of identified DUBs belonging to several distinct protein families suggests that there is a specialization of their functions and specificity. Indeed, it has been demonstrated that DUBs target distinct pathways and their localization may be limited to certain subcellular compartments [(Nijman et al, 2005), (Sowa et al, 2009)]. The specificity of DUBs can be discussed on two levels: their specificity towards different types of ubiquitin conjugates and their specificity towards substrates.

Although more comprehensive studies are required for the majority of DUBs, many of them are unspecific in terms of recognition of different ubiquitin linkages. An example is IsoT (Usp5), one of the first characterized DUBs. It is capable of cleaving lys^{29}-, lys^{48}-, and lys^{63}- linked polyubiquitin chains and it maintains the free ubiquitin pool (Raasi et al, 2005). Interestingly, within the USP family, CYLD is specific for lys^{63}- polyubiquitin and linear ubiquitin [(Komander et

al, 2008), (Komander et al, 2009b)], while USP14 preferentially cleaves lys^{48}-linked ubiquitin chains (Hu et al, 2005). The OTU family also contains DUBs with affinities towards particular ubiquitin linkages, including Trabid that displays a preference for lys^{63}-linkages (Tran et al, 2008). One explanation for the ability of DUBs to distinguish between different ubiquitin linkages suggests that DUBs can recognize several ubiquitins in a chain, what is mediated by the presence of several **u**biquitin-**b**inding **d**omains (UBDs). Another model proposes that this specificity relies on the structural flexibility of the UBD motion [(Komander et al, 2009b), (Sato et al, 2008), (Reyes-Turcu et al, 2009)]. Both hypotheses may be correct but to evaluate them more thoroughly, structures and specificities of more DUBs need to be examined, combined with mutational analysis of the UBDs.

Despite the broad palette of proteomic methods available for interaction studies, substrates remain unidentified for the majority of DUBs. Similarly to other enzyme—substrate pairs, an interaction between DUB and the target protein is transient, therefore making the identification challenging [(Nijman et al, 2005), (Sowa et al, 2009)]. Some insight on the target recognition is provided by USP7 (**H**erpes-**a**ssociated **USP**, HAUSP) that interacts with p53, Mdm2 and Mdmx, and its deubiquitinating function on p53 protects cells from apoptosis (Li et al, 2002). The N-terminal domain of USP7 interacts with p53 and Mdm2 by binding two closely spaced four-residue sites of both substrates. The experimental data and structural details indicate preference of USP7 for a P/AXXS binding motif (Sheng et al, 2006). For many DUBs there is a clear affinity for specific targets (Sowa et al, 2009), therefore such binding motifs may be a common feature. Moreover, the substrate specificity can be enhanced or triggered by macromolecular complexes in which they exist (Ventii & Wilkinson, 2008).

1.4.5. Regulation of enzymatic activity of deubiquitinating enzymes

DUBs are generally expressed as active enzymes, rather than inactive precursors. However, certain DUBs require ubiquitin binding to obtain their active conformations and that prevents their uncontrolled proteolytic activity. The structural data for several DUBs [(Edelmann et al, 2009), (Hu et al, 2005), (Johnston et al, 1997), (Johnston et al, 1999), (Lin et al, 2008), (Misaghi et al, 2005)] reveal that the ubiquitin-binding by DUBs is accompanied with the active site rearrangements and that such conformational alterations enhance their hydrolytic activity.

Another way of modulation of the DUB activity is through binding of scaffold and adaptor proteins. Despite their good catalytic capability, some DUBs display low affinity for ubiquitin and therefore require additional interactors for the substrate binding (Ventii & Wilkinson, 2008). DUBs may require incorporation into large macromolecular complexes to attain the active state, exemplified by USP14 or POH1 that are activated by their binding to the 26S proteasome [(Borodovsky et al, 2001), (Verma et al, 2002), (Reyes-Turcu et al, 2009)]. Activation of USP8 and AMSH is facilitated by **s**ignal **t**ransducing **a**daptor **m**olecule **2** (STAM2) and both proteins are involved in regulation of the endocytic traffic (McCullough et al, 2006). Protein—protein interactions can also inhibit protease activity, for example UCH37 activity is inhibited by its binding to chromatin-remodeling complex (Yao et al, 2008).

It has been demonstrated that some DUBs undergo post-translational modifications, which represent an effective means of regulation of their activity. CYLD, for instance, is transiently modified by phosphorylation, which inhibits its deubiquitinating activity on TRAF2 (Reiley et al, 2005). USP8 (UBPY) activity is

inhibited by phosphorylation induced by 14-3-3 proteins during the interphase (Mizuno et al, 2007). USP7 (HAUSP) was also shown to be phosphorylated, although any relation of this modification to its activity has not been yet demonstrated [(Beausoleil et al, 2004), (Fernandez-Montalvan et al, 2007)]. Another example of the post-translational modification regulating DUB activity is monoubiquitination of UCH-L1, a DUB involved in Parkinson's disease. Monoubiquitination of UCH-L1 in close proximity to its active site inhibits its activity through restriction of binding to ubiquitinated substrates (Meray & Lansbury, 2007). Moreover, modification with SUMO molecules was shown to inhibit activity of USP25 imposed by reduced binding to polyubiquitin chains (Meulmeester et al, 2008).

Last but not least, proteolytic cleavage of DUBs may also regulate their function. This can be illustrated by USP1, which undergoes the autoproteolysis that in turn inactivates this enzyme (Huang et al, 2006).

1.5. Deubiquitinating enzymes targeted by infectious pathogens

In the course of evolution viral and bacterial pathogens have acquired a series of molecular strategies to promote infection, proliferation and survival during the host—pathogen interactions. This aspect has been of considerable interest over many years, since by observing how pathogens are capable of manipulating the host cell pathways to their advantage much can be learned about cell biology in general. In addition, a greater understanding of the underlying molecular mechanisms of these processes will continue to provide entry points for drug targets and therapeutic treatment strategies, for which there is a great demand

since many infectious diseases are still not curable or there is a lack of satisfactory treatment options.

Pathogens are able to corrupt numerous cellular pathways of the host, one of which is the ubiquitin system. A reason for this is the fact that to date, over a thousand proteins have been associated with this pathway [(Kerscher et al, 2006), (Welchman et al, 2005)]. Modification by ubiquitin and Ubls is now recognized to be essential for the fate and function of the majority of cellular proteins and it can regulate various biological processes including protein turnover, receptor internalization, protein targeting to different cellular compartments, regulation of cell function and cell signaling. There are more than 500 proteins that are known to recognize ubiquitin and attach it to specific substrates, and over 90 deubiquitinating enzymes that can reverse this reaction (Nijman et al, 2005). This extraordinary diversity reflects the fact that ubiquitin conjugation and deconjugation is a very general regulatory process, which needs to be tightly controlled and the ubiquitination status of such a wide variety of substrates requires specific and targeted regulation. Furthermore, additional complexity of this pathway is based on the existence of many Ubls, such as SUMO-1/-2/-3/-4, NEDD8, ISG15, FAT10 and others [(Kerscher et al, 2006), (Herrmann et al, 2007)]. Targeting Ub/Ubl conjugation via the E1/E2/E3 enzymatic cascade by viral and bacterial pathogens has been a well-recognized theme and the subject of recent reviews [(Chen & Gerlier, 2006), (Munro et al, 2007), (Rytkonen & Holden, 2007), (Randow & Lehner, 2009)]. Removal of ubiquitin or Ubls from substrates by DUBs also appears to be an important biochemical step that viruses, bacteria and other pathogens can interfere with [(Rytkonen & Holden, 2007), (Lindner, 2007), (Randow & Lehner, 2009)] and will be discussed here in detail.

In general, one can distinguish two distinct ways in which pathogens interfere with the function of Ub/Ubl-specific proteases. First, the host cell-encoded Ub/Ubl-specific proteases are manipulated by pathogens during the infection process (Table 1.4). Second, pathogens encode for proteases that have Ub/Ubl-specific activities towards the host–encoded proteins (Table 1.5). In this chapter I argue that some of them have a common origin with mammalian counterparts, whereas others might have evolved completely independently for different functions within pathogen biogenesis, and adapted to the host—pathogen interactions throughout evolution.

1.5.1. Host encoded UB/Ubl-specific proteases modulated by pathogens

1.5.1.1. Proteases specific for ubiquitin-like proteins stimulated by interferons

Infection of most cell types by viruses or bacteria leads to expression of IFN-stimulated genes, which include the ubiquitin-like protein ISG15 and one of its specific proteases UBP43 [USP18; reviewed in (Kim et al, 2005)]. This suggests that they are components of the innate immune system and can contribute to an inherent antimicrobial response. During infection, ISG15 is conjugated to the substrate proteins by UBE1L, an ISG15-activating enzyme. This process can be specifically blocked by the influenza B virus encoded protein NS1 (Yuan & Krug, 2001). Furthermore, ISG15−/− mice have an increased susceptibility to influenza A/B, herpes simplex virus type 1, murine gammaherpesvirus 68 and sindbis virus (Lenschow et al, 2007).

Organism	Pathogen	Protein	Biological function	Host DUB	Biological function of the target	Physiological effect	References
Viruses	Herpes simplex virus type 1	ICP0	Maintaining balance between the latent and lytic states of a virus	USP7	Deubiquitination	Stabilization of ICP0	(Everett et al, 1997), (Geoffroy et al, 2006)
	Epstein–Barr virus	EBNA1	Replication, segregation, transcriptional activation of latent virus genomes	USP7	Deubiquitination	Stabilization of EBNA1, regulation of its replication function	(Holowaty et al, 2003), (Saridakis et al, 2005)
	LCMV, VSV	N.D.	N.D.	USP18	DeISGylation	Regulation of interferon signaling	(Ritchie et al, 2004), (Malakhova et al, 2006)
Bacteria	Non-Typeable *Haemophilus influenza*	N.D.	N.D.	CYLD	Deubiquitination	Negative regulation for NF-kB dependent inflammation, protection of the host against response to infection	(Lim et al, 2007a), (Sakai et al, 2007)

Organism	Pathogen	Protein	Biological function	Host DUB	Biological function of the target	Physiological effect	References
	Streptococcus pneumoniae	N.D.	N.D.	CYLD	Deubiquitination	Acute lung injury, increased mortality, CYLD deubiquitinates TAK1	(Lim et al, 2007b), (Koga et al, 2008)
	Escherichia coli	N.D.	N.D.	CYLD	Deubiquitination	Negative regulation for NF-kB dependent inflammation, protection of the host against response to infection	(Lim et al, 2008)

Table 1.4. ***Host encoded Ub/Ubl-specific proteases (N.D.: not determined).***

Organism	Pathogen	Ub/Ubl-specific protease	Specificity	Target	Physiological effect	References
Viruses	Coronavirus NL63	PLP2	Deubiquitination, deISGylation	N.D.	N.D.	(Nicholson et al, 2008), (Chen et al, 2007), (Sulea et al, 2006)
	Severe Acute Respiratory Syndrome Coronavirus	SARS-CoV PLpro	Deubiquitination, deISGylation, deneddylation	N.D.	N.D.	(Barretto et al, 2005), (Lindner et al, 2005), (Lindner et al, 2007), (Sulea et al, 2005), (Ratia et al, 2006), (Harcourt et al, 2004), (Devaraj et al, 2007)
	Adenovirus	Adenain (Avp, L3 23K proteinase)	Deubiquitination, deISGylation	N.D.	N.D.	(Balakirev et al, 2002), (Mangel et al, 2003), (Cao et al, 2003)
	Crimean–Congo Hemorrhagic Fever virus	OTU L (CCHFV-L)	Deubiquitination, deISGylation	N.D.	N.D.	(Frias-Staheli et al, 2007)
	Nairobi Sheep Disease virus	OTU L (NSDV-L)	Deubiquitination, deISGylation	N.D.	N.D.	(Frias-Staheli et al, 2007)
	Dugbe virus	OTU L (DUGV-L)	Deubiquitination, deISGylation	N.D.	N.D.	(Frias-Staheli et al, 2007)

Organism	Pathogen	Ub/Ubl-specific protease	Specificity	Target	Physiological effect	References
	Porcine Respiratory and Reproductive Syndrome virus	OTU L (PRRSV-L, Nsp2)	Deubiquitination, deISGylation	N.D.	N.D.	(Frias-Staheli et al, 2007), (Honig et al, 2004)
	Equine Arteritis virus	OTU L (EAV-L, Nsp2)	Deubiquitination, deISGylation	N.D.	N.D.	(Frias-Staheli et al, 2007), (Honig et al, 2004)
	Rice-Stripe virus Zhejiang	OTU L (RSV-L, pc1)	Predicted deubiquitination	N.D.	N.D.	(Zhang et al, 2007)
	Herpes simplex virus 1	UL36	Deubiquitination	N.D.	N.D.	(Kattenhorn et al, 2005)
	Murine cytomegalo-virus	M48	Deubiquitination	N.D.	N.D.	(Schlieker et al, 2005)
	Epstein–Barr virus	BPLF1, BSLF1, BXLF1	Deubiquitination	N.D.	N.D.	(Schlieker et al, 2005), (Sompallae et al, 2008)

Organism	Pathogen	Ub/Ubl-specific protease	Specificity	Target	Physiological effect	References
	Human cytomegalo-virus	pUL48	Deubiquitination	N.D.	N.D.	(Wang et al, 2006b)
	Murine gammaherpes virus 68	ORF64	Deubiquitination	N.D.	N.D.	(Gredmark et al, 2007)
	Pseudorabies virus	pUL36	Predicted deubiquitination	N.D.	Viral replication and neuroinvasion	(Bottcher et al, 2008)
	Marek's disease virus	MDV^{USP}	Deubiquitination	N.D.	Viral neuroinvasion	(Jarosinski et al, 2007)
Bacteria	*Salmonella sp*	AvrA	Deubiquitination	IκBα, β-catenin	Inhibition of the inflammatory responses	(Ye et al, 2007)
	Salmonella sp	SseL	Deubiquitination	N.D.	Delayed cytotoxicity of macrophages	(Le Negrate et al, 2008a), (Rytkonen et al, 2007)
	Yersinia sp	YopJ	Acetylation, deubiquitination, desumoylation	TRAF2/ 3/6, IκBα	Inhibition of the host inflammatory responses	(Sweet et al, 2007), (Zhou et al, 2005a), (Orth et al, 2000), (Mukherjee et al, 2006), (Orth, 2002)

Organism	Pathogen	Ub/Ubl-specific protease	Specificity	Target	Physiological effect	References
	Chlamydia trachomatis	ChlaDub1, ChlaDub2	Deubiquitination, deneddylation	N.D.	Suppression of NF-κB activation, stabilization of IκBα	(Misaghi et al, 2006) (Le Negrate et al, 2008b)
	Escherichia coli	ElaD	Deubiquitination	N.D.	N.D.	(Catic et al, 2007b)
Protozoa	*Plasmodium falciparum*	PfUCH54	Deubiquitination, deneddylation	N.D.	N.D.	(Artavanis-Tsakonas et al, 2006)
	Plasmodium falciparum	PfUCHL3	Deubiquitination, deneddylation	N.D.	N.D.	(Artavanis-Tsakonas et al, 2006), (Frickel et al, 2007)
	Toxoplasma gondii	TgUCHL3	Deubiquitination, deneddylation	N.D.	N.D.	(Frickel et al, 2007)

Table 1.5 ***Pathogen encoded Ub/Ubl-specific proteases (N.D.: not determined).***

Interference with deISGylation also affects susceptibility to viral infection, as demonstrated in USP18−/− mice. Cells derived from these animals did not support replication of a number of viruses, and animals infected with bacteria succumbed to an overshooting immune reaction. This event was accompanied by increased levels of ISGylation, suggesting a possible involvement of USP18-mediated isopeptidase activity in this process (Ritchie et al, 2004). However, an increased susceptibility to infection may be explained through multiple mechanisms, since USP18 was also reported to regulate IFN signaling independently of its ISG15 isopeptidase activity (Malakhova et al, 2006). No example has yet been reported in which USP18 is targeted by pathogens directly, but a number of viral gene products have recently been described to have the ISG15 deconjugating activity, therefore dampening the IFN response.

In addition, several DUBs encoded by mammalian cells can cross-react with ISG15-specific active-site probe, such as USP2, IsoT1 (USP5), IsoT3 (USP13) and USP14, suggesting potential specificity for ubiquitin and ISG15 (Catic et al, 2007a). However, in the case of USP2 and USP5, no significant ISGylating activity was detected using fluorescence-based substrates (Nicholson et al, 2008). The functional relevance of the potential dual specificity of these enzymes still awaits confirmation with protein-based substrates.

A second ubiquitin-like protein, FAT10, is also up-regulated upon exposure to IFN or TNFα (Groettrup et al, 2008). Overexpression of FAT10 can induce apoptosis, and was reported to target substrates for proteasomal proteolysis as an alternative pathway to ubiquitin (Hipp et al, 2005). The IFN dependence of FAT10 expression suggests that this molecule also represents a component of the innate

immune defense against pathogens, but its specific role in this process as well as FAT10-specific proteases remain to be examined.

1.5.1.2. USP7 targeted by herpesviruses

The **u**biquitin **s**pecific **p**rotease **7** (USP7) was initially described as **h**erpes **a**ssociated **USP** (HAUSP), based on the initial observation that it interacted with the **E**pstein–**b**arr **n**uclear **a**ntigen **1** (EBNA1) and the herpes simplex virus type 1 regulatory protein ICP0 (Everett et al, 1997). However, despite that these interactions have been studied in detail (Holowaty et al, 2003), their functional significance is not yet completely understood. In the case of EBNA1, the USP7 interaction domain overlaps with the one of the tumor suppressor gene p53, suggesting that EBNA1 may interfere with p53-mediated effects, such as premature apoptosis of infected cells (Saridakis et al, 2005). USP7 also contributes to ICP0 induced reactivation of gene expression from integrated adeno-associated virus AAV (Geoffroy et al, 2006). USP7 is generally involved in processes such as transcriptional regulation, DNA replication, apoptosis, and possibly in endosomal organization (Kessler et al, 2007). It appears that these viruses exploit multiple functions of USP7 and further experimental work is required to shed more light on these mechanisms.

1.5.1.3. CYLD and bacterial infections

Cylindromatosis is a benign skin tumor also referred to as turban tumor, and is originated by mutations in the CYLD gene (Bignell et al, 2000). Biochemical studies revealed that CYLD is a deubiquitinating enzyme acting on TRAF2 and TRAF6,

which results in a negative regulation of NF-κB, suggesting that CYLD plays an important role in controlling inflammation [(Kovalenko et al, 2003), (Brummelkamp et al, 2003), (Trompouki et al, 2003)]. Recent work uncovered that CYLD−/− mice are hypersensitive to infection by non-typeable *Haemophilus influenzae* [(Lim et al, 2007a), (Sakai et al, 2007)]. CYLD expression is induced in cells infected by this pathogen, and it was suggested to down-regulate NF-κB activation through deubiquitination of TRAF6 and TRAF7, therefore preventing the formation of a host antiviral response. A similar observation was made in an infection model using *Escherichia coli* pneumonia, in which an enhanced innate immune response was observed in CYLD−/− mice (Lim et al, 2008). An adverse effect was observed when CYLD−/− mice were challenged with *Streptococcus pneumoniae* (Lim et al, 2007b). In this case, CYLD was shown to provoke acute lung injury in the context of infection and therefore contributing to lethality. CYLD was suggested to interfere with the NFAT signaling pathway by deubiquitinating the **t**ransforming growth factor-β-**a**ctivated **k**inase **1** (TAK1), and to negatively regulate KK3-p38 kinase-dependent expression of plasminogen activator inhibitor-1 [(Lim et al, 2007b), (Koga et al, 2008)].

The example of CYLD having opposing effects in three different infection models particularly highlights the potential challenges that will be faced by the development of novel therapeutic approaches, which may interfere with one infectious agent, but may promote sensibility to another. Since there are other Ub/Ubl processing enzymes that are involved in regulating inflammation and the function of immune cells, such as A20 (Boone et al, 2004), it is expected that additional pathogen derived molecular mechanisms might exist that interfere with ubiquitin mediated activation of immune responses.

1.5.2. Ub/Ubl proteases in viral pathogens

1.5.2.1. Coronaviruses (SARS)

An example of a viral DUB that was discovered through structural bioinformatics is the **p**apain-**l**ike **pro**tease (PLpro) encoded by **s**evere **a**cute **r**espiratory **s**yndrome **co**rona**v**irus (SARS-CoV). Following this prediction, it has been demonstrated that PLpro can indeed cleave lys^{48}-linked polyubiquitin chains as well as ISG15 and Nedd8 conjugates *in vitro*, but with a preference for ISG15 [(Barretto et al, 2005), (Lindner et al, 2005), (Lindner et al, 2007)]. Crystallographic studies confirmed that PLpro shares considerable similarities to the catalytic core domain of USP7, USP14 and other already characterized DUBs [Figure 1.2; (Lindner et al, 2005), (Sulea et al, 2005)]. PLpro was therefore suggested to belong to the USP family (Ratia et al, 2006). The role of this enzyme is to process nsp1 to nsp3 from the viral replicase polyprotein (Harcourt et al, 2004).

Additionally, PLpro has been shown to block IRF3-dependent antiviral responses through inhibition of phosphorylation and translocation of IRF3. These two actions were suggested not to be attributable to the protease domain of PLpro, although the results obtained with the catalytic mutants used in this study were inconclusive (Devaraj et al, 2007). Interestingly, these events disturb activation of the type I IFN responses, which suggests that PLpro is an important molecule in the infection process.

It still remains a challenge to functionally distinguish between the general proteolytic activity and specific isopeptidase activity of PLpro, although more recent protease protection experiments suggest that the putative cytoplasmic domain of nsp3, which includes the PLpro domain, might be exposed and therefore

potentially capable of processing ubiquitinated or ISGylated host cell substrates (van Hemert et al, 2008).

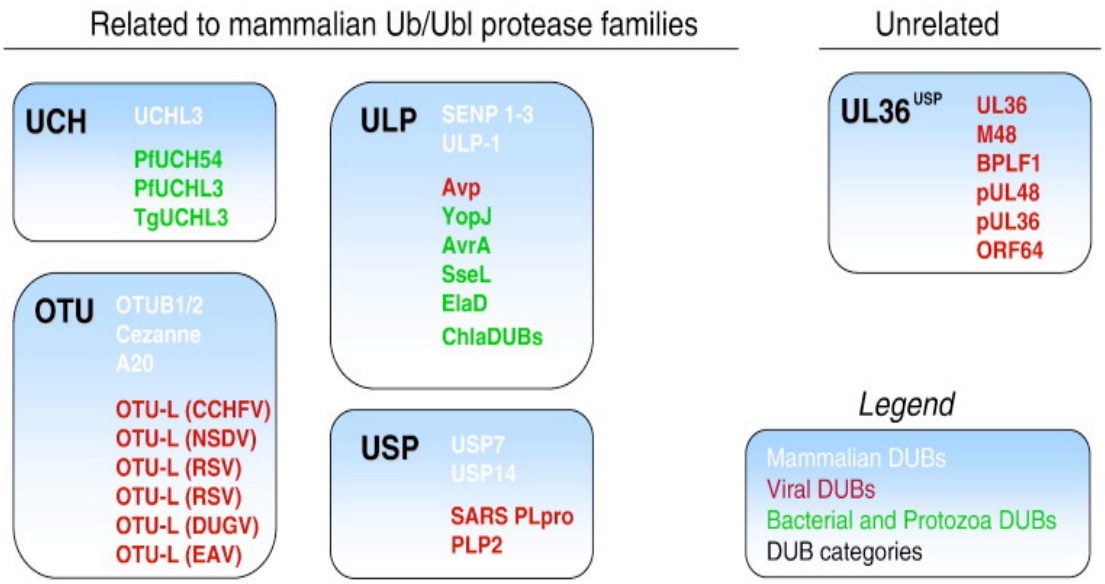


Figure 1.2. ***Pathogen encoded Ub/Ubl proteases and their relation to mammalian enzymes.*** Although many proteolytic enzymes derived from pathogens such as viruses (red), bacteria and protozoa (green) have unique properties, most of them are related to mammalian Ub/Ubl protease families (white) according to the sequence or structure-based similarities. Proteins with DUB activity encoded by *Herpesviridae* appear to be unique with little or no resemblance to any other Ub/Ubl protease families.

In another human coronavirus, NL63, two **p**apain-**l**ike **p**roteases (PLPs) PLP1 and PLP2 have been characterized, which process viral replicase polyproteins essential for RNA synthesis and viral replication (Chen et al, 2007). PLP2 shares some similarity with PLpro encoded by SARS-CoV, and has been shown to cleave lys^{48}-polyubiquitin chains *in vitro* (Sulea et al, 2006). Moreover, PLP2 has deISGylation activity, although it has a preference for ubiquitin over ISG15 (Nicholson et al, 2008). In this case, bioinformatics studies revealed that all coronaviruses encode for at least one papain-like protease with the deubiquitinating activity (Sulea et al, 2006).

1.5.2.2. Adenoviruses

Biochemical experiments on adenovirus-infected cells performed using **ub**iquitin **al**dehyde (Ubal) as a bait suggested deubiquitinating properties of Avp (23K proteinase, Ad L3, adenain). During infection, Avp processes viral proteins and appears to deubiquitinate some cellular proteins (Balakirev et al, 2002). In order to perform this task, it requires activation through viral DNA, an 11-amino acid viral peptide and actin as cofactors (Mangel et al, 2003). Additionally, Avp has been shown to cleave the peptide bond of an ISG15 precursor protein. Despite its structural similarity to Ulp1, a SUMO-specific protease, Avp does not have SUMO deconjugating properties (Balakirev et al, 2002). This viral proteinase plays a crucial role in the viral infection process by cleaving virion precursor proteins essential for the infectivity of the virus. In addition, Avp disrupts the cytoskeleton by cleavage of cytokeratin 18 in the cytosol, thereby provoking more efficient cell lysis (Mangel et al, 2003). Nevertheless, the deubiquitinating function of Avp has not yet been implicated in any physiological processes.

1.5.2.3. Viruses encoding for ovarian tumor (OTU)-like proteases

In the family of nairoviruses that includes **C**rimean-**C**ongo **h**emorrhagic **f**ever (CCHF), **dug**be (DUG) and **N**airobi **s**heep **d**isease (NSD) viruses, an OTU-like domain is present within their **l**arge (L) protein that also contains the RNA polymerase domain [(Frias-Staheli et al, 2007), (Honig et al, 2004)]. The L protein of CCHFV has been shown to posses a deubiquitinating and deISGylating activity. It has been demonstrated that this protein directly deconjugates polyubiquitin chains and ISG15-conjugated proteins *in vitro* and when expressed in mammalian

cells. All these properties have been observed in L proteins of NSDV and DUGV, when these were expressed in mammalian cells. Furthermore, sindbid viruses overexpressing the OTU-like domain of CCHFV-L showed an increase in infectious pathogenicity in mice. This result has been attributed to neutralization of the protective effect of ISG15 synthesis in infected cells (Frias-Staheli et al, 2007).

Another family of viruses, arteriviruses, including **e**quine **a**rteritis (EAV) as well as **p**orcine **r**espiratory and **r**eproductive **s**yndrome (PRRSV) viruses, encode for an OTU-like domain within the **n**on**s**tructural **p**rotein **2** [nsp2; (Frias-Staheli et al, 2007), (Honig et al, 2004)]. Nsp2 cleaves the large viral polyprotein in the nsp2/nsp3 site (Snijder et al, 1995). When overexpressed in mammalian cells, nsp2 decreases the level of ubiquitin and ISG15 conjugates (Frias-Staheli et al, 2007).

The OTU-like domain was also found in the **r**ice **s**tripe **v**irus (RSV) Zhejiang isolate as well as other RSV isolates that belong to the family of furoviruses, and it is present in the pc1 protein encoded by RNA1 (Zhang et al, 2007). It has been suggested that a putative role of this domain is the auto-proteolytic cleavage of pc1, which leads to activation of the viral polymerase (Zhang et al, 2007). Additionally, the DUB function of pc1 would provide a way to interfere with the host proteolytic pathway.

1.5.2.4. Herpesviruses

Activity-based profiling of cells infected with HSV-1 (**h**erpes **s**implex **v**irus-**1**) using active site-directed probes with a ubiquitin scaffold, combined with tandem mass spectrometry has revealed a virus-encoded USP, $UL36_{USP}$, that corresponds to the

N-terminal fragment of the large viral tegument protein UL36 (Kattenhorn et al, 2005). $UL36_{USP}$ cleaves ubiquitin, but not SUMO, ISG15 or Nedd8, and it has specificity towards lys^{48}-linked polyubiquitin (Kattenhorn et al, 2005). The catalytic site of $UL36_{USP}$ is conserved throughout the whole family of *Herpesviridae* (Kattenhorn et al, 2005), but there are no homologues of $UL36_{USP}$ in *Eukaryota*. Other members of the *Herpesviridae* family such as human **c**yto**m**egalo**v**irus (CMV), **m**urine **c**yto**m**egalo**v**irus (MCMV), **m**urine gamma**h**erpes**v**irus 68 (MHV), **M**arek's **d**isease **v**irus (MDV) and **p**seudo**r**abies **v**irus (PrV) contain $UL36_{USP}$ homologues. In most of these viruses it has been shown that the $UL36_{USP}$ homologues have deubiquitinating activity *in vitro* [(Schlieker et al, 2005), (Wang et al, 2006b), (Bottcher et al, 2008), (Jarosinski et al, 2007)]. The physiological relevance of these viral DUBs is not yet fully understood, although in MDV and PrV, a mutation of the catalytic site cysteine of $UL36_{USP}$ homologues affected the rate of viral replication. The expression of MDV_{USP} is additionally associated with lymphoma formation and metastasis in chicken (Jarosinski et al, 2007), whereas mutagenesis of the active site of PrV_{USP} inhibits viral neuroinvasion (Bottcher et al, 2008), which may be linked to a possible defect in transporting membrane-associated virus particles along microtubules (Shanda & Wilson, 2008).

1.5.3. Ub/Ubl proteases in bacteria and protozoa

1.5.3.1. Bacteria

Although bacteria do not have an intrinsic ubiquitin system, nor do they encode for ubiquitin or Ubls, with exception of recently characterized Pup (Burns & Darwin), it was found that many bacterial strains contain Ub/Ubl-specific proteases. For

instance, the *Salmonella* genome encodes for two different proteins with deubiquitinating activities, AvrA and SseL. Both are effector proteins translocated through the **t**ype **t**hree **s**ecretion **s**ystem (TTSS) of *Salmonella* [reviewed in (Munro et al, 2007)]. AvrA deubiquitinates IκBα and β-catenin as demonstrated with GST-tag purified enzyme, thus stabilizing IκBα and β-catenin and inhibiting the NF-κB inflammatory responses of the host (Ye et al, 2007). Similarly, SseL through its DUB activity suppresses IκBα ubiquitination and thereby prevents NF-κB activation (Le Negrate et al, 2008a). SseL can deubiquitinate mono- and poly-ubiquitinated substrates and has affinity towards lys^{63}-polyubiquitin chains (Rytkonen et al, 2007), indicating a role in the cellular signaling. In addition, SseL might be involved in delayed cytotoxicity of macrophages that constitutes an important mechanism of *Salmonella* virulence (Le Negrate et al, 2008a).

During infection with *Yersinia*, another enterobacterium that uses a TTSS, a virulence factor YopJ promotes intracellular survival of the bacterium via inhibition of the NF-κB, MAPK and IRF3 signaling pathways (Sweet et al, 2007). YopJ was first described to act as a DUB cleaving lys^{63}-polyubiquitin chains of TRAF2, TRAF3 and TRAF6 [(Sweet et al, 2007), (Zhou et al, 2005a)]. YopJ can also deubiquitinate lys^{48}-polyubiquitin chains of IκBα, thereby preventing its degradation and translocation of NF-κB (Orth et al, 2000). In addition, it has been proposed that YopJ might have a desumoylating activity (Orth et al, 2000), and more recent studies revealed that it additionally acts as an acetyltransferase to modify serine and threonine residues in the activation loop of MAPKK6, thereby blocking its phosphorylation and activation (Mukherjee et al, 2006). Consequently, YopJ interferes with the host pro-inflammatory responses, most likely via its Ub/Ubl processing properties as well as its acetylating activity [(Mukherjee et al,

2006), (Orth, 2002)]. This finding may represent a common feature that is also present in other proteolytic enzymes, such as the *E. coli*-derived TAP protein (Lee et al, 2006).

Activity-based profiling studies in the *Chlamydia* proteome using the ubiquitin-based active-site probes identified two DUBs of *Chlamydia trachomatis*, ChlaDub1 and ChlaDub2. They have been reported to cleave ubiquitin and Nedd8 conjugates (Misaghi et al, 2006). ChlaDub1 has been shown to suppress NF-κB activation and inhibit IκBα degradation, therefore interfering with the host inflammatory response (Le Negrate et al, 2008b).

The *E. coli*-encoded protease ElaD has been identified as a DUB specific for ubiquitin, but not for ISG15, Nedd8 or SUMO. ElaD is found in all intestinal strains, but absent from extraintestinal strains of *E. coli*, an observation that may point towards the significance of ElaD in the infection process (Catic et al, 2007b).

1.5.3.2. Parasitic protozoa

Although there have been predictions of many putative DUBs [reviewed in (Ponder & Bogyo, 2007)] encoded by medically relevant parasitic protozoa using bioinformatics studies, only three DUBs have been reported to date [(Artavanis-Tsakonas et al, 2006), (Frickel et al, 2007)]. All of them were found via an activity-based Ub/Ubl probe approach and no alternative validation of their DUB activity has been yet carried out. *Plasmodium falciparum* encodes for two deubiquitinating enzymes, Pf_{UCHL3} and Pf_{UCH54}, the latter being a homolog of human deubiquitinating enzymes UCH37, UCH-L1 and UCH-L3 (Figure 1.2). Although the sequence did not reveal a high sequence identity between Pf_{UCH54} and human DUBs, all the active site

residues have been preserved (Artavanis-Tsakonas et al, 2006). Interestingly, Pf_{UCHL3} and Pf_{UCH54} have both deubiquitinating and deneddylating activity [(Artavanis-Tsakonas et al, 2006), (Frickel et al, 2007)], as shown using activity-based Ub/Ubl probes. Because Pf_{UCH54} does not have a specific N-terminal sequence that allows it to be secreted, it has been hypothesized that this protein has an important function within the parasite itself, and is not host-targeted (Artavanis-Tsakonas et al, 2006).

Toxoplasma gondii encodes for Tg_{UCHL3} that is also a homologue of human DUB, UCH-L3. Tg_{UCHL3} was found to be specific to Nedd8 and ubiquitin active-site probes, but not SUMO (Frickel et al, 2007). However, the probes that were used for studying Tg_{UCHL3} and Pf_{UCHL3} reactivity to Nedd8 and SUMO were prepared using Ubl proteins that are more closely related to the human sequences than to their parasitic homologues, therefore the observed reactivities towards the different Ubls should be interpreted with caution. In all these cases, the precise role of these proteases within the context of pathogenicity has not yet been elucidated.

1.5.4. Common ancestry patterns of Ub/Ubl-specific proteases involved in host-pathogen interactions

Although the bacterial proteasome has been only identified in the order *Actinomycetales* (Burns & Darwin), many pathogens of prokaryotic origin seem to encode for proteins with Ub/Ubl recognition and processing properties. This is also the case for viral pathogens, in which the presence of Ub/Ubl-specific proteases appears to be more widespread than previously anticipated. Pathogens with larger genomes contain most likely a larger number of Ub/Ubl-specific

proteases, and more advanced bioinformatics approaches reveal novel members, as exemplified by the recent discovery of bona fide DUBs encoded by EBV (Sompallae et al, 2008). For many of these, we are at an early stage of deciphering their precise functions, but sequence and structure-based comparisons with mammalian Ub/Ubl-specific protease families may provide some clues about their biology. Based on this, pathogen encoded Ub/Ubl proteases would appear to fall into two general categories.

The first one consists of Ub/Ubl proteases that can be added to the list of already characterized superfamilies (Figure 1.2). For instance, the family of UCH contains three mammalian hydrolases, and the more recently characterized proteases encoded by the parasites *Plasmodium falciparum*, *Toxoplasma gondii* and related organisms may form an extended UCH superfamily. The second example is the adenovirus encoded protease adenain, the *Chlamydia* derived ChlaDUB-1/-2 proteases, *E. coli* derived ElaD, *Salmonella* encoded SseL, *Yersinia* encoded YopJ and similar sequences observed in other pathogens, all of which share some similarities with ULP/SENP-related proteases that are specific for SUMO and Nedd8 (Figure 1.2). This superfamily of cysteine proteases has been classified as the CE clan, which targets ubiquitin and Ubls [MEROPS database; (Catic et al, 2007b)], but potentially harbors other enzymatic functions, as illustrated in the cases of YopJ (Trosky et al, 2008) and adenain (Balakirev et al, 2002). The third example is the SARS coronavirus encoded protein PLpro that contains a catalytic core that is structurally related to the ones observed in USP7 and USP14 (Sulea et al, 2005). A fourth example is the protein family sharing the OTU domain, which contains approximately 80 members derived from plants, *Drosophila*, bacteria, viruses, yeast and higher eukaryotes [(Nijman et al, 2005), (Makarova et al, 2000)].

In all these cases this may point towards common fundamental ancestry functions that are essential within these organisms. Adaptation mechanisms in the context of host—pathogen interactions may have further diversified their functions, as exemplified by viral OTU domain-containing proteases that have acquired specificity for ISG15, a trait that is not observed for their mammalian counterparts (Frias-Staheli et al, 2007).

The second category is represented by novel and unique classes of pathogen encoded Ub/Ubl-specific proteases (Figure 1.2). This is most likely the case for herpes virus encoded DUBs [reviewed in (Sulea et al, 2006)]. The primary function of these proteases may involve processing of the viral gene products necessary for virus life cycle. Adaptation to ubiquitin specificity as a dual function may have evolved in an evolutionary process to increase the pathogenicity of the host—pathogen interactions. Unique structural features of pathogen encoded Ub/Ubl-specific proteases are attractive entry points for the development of new antimicrobial drugs, and research efforts towards this direction may soon provide novel alternatives to combat a variety of infectious diseases.

1.6. Objectives of this study

DUBs constitute a diverse protein family, many of which remain yet to be characterized. The specificities, functions, and substrates of the majority of DUBs are still unknown (Reyes-Turcu et al, 2009), although their impact on numerous biological and pathological processes is now widely appreciated [(Komander et al, 2009a), (Reyes-Turcu et al, 2009)].

This study focuses on the characterization of the human deubiquitinating enzyme OTUB1 (Balakirev et al, 2003), which belongs to the DUB superfamily of OTU-domain containing proteases (Makarova et al, 2000). Amongst other functions, OTUB1 has been suggested to be a substrate of YpkA (Juris et al, 2006) – a bacterial kinase that represents an important virulence factor of *Yersinia*. As described earlier (Chapter 1.5), DUBs are potentially important players in microbial infection, yet the functions of OTUB1 in *Yersinia* infection and the details of its molecular interaction with YpkA have not been yet demonstrated.

I have aimed to reveal the biochemical properties of OTUB1, in particular its structure and specificity for different ubiquitin chains and Ubls. Next, I localized OTUB1 in subcellular compartments using microscopy and fractionation methods and screened for substrates of OTUB1 using proteomics-based technique (Chapter 4). I then investigated the function of OTUB1 in *Yersinia* infection and its effect on RhoA. Moreover, since OTUB1 proved to be post-translationally modified by phosphorylation, I attempted to map the phosphorylated residues (Chapter 5) using previously established mass spectrometric methods (Chapter 3). Finally, I examined the effect of phosphorylation on the enzymatic activity of OTUB1 and set out to identify kinases responsible for this modification (Chapter 5, Chapter 6).

Please note that only the abstract, table of contents and first chapter of this thesis can currently be made available in ORA.