Thesis for doctoral degree (Ph.D.) 2007

MODULATION OF THE DEUBIQUITINATING SYSTEM IN VIRAL INFECTION

Ulrika Rolén

Karolinska Institutet

Thesis for doctoral degree (Ph.D.)
2007

MODULATION OF THE DEUBIQUITINATING SYSTEM IN VIRAL INFECTION, LYMPHOID CELL ACTIVATION AND MALIGNANT TRANSFORMATION

Ulrika Rolén

Karolinska Institutet

From the Department of Microbiology, Tumor and Cell Biology,
Karolinska Institutet, Stockholm, Sweden

MODULATION OF THE DEUBIQUITINATING SYSTEM IN VIRAL INFECTION, LYMPHOID CELL ACTIVATION AND MALIGNANT TRANSFORMATION

Ulrika Rolén

Karolinska Institutet

Stockholm 2007

Published by Karolinska Institutet
Box 200, SE-171 77 Stockholm, Sweden

ISBN 978-91-7357-389-4

Printed by
REPROPRINT AB
Stockholm 2007
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

“A teacher affects the eternity; he never knows where his influence ends.”
Henry Adams

Abstract

Most cellular proteins are affected by the conjugation of Ubiquitin (Ub), which may regulate their stability, activity or localization. The family of deubiquitinating enzymes (DUBs) removes Ub from conjugates and regulates the production and recycling of Ub. DUBs are thereby critically involved in the control of important functions such as cell growth, differentiation and apoptosis. Increasing evidence implicates deregulation of DUBs in malignant transformation. The human genome contains many putative DUB-encoding genes but little is known about their tissue distribution, pattern of expression, activity and substrate specificity. This thesis describes the use of a chemistry-based functional proteomics approach to identify active DUBs in human tumor cells of different tissue origin, in primary resting and mitogen stimulated cells, in Epstein-Barr virus (EBV) infected B lymphocytes, as well as in human papilloma virus (HPV) E6/E7 immortalized keratinocytes. The role of the ubiquitin C-terminal hydrolase (UCH)-L1 in the EBV-related B cell tumor Burkitt´s lymphoma (BL) is further investigated.

Both tumor specific and tissue specific patterns of DUB activity were identified in a panel of tumor cell lines of different tissue origin. HPV carrying cervical carcinomas showed a diverse activity pattern when compared to the adjacent normal tissue, suggesting a role for some of the DUBs in the development of this tumor. The activity of specific enzymes, including USP5, -7, -9, -13, -15 and -22, was upregulated by mitogen activation or virus infection in normal T- and B-lymphocytes. UCH-L1 was highly expressed in tumor cell lines of epithelial and hematopoietic cell origin but was not detected in freshly isolated and mitogen activated cells. Upregulation of this DUB was a late event in the establishment of EBV immortalized lymphoblastoid cell lines (LCLs) and correlated with enhanced proliferation suggesting a possible role in growth transformation.

UCH-L1 was shown to regulate lymphocyte function-associated antigen (LFA)-1 dependent homotypic adhesion in B-lymphocytes. Integrin-mediated cell adhesion is essential in the development and activation of B-cells, and tight LFA-1 mediated adhesion may hinder malignant cell growth and invasion. LFA-1 dependent homotypic adhesion was activated following UCH-L1 knockdown in BL cells and this correlated with slow-down of BL cell proliferation in suspension and in semisolid agar and an accumulation in the G1 phase of the cell cycle. This suggests that UCH-L1 is required for maintaining LFA-1 in a low-avidity, non-adhesive state that favors the proliferation of malignant B-cells.

List of publications

This thesis is based on the following papers that will be referred to in the text by their Roman numerals:

I Huib Ovaa*, Benedikt M. Kessler*, **Ulrika Rolén***, Paul J. Galardy, Hidde L. Ploegh and Maria G. Masucci. Activity-based ubiquitin-specific protease (USP) profiling of virus-infected and malignant human cells. PNAS, vol. 101, no. 8, 2253–2258, 2004.

II **Ulrika Rolén***, Vera Kobzeva*, Natalja Gasparjan, Huib Ovaa, Gösta Winberg, Fjodor Kisseljov and Maria G. Masucci. Activity profiling of deubiquitinating enzymes in cervical carcinoma biopsies and cell lines. Molecular Carcinogenesis, vol. 45, no. 4, 260-269, 2006.

III **Ulrika Rolén**, Elio Freda, Vanessa Vasques-Leiva, Jianjun Xie and Maria G. Masucci. The ubiquitin C-terminal hydrolase UCH-L1 regulates LFA-1 dependent homotypic adhesion and B-lymphocyte proliferation. *Submitted*

* These authors contributed equally to the work

Abbreviations

ATP	Adenosine triphosphate
BCR	B cell receptor
BL	Burkitt´s lymphoma
CIN	Cervical intraepithelial neoplasia
CTL	Cytotoxic T lymphocyte
CSN	COP9 signalosome
DUB	Deubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
E6AP	E6 associated protein
E6TP	E6 targeting protein
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complex required for transport
FAM	Fat facets in mouse
GAP	GTPase activating protein
GEF	Guanine exchange factor
Gly-ala	Glycine-alanine
GPCR	G-protein coupled receptor
HAUSP	Herpes virus associated USP
HCMV	Human cytomegalovirus
HD	Hodkin´s disease
HECT	Homology to E6AP C-terminus
HPV	Human papilloma virus
HRS	Hodkin´s Reed Sternberg
HSV	Herpes simplex virus
ICAM	Intercellular adhesion molecule
IFN	Interferon
ITAM	Immunoreceptor tyrosine-based activation motif
KSHV	Kaposis sarcoma herpes virus
LCL	Lymphoblastoid cell line
LFA	Lymphocyte function-associated antigen
LMP	Latent membrane protein
LSD	Lysosomal storage disease
MHC	Major histocompatibility complex
MJD	Machado-Joseph disease protease
MVB	Multivesicular body
NPC	Nasopharyngeal carcinoma
OTU	Ovarian tumor
PLpro	Papain-like protease
RAPL	Regulator of adhesion and cell polarization enriched in lymphoid tissues
RIAM	Rap-interacting adapter molecule
RING	Really interesting new gene
RNAi	RNA interference
RTK	Receptor tyrosine kinase
SARS-CoV	Severe acute respiratory syndrome corona virus
SDS-PAGE	SDS polyacrylamide gel electrophoresis
TAP	Transporter associated with antigen processing
TGF	Transforming growth factor
TNFR	Tumor necrosis factor receptor
TPP II	Tripeptidyl-peptidase II
TRAF	TNFR associated factor
Ub	Ubiquitin
UBD	Ubiquitin binding domain
UBL	Ubiquitin like protein
UCH	Ubiquitin carboxy-terminal hydrolase
UIM	Ubiquitin interacting motif
UPS	Ubiquitin proteasome system
USP	Ubiquitin specific protease
VHL	Von Hippel Lindau

Contents

Aims of this study

The general aim of the work presented in this thesis was to investigate the importance of the deubiquitinating system during lymphoid cell activation, viral infection, immortalization and malignant transformation.

The specific aims were to:

- Investigate how infection with the Epstein-Barr virus affects the activity profile of deubiquitinating enzymes in B lymphocytes.

- Investigate the pattern of activity of deubiquitinating enzymes in EBV and HPV carrying malignant cells in comparison with the pattern seen in normal tissue.

- Investigate the role of the deubiquitinating enzyme UCH-L1 in the tumor phenotype of Burkitt´s lymphoma.

General introduction to ubiquitination and deubiquitination

All processes that take place in a cell are, directly or indirectly, affected by ubiquitination and deubiquitination. Cell proliferation, differentiation, immune response and apoptosis are examples of important systems regulated by ubiquitination. The most explored outcome of ubiquitination is degradation of the ubiquitinated protein by the proteasome, and the list of cellular substrates of the ubiquitin proteasome system (UPS) is growing rapidly to include cell cycle regulators such as cyclins, cyclin-dependent kinase inhibitors, proteins involved in sister chromatid separation, tumor suppressors, oncoproteins, transcriptional activators and their inhibitors. Also mutated and denatured or misfolded proteins are recognized and removed efficiently by the UPS, a process referred to as quality control.

Post-translational modification by ubiquitination is also associated with non-proteolytic outcomes, such as DNA repair, histone regulation, endocytosis and protein sorting. The process of ubiquitination has been extensively studied, and mainly involves enzymes from three groups: E1, E2 and E3. The reversal of ubiquitination by the action of deubiquitination is an equally important step in regulating ubiquitination, and the functions and specificities of an increasing list of deubiquitinating enzymes (DUBs) are being elucidated. Considering the important regulatory role of ubiquitination in diverse signaling cascades in the cell, it is not surprising that increasing evidence supports a role of several DUBs in diseases such as malignancies.

Processes regulated by ubiquitination and deubiquitination are being exploited by pathogens such as viruses. Viral entry and budding, and interference with the immune system, are all processes where viruses take advantage of or interfere with the host cell functions involving ubiquitination. In many cases, viral interference with the infected host cell may eventually lead to malignant transformation. Some examples of viruses expressing proteins with ubiquitinating capacity, or regulating the activity or specificity of ubiquitinating enzymes of the infected cells have been widely studied. Although it is likely that viruses interfere also with deubiquitination, so far not much is known about the extent and specificity of such interference.

Ubiquitination and proteasomal degradation

Ubiquitination occurs through the concerted action of several enzyme groups. One or possibly more ubiquitin (Ub) activating enzymes (E1) [1, 2], several Ub conjugating enzymes (E2) and hundreds of Ub ligases (E3) cooperate in this multi-step process (reviewed in [3]). Figure 1 illustrates the ubiquitination of a protein. In the first step, Ub is activated by the E1, forming a thioester between the catalytic cysteine of the E1 and the C-terminal glycine of Ub. After this, Ub is transferred to an E2, forming a new thioester. Ub can then be transferred directly to the substrate protein, as demonstrated only in a few cases where ubiquitin binding domain (UBD)-containing proteins cooperate with the Ub-binding E2 to promote their own ubiquitination [4]. More commonly however, Ub is transferred to the substrate protein from the E2 through the action of an E3 ligase. The E3 enzymes are grouped into families depending on homology and function. The homology to E6-AP C-terminus (HECT) family of E3s normally accepts Ub from the E2, forming a thioester, followed by donation of Ub to the target protein. The really interesting new gene (RING) family of E3s brings an E2-Ub thioester conjugate and the substrate in close proximity to facilitate the transfer of Ub from the E2 to the substrate. (Reviewed in [5])

The multitude of E3s suggests that it is in these and possibly to some extent in the E2s that the specificity for the substrates to be ubiquitinated is retained. Indeed, most E3 ligases have been identified as specifically targeting one or a subset of proteins for degradation (reviewed in [6]). Substrates of the E3s include tumor suppressor proteins and oncoproteins. An increased degradation of a tumor suppressor or a decreased degradation of an oncoprotein might contribute to tumorogenesis.

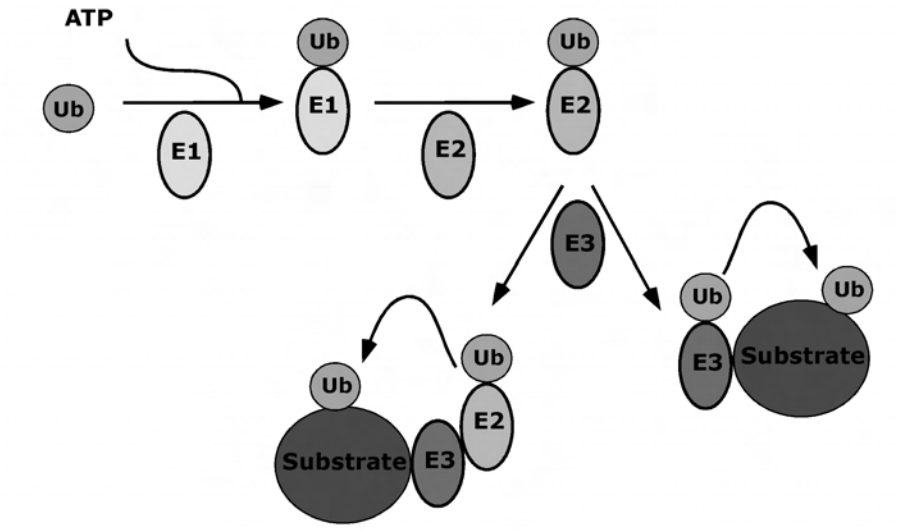

Figure 1. Ubiquitination of proteins is a multistep process, involving the action of E1, E2 and E3 enzymes. E1 activates Ub in an energy-dependent manner. Ub is then transferred to an E2, and to the substrate via an HECT E3 (right) or directly to the substrate from the E2 through the action of a RING finger E3 (left).

Proteins are commonly ubiquitinated by the formation of an isopeptide bond between the C-terminal glycine of Ub and the ε-amino group of a lysine (K) in the target protein (Reviewed in [6]). Proteins may also be ubiquitinated on their N-terminal amino group, by N-terminal ubiquitination (reviewed in [7]). Examples of naturally occurring lysine-less proteins are the human cell cycle regulator p16 and the human papilloma virus (HPV) oncoprotein HPV-58 E7 [8]. Other examples of proteins that undergo N-terminal ubiquitination and consequent degradation are the Epstein-Barr virus (EBV) latent membrane protein (LMP)-1 [9] and LMP-2A [10]. Ubiquitination may also occur on amino acids other than lysine: cysteine is ubiquitinated by the action of the Kaposi's sarcoma herpes virus (KSHV) protein K3 [11], and formation of an ester bond between Ub and serine or threonine residues by the action of the mK3 has been proposed to promote polyubiquitination and degradation of the heavy chain of the major histocompatibility complex (MHC) class I [12].

After the first Ub molecule is attached, a chain consisting of several Ub molecules may be constructed by the concerted action of an E2 and an

E3. An isopeptide bond between the ε-amino group of one of the seven internal lysine residues of the already attached Ub and the C-terminal glycine residue of the next Ub is formed. By repeating this step, eventually a polyubiquitin chain is formed. Each of the lysine residues present within Ub can be targeted by another Ub molecule [13, 14]. Normally a chain of Ub moieties attached through lysine 48 (K48-linked chains) targets the protein for degradation by the proteasome. (Reviewed in [5])

Intracellular degradation of proteins is a highly specific, tightly regulated process. Ubiquitination of proteins is followed by the recognition and consequent degradation of the protein by the proteasome (reviewed in [5]). Proteolysis is an energy-demanding process, dependent on several different factors [15, 16], including Ub [17, 18]. In a model postulated by Herschko et al [16], at least four different steps are important for the Ub- and energy dependent proteolysis of proteins to occur. Figure 2 shows a modification of the model with the addition of the important newly identified factors at each step. Step 1 involves the ATP-dependent ubiquitination of a protein. Step 2 represents the rescue of proteins from degradation by deubiquitination. In step 3 the ubiquitinated protein is recognized and degraded by the proteasome, while step 4 illustrates the recycling of Ub molecules to the free Ub pool by DUBs. The proteasomal products are peptides that can be further processed by peptidases in the cell. (Reviewed in [3])

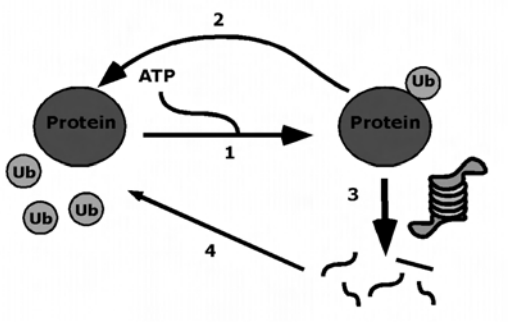

Figure 2. Proteins are tagged for degradation, in an ATP-dependent manner, by modification with Ub (step 1). DUBs may trim or reverse the ubiquitination (step 2), that otherwise leads to proteasomal degradation of the modified protein into peptides (step 3). Ub is recycled into the free Ub pool by the action of DUBs (step 4).

The proteasome is a big, barrel-shaped, multi-subunit complex consisting of a 19S regulatory particle (RP) and a 20S core particle (CP). To avoid unspecific cleavage, the proteolytic activity of this big protease is hidden within the 20S CP. After recognition by the 19S RP, the substrate is deubiquitinated by the action of DUBs associated with the 19S RP. The protein is unfolded by the AAA-ATPase subunits of the 19S RP, and can thereafter enter into the core of the proteasome. The 20S CP consists of two α-heptamers forming the outer rings, and two β-heptamers forming the inner rings of the CP. The proteolytic activities of the proteasome reside in the β-heptamer subunits. Hydrolysis preferentially occurs on the carboxyl side of hydrophobic, basic, or acidic residues. (Reviewed in [5]) Interferon (IFN)-γ alters the composition of the enzymatic subunits and causes changes in the peptidase activity that favors the production of peptides with hydrophobic or basic carboxyl termini, i.e. the types found on MHC class I molecules. This new composition is called the immunoproteasome (reviewed in [19]). Interfering with the proteasome to avoid production of immunogenic epitopes might be one way for viruses and tumor cells to escape immune response. In a comparison of phenotypic characteristics between in vitro EBV-immortalized lymphoblastoid cell lines (LCLs) and EBV positive cell lines derived from Burkitt´s lymphoma (BL) it was demonstrated that BLs are resistant to cytotoxic T lymphocyte (CTL) dependent killing in vitro, partly explained by a lower activity of the immunoproteasome leading to a lower production of immunogenic epitopes [20, 21].

Non-proteolytic consequences of ubiquitination

The most extensively studied consequence of ubiquitination is proteasomal degradation. However, ubiquitination accounts for regulation of other less well-characterized processes such as endocytosis of membrane-bound receptors and DNA repair. The seven lysine residues in Ub are located at positions 6, 11, 27, 29, 33, 48 and 63, and polyubiquitin chains could form through the subsequent attachment of Ub to any of these [13, 14]. K6 linked chains have been reported to occur by the action of the tumor suppressor BRCA1 that works as an E3 ligase when in complex with BARD1 [22]. BRCA1 is suggested to play a role in DNA damage response. When absent or mutant, as is the case for example in

breast cancer, DNA damage repair does not occur, allowing for tumor development through the accumulation of mutations (reviewed in [23]). K63-linked chains are involved in DNA repair [24] and signal for activation of the IκBα kinase (IKK) in inflammatory signaling pathways (reviewed in [25]).

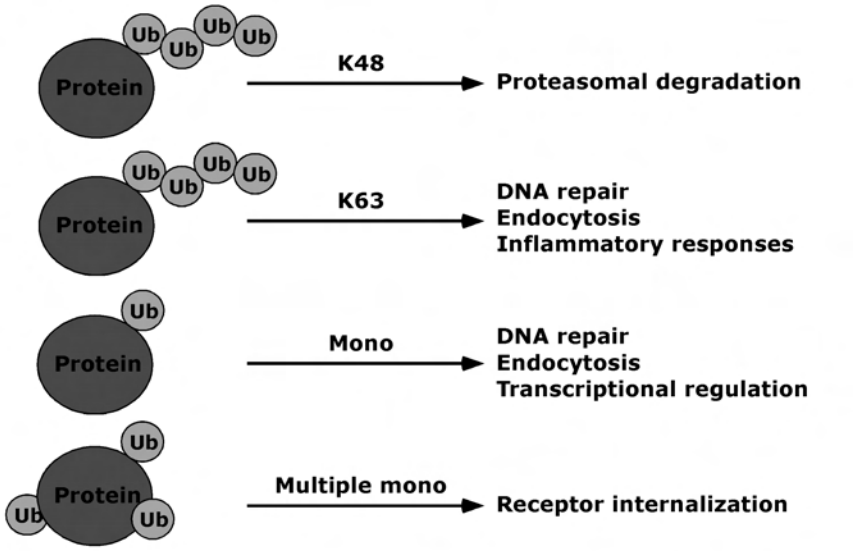

Figure 3. Ubiquitination has diverse consequences depending on the type of modification that takes place. Polyubiquitination via K48 linked chains dictates degradation via the proteasome, while polyubiquitination via K63 linked chains, and single or multiple monoubiquitination regulates processes such as endocytosis, DNA repair and transcription.

Monoubiquitination, i.e. attachment of only one Ub, of histone H2A upon DNA damage seems to be involved in facilitating an appropriate DNA damage response [26]. Also several actors of the UPS, such as Rad23 [27], a functional proteasome [28], and several E3 ligases have been shown to play a role in DNA repair. Monoubiquitination of histone H2B has been implied in regulation of transcriptional activity [29].

Monoubiquitination, multiubiquitination, or K63-linked Ub chains regulate endocytosis. Endocytosis is used by the cell to regulate for example the composition of the plasma membrane and cell migration. Also different aspects of signal transduction, such as intensity and duration, are regulated through endocytosis. Monoubiquitination and consequent endocytosis of membrane bound receptors, following their activation, is used as a mean of terminating the signaling of receptor

tyrosine kinases (RTKs) (reviewed in [30]). The E3 ligase of several RTKs is Cbl, and the function and interacting proteins of the Cbl proteins have been extensively studied (reviewed in [31]). Ubiquitination seems to be involved in regulating the choice of endocytic pathway and the consequences of endocytosis. The epidermal growth factor receptor (EGFR) is internalized through clathrin-mediated endocytosis when stimulated with low levels of ligand. High levels of EGF lead to monoubiquitination of EGFR and internalization also through clathrin-independent endocytosis [32]. It is speculated that the clathrin-mediated endocytosis allows prolonged signaling, and is therefore preferred when low levels of the ligand is present, while upon high levels of ligand, the clathrin-independent pathway is chosen as it allows for receptor degradation (reviewed in [33]).

Not only endocytosis, but also sorting of an internalized protein is regulated by ubiquitination. One mechanistic explanation lies in the finding that many of the endocytic adapter proteins carry UBDs allowing for their interaction with ubiquitinated proteins. Eps15 and epsin are adaptor proteins suggested to couple ubiquitinated cargo to clathrin-independent internalization. UBD containing proteins often have more than one UBD, and this could allow for the recognition of multiubiquitinated proteins or K63-linked chains. They also often contain other protein-interaction sites, through which they may interact with other proteins involved in the endocytic process. (Reviewed in [30]) The adaptor proteins are ubiquitinated themselves, and the attached Ub seems to bind intramolecularly to the UBD within the same adaptor protein [34]. This binding has been suggested to inhibit the interaction of adaptor proteins with ubiquitinated cargo, and thereby work as a control mechanism.

Sorting of endosomal cargo into the multivesicular body (MVB) is regulated by mono- or multiubiquitination, while proteins that are not ubiquitinated are likely to be sorted back to the membrane to be displayed on the cell surface once more. Ub is recognized by the vacuolar protein sorting-associated protein (Vps27) that brings together ubiquitinated cargo with the endosomal sorting complex required for transport (ESCRT)-I complex. The ubiquitinated cargo is then passed on to ESCRT-II and then ESCRT-III, allowing for concentration of cargo

proteins in the MVB vesicles. This maturation eventually leads to the formation of lysosomes in which the endosomal cargo is degraded. (Reviewed in [30]) Ubiquitination thereby seems to target proteins not only for proteasomal degradation, but also to degradation of proteins in the lysosome. Compromised Ub-mediated transport of certain signaling receptors is associated with disease states, including oncogenic transformation (reviewed in [35]).

Deubiquitination

DUBs are crucial in regulating the efficiency and specificity of ubiquitination, in that they mediate the removal and processing of Ub. They are therefore likely to play important roles in regulating cellular processes such as cell cycle progression, DNA repair, endocytosis and apoptosis. Indeed changes in DUB activities are implicated in disease states such as neurodegeneration and malignancies.

The human genome encodes for more than 90 putative DUBs and many of them contain domains involved in protein-protein interactions [36]. Most DUBs are cysteine proteases, where the catalytic activity relies on a cysteine residue, an adjacent histidine and an aspartate residue. The cysteine protease DUBs have further been divided into four subclasses depending on their protease domain: Ub specific protease (Usp), Ub carboxy-terminal hydrolase (UCH), Ovarian tumor domain containing protease (OTU) and Machado-Joseph disease protease (MJD). Metalloproteases lack the cysteine protease signature and was shown to cleave Ub from substrates in a Zn^{2+}- and ATP-dependent manner [37]. They are all called JAMM (JAB1/MPN/Mov34 metalloisopeptidase) motif proteases. A resent report identified 58 USP, 4 UCH, 5 MJD, 14 OTU and 14 JAMM domain-containing genes in the human genome [36].

DUBs are important for generating free Ub from pro-Ub precursors and Ub-protein conjugates coded for by the Ub genes. This function is mainly attributed to the small DUBs of the UCH family. They are also thought to recycle Ub from inappropriate small conjugates that form between Ub and nucleophiles such as glutathione and polyamines. (Reviewed in [38]) Due to the small size it is thought that UCH proteases are unable of recognizing specific ubiquitinated proteins. However, some of them have been shown to display specific functions. DUBs contribute to

protein degradation by detaching the polyubiquitin tag from proteasome-targeted substrates, allowing for the entry of unfolded polypeptides to the proteolytic chamber. In addition, DUBs catalyze the disassembly of polyubiquitin conjugates, which allows the recycling of monomeric Ub (reviewed in [39]). UCH37, also called UCH-L5, interacts with the novel proteasomal subunit human Rpn13 (or ADRM1) [40-42]. This interaction increases the isopeptidase activity of UCH37, and seems to be involved in regulating overall protein-degradation by the proteasome. Also, UCH37 has been shown to interact with the S14 subunit of the proteasome, an interaction that is blocked by a protein called UIP1 (UCH37 interacting protein 1) [43].

Deubiquitination may also rescue polyubiquitinated proteins from degradation by the proteasome, and revert ubiquitination involved in regulation of endocytosis, protein trafficking and protein activity.

The USP proteases have a catalytic domain containing two well-conserved motifs, the Cys and the His boxes, parted by a region of diverse size. USPs are generally larger proteins than the UCH proteases, and for many of them substrate specificity has been detected. USP9X/FAM (fat facets in mouse) is suggested to be involved in regulating cell adhesiveness and mobility, through its interactions with β-catenin [44], E-cadherin [45] and the GTPase Ras effector protein AF-6 [46, 47]. This DUB is upregulated in breast cancer tissues as compared to adjacent normal tissue [48].

One DUB that has been extensively studied is Usp7, a regulator of the tumor suppressor p53. Usp7 is important for the stability of both p53 and its regulators Mdm2 and Mdmx through deubiquitination (reviewed in [49]).

The DUB family containing the OTU domain was discovered using a proteomics approach [50], and several active members have been identified since [51, 52]. Otubain1 associates with GRAIL, an E3 ligase important in regulating CD4 T cell anergy [53]. Otubain1 expression correlates with low levels of GRAIL, and proliferating and functionally active cells, while expression of an alternatively spliced isoform of Otubain1 correlates with high GRAIL levels, and poor proliferation and function of T cells [53]. The two isoformes might therefore have opposing roles in controlling GRAIL stability and anergy in T cells.

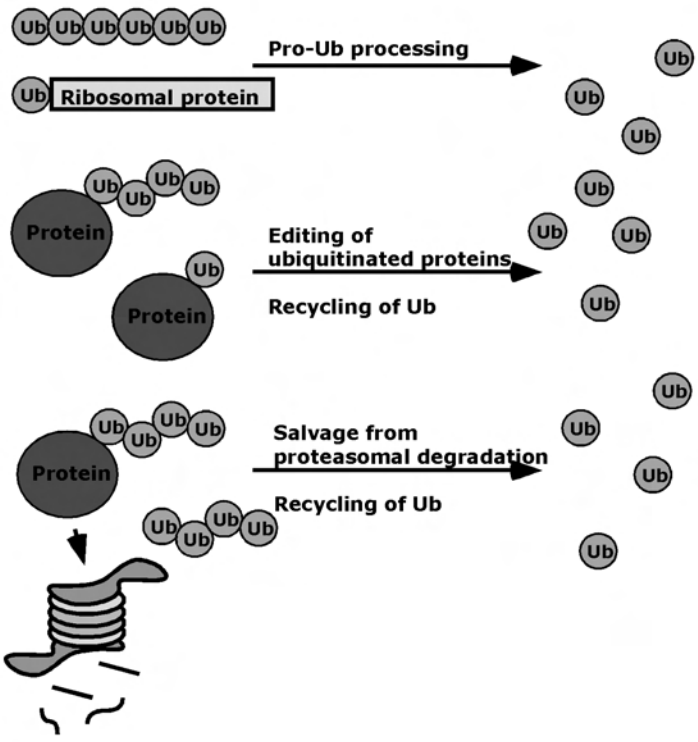

Figure 4. DUBs are involved in several steps of the ubiquitination. They are responsible for generating free Ub from the pro-Ub products produced by Ub genes, and involved in the editing of ubiquitination, salvage of proteins targeted for proteasomal degradation as well as recycling of Ub.

It was recently shown that two deubiquitinating enzymes, AMSH and USP8/UBPY, are involved in regulating endocytosis through their interaction with ESCRT protein components. AMSH has been suggested to rescue ubiquitinated cargo from lysosomal degradation through disassembly of K63-linked polyubiquitin chains, while UBPY might exert dual Ub-chain specificities, degrading both K63- and K48-linked chains, and seems to be necessary for lysosomal sorting [54]. UBPY might work by regulating the stability of ESCRT-associated proteins by reverting their ubiquitination (reviewed in [55]). In EGF-stimulated cells, UBPY is ubiquitinated and binds to the EGFR. UBPY overexpression leads to reduced ubiquitination levels of EGFR, while depletion of UBPY results in increased ubiquitination and degradation of EGFR [56]. Thus, UBPY seems

to negatively regulate the rate of EGFR down-regulation by deubiquitinating the EGFR on the endosome. UBPY depletion is associated with swelling and changes in the morphology of endosomes, and leads to elevated levels of monoubiquitinated Eps15 and the accumulation of Eps15 in the formed endosomal aggregates [57]. Eps 15, that is important for functional endocytic activities, was therefore suggested to be a substrate of UBPY.

In summary, deubiquitinating enzymes are implied in the regulation of processes such as cell cycle progression and endocytosis, suggesting an important role for DUBs in cell survival and tumor development. Indeed, deregulation of several DUBs has been associated with tumors. It is also likely that DUBs are targeted by pathogens to influence or interrupt host cell functions.

Table 1. Summary of the DUBs mentioned in this thesis.

DUB	Substrate	Function
UCH-L1	Not known	Important for neuronal functions. Associated with malignancies. Implied function in spermatogenesis, oocyte maturation and fertilization. Regulation of $p27^{Kip1}$ stability. Possible role in NFκB signaling.
UCH-L3	Not known	Associated with certain malignancies. Involved in spermatogenesis and fertilization. Possible role in mitochondrial functions. Antiapoptotic. Role in neuronal functions.
UCH37	Proteins targeted for proteasomal degradation. TGF-β receptor.	Interacts with the proteasome - regulates overall protein degradation by the proteasome. Positively regulates TGFβ-dependent signaling
Otubain1	Associated with the E3 ligase GRAIL.	Involved in regulation of CD4 T cell anergy.
Usp5	Not known	No known function. Interacts with ISG15 and with K29-linked chains.
Usp7	p53 Mdm2 Mdmx Histone H2B Chfr	Interacts with the HSV-1 immediate early protein ICP0 - stimulates the viral proteins function in gene expression and viral lytic cycle. Interacts with the EBV protein EBNA1. Deubiquitinates and stabilizes p53, Mdm2 and Mdmx. Regulates monoubiquitination of histone H2B. Negative regulator of FOXO-regulated transcription. Stabilizes the mitotic check point protein Chfr.
Usp8	Interacts with ESCRT proteins. Eps15? EGFR Nrdp1	Involved in regulating endocytosis. Deubiquitinates K63- and K48-linked chains. Negatively regulates EGFR-downregulation, by deubiquitinating EGFR in the endosome. Interacts with and stabilizes the E3 ligase Nrdp1 - important for regulation of RTKs.
Usp9X	β-catenin AF6 Itch Interacts with E-cadherin Interacts with doublecortin	Works pro-adhesive, possibly through its interaction with the Ras effector protein AF-6, β-catenin or E-cadherin. Suggested involvement in migration regulation. Deubiquitinates the E3 ligase Itch, suggesting a role in endocytosis and endocytic sorting.
Usp13	Not known	No known function. Interacts with modified ISG15 probes.
Usp15	IκBα ?	Associates with the COP9 signalosome, and is involved in the regulation of IκBα degradation and thus NFκB signaling.
Usp22	Not known	No known function.

The Ubiquitin C-terminal Hydrolase UCH-L1

UCH-L1, also known as PGP9.5, belongs to the UCH proteases. It is highly abundant in neurons, where it seems to have an important function. Mutations in the protein have been associated with familiar Parkinson disease [58] and Huntington's disease [59]. Despite intensive research aiming at elucidating the role of this DUB, the specific functions and substrates of UCH-L1 are so far unknown.

Parkinson disease (PD) is the second most common neurodegenerative disease, characterized by the progressive loss of dopamine neurons and the accumulation of Lewy bodies. One of the major molecular pathways commonly underlying the pathogenesis is a dysfuntion in the ubiquitin proteasome system. Several lines of evidence support a role for genetic risk factors in PD. α-synuclein is a protein abundantly expressed in the mammalian brain that has been genetically linked to PD. It is a plastic protein that can form aggregates. These fibrillar moieties form the major components of the intracellular proteinaceous inclusions known as Lewy bodies found in PD. There is a correlation of PD with high levels of α-synuclein. (Reviewed in [60]) UCH-L1 has been found in Lewy bodies of PD cases, and has been shown to promote the accumulation of α-synuclein [61, 62]. A heterozygous I93M substitution in the UCH-L1 gene was identified in a sibling pair both affected by PD. In vitro studies of this mutant showed a decrease of hydrolytic activity with approximately 50% [62, 63]. A S18Y substitution may be associated with a decreased risk of idiopathic PD [63, 64]. Interestingly, UCH-L1 overexpression was demonstrated to correlate with the generation of larger ATP-induced inward currents in neuronal cells, independently of its deubiquitinating capacity [65], although the mechanism is still unclear. The S18Y variant seems to have a decrease in the reported ligase activity and an increased hydrolase activity [63, 66].

Further strengthening the role of UCH-L1 in neuronal functions is its involvement in the phenotype of the gracile axonal dystrophy (gad)-mouse. The gad-mouse shows sensory ataxia at an early stage, followed by motor ataxia at a later stage. The mouse is characterized by a "dying back" axonal degeneration and formation of spheroid bodies in nerve terminals. Accumulation of amyloid β-protein and Ub-positive deposits occur retrogradely along the sensory and motor nervous systems. The

gad mutation was shown to depend on an inframe deletion including exon 7 and 8 of the UCH-L1 gene [67]. The truncated protein was not detected in tissue from gad mice, although the mRNA was still present, suggesting that the mutation leads to an increased degradation of UCH-L1 [67]. The mice seem to have a dysfunctional reusage of Ub, resulting in the accumulation of abnormal proteins in the brain. However, none of the pathological features of PD are recapitulated.

In microarray expression analysis studies of gad mice, 103 genes were found upregulated and 43 genes downregulated. The analysis does not allow any firm conclusions on signaling pathways affected by the lack of UCH-L1, but proteins involved in processes such as RNA-metabolism, membrane- and vesicle-transport, cellular structure, signal transduction, transcriptional regulation and protein-degradation were identified [68].

Despite the obvious important function of UCH-L1 in neuronal functions, and the many attempts to elucidate the function of UCH-L1, so far very little is now on the mechanistic role of UCH-L1 in different cell functions. The enzyme seems to have a role also in tumor development and cell survival, again strengthening the need of more information of the functions of this protease.

In wild type mouse retina, light stimuli and ischemic retinal injury induce neural cell apoptosis. This correlate with increased Ub levels and increased caspase activity. In gad mice, there is a reduced Ub induction after light stimuli and ischemia, an increased expression of the antiapoptotic proteins Bcl-2 and XIAP and prosurvival signals (pCREB and BDNF), as well as a decreased caspase 3 activity [69]. Similar results were obtained in the testis upon exposure to higher body temperature via experimentally induced cryptorchidism [70]. These studies suggest that UCH-L1 is involved in the regulation of Ub levels as well as apoptosis.

A genetically determined deficiency of lysosomal enzymes and proteins, or of proteins involved in lysosomal biogenesis, results in lysosomal storage diseases (LSDs). LSDs are characterized by the increase of lysosomes in size and number, the storage of macromolecules, such as sphingolipids, mucopolysaccharides and glycoproteins, and often with the aggregation of ubiquitinated proteins. This affects cell turnover and may lead to hepatosplenomegaly, corneal clouding, skeletal deformation and central nervous system degeneration. LSDs were shown

to associate with reduced UCH-L1 levels [71]. Experimental induction of lysosomal storage reduced mRNA and protein levels of UCH-L1, and induced apoptosis. Overexpression of UCH-L1 reversed apoptosis, while downregulation of UCH-L1 using RNAi increased caspase-activity and expression of the proapoptotic proteins Bim and Bax, accompanied by increased apoptosis [71]. In contrast to the studies in gad-mice testis and retina, this suggests UCH-L1 to have an antiapoptotic effect. It also suggests a plausible role of UCH-L1 in lysosomal trafficking.

UCH-L1 overexpression is related to tumors and in some cases even to a more advanced stage of the cancer. One study reports on a correlation between UCH-L1 and the tumor suppressor Von Hippel Lindau (VHL). Through deubiquitination of HIF-1α, UCH-L1 is suggested to oppose the ubiquitination of this transcription factor by VHL [72]. HIF-1α is important for de novo synthesis of blood vessels, and it is plausible that UCH-L1 through the stabilization of HIF-1α may stimulate vessel growth. UCH-L1 is also implied in remodeling of blood vessels. Through inhibition of the NFκB pathway, UCH-L1 was suggested decrease the levels of several NFκB-driven genes important for vascular remodeling [73]. UCH-L1 interacts with Jab1, a regulator of $p27^{Kip1}$ nuclear export and consequent degradation [74]. Through the interaction with Jab1 and $p27^{Kip1}$, UCH-L1 might regulate progression through the cell cycle. A summary of the suggested functions of UCH-L1 can be seen in Figure 5.

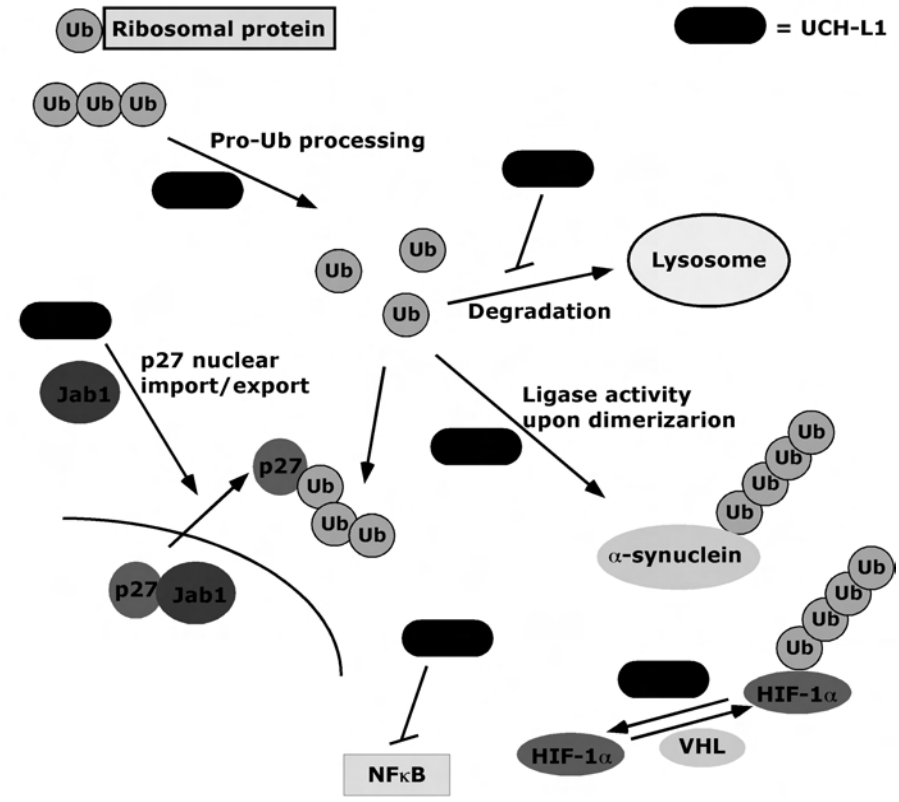

Figure 5. Summary of some of the suggested functions of UCH-L1. UCH-L1 has been suggested to keep a free ubiquitin pool, by processing pro-Ub and rescuing Ub from lysosomal degradation by binding to Ub. UCH-L1 also seems to regulate $p27^{Kip1}$ nuclear import and export through interactions with Jab1. Upon high UCH-L1 levels, the enzyme forms homodimers that form K63 linked chains using α-synuclein as a substrate. UCH-L1 has been suggested to inhibit NFκB signaling, although the mechanism is unclear. HIF-1α might get deubiquitinated and rescued from proteasomal degradation by the action of UCH-L1

Viruses and how they exploit the ubiquitin system

Considering the wide range of substrates and the amount of important cellular functions affected by and regulated through ubiquitination and deubiquitination, it is not surprising that several viruses have evolved ways of mimicking, redirecting or inhibiting the regulation of cellular and viral processes by these mechanisms. Viral entry and budding use host cell functions dependent on ubiquitination. Also, in order to overcome the immune system, many viruses interfere with antigen processing, MHC class I antigen presentation and apoptosis. The known viral strategies to interfere with these processes often involve either interaction of a viral protein with a cellular E3 or expression of a viral protein with E3 activity. A few recent studies describe viral interference with the deubiquitinating system, including expression of viral proteins with DUB activity.

USP7 was first identified as an interacting protein of the Herpes simplex virus (HSV)-1 immediate early protein ICP0, and the two proteins were shown to colocalize to some extent in PML bodies [75]. PML bodies are nuclear structures implicated in processes such as response to stress and interferons, oncogenesis and viral infections. The interaction of USP7 with ICP0 seems to contribute to the ability of ICP0 to stimulate gene expression and viral lytic cycle [76]. Because of the interaction, USP7 is commonly called Herpes virus associated USP (HAUSP). In 2003 it was shown that ICP0 is an E3 ligase that interacts with and ubiquitinates the tumor suppressor protein p53 [77]. The ability of ICP0 to bind to HAUSP rescues ICP0 from autoubiquitination and consequent degradation [78], suggesting a mechanism by which HAUSP may affect the activity of ICP0. ICP0 has also been shown to act as an E3 ligase of HAUSP, inducing its degradation [79], implying that a balance between the two proteins in important during HSV-1 infection.

The severe acute respiratory syndrome corona virus (SARS-CoV) encodes a protein with sequence similarity to HAUSP. This protein is a papain-like protease (PLpro), and was shown to act on Ub and ISG15 [80]. ISG15 is a Ub like molecule, induced upon IFN stimulation, that is believed to have important functions in inflammatory responses to infection. This might be a way for the CoV to inhibit inflammatory reactions.

Infection with adenovirus is associated with an increased DUB activity. This was shown do depend on the adenoviral protein Avp displaying DUB activity [81]. Likewise, the N-terminal domain of the UL36 gene product of HSV-1 was identified as a protein with deubiquitinating activity [82]. This region of UL36 shows no homology to previously known DUBs, but other herpes viruses all possess a UL36 homolog with sequence similarity to HSV-1 UL36. This suggests that proteins from other herpes viruses, such as the EBV BPLF1, may also display DUB activity. Indeed, the human cytomegalovirus (HCMV) homolog, high-molecular-weight protein (HMWP), displays DUB activity [83]. The lack of sequence similarity with human DUBs identifies these viral enzymes as potential targets for selective inhibition.

Other examples of tumor viruses exploiting the ubiquitin proteasome system are EBV and HPV. These are discussed in more detail below.

The Epstein-Barr Virus

EBV is a γ-herpes virus that normally establishes primary infections during childhood, giving no symptoms. When infecting in adulthood the symptoms can be more severe and lead to infectious mononucleosis, with glandular swelling, sore throat and high fever. EBV preferentially infects B cells, and enters the target cells by binding of the viral glycoprotein gp350 to CD21 and gp42 to the HLA class II molecules on the cell surface of resting B cells. The virus may also infect epithelial cells. To sustain persistent infections and in order to avoid elimination of the infected cells by immune responses, it alternates between latent, non-virus producing, and lytic, virus producing phases. (Reviewed in [84]) Healthy EBV-positive adults carry between one and 50 EBV-infected B lymphocytes per million cells in the peripheral blood [85]. There is a constant viral shedding through the saliva of infected individuals, thanks to a low level of reactivation of the virus into lytic phase, facilitating the spreading of the virus.

EBV infection is followed by expression of a distinct set of viral proteins, where the resting memory B cells support the latent cycle while terminally differentiated plasma cells, already set to die, favor the lytic, productive cycle (Reviewed in [86]). All EBV related tumors express

different sets of latent EBV proteins, which seem to contribute to the malignant phenotype. The latent EBV genes encode six nuclear antigens (EBV nuclear antigens, EBNA) –1, -2, -3, -4, -5 and –6, and three membrane associated proteins known as latent membrane proteins (LMP)-1, -2A and –2B. Two untranslated RNAs, EBER-1 and EBER-2, and other RNAs of unknown function are detected in infected cells. (Reviewed in [86]).

The latent state is normally divided into four different stages, depending on the expression pattern of viral proteins. In latency 0, seen in long-lived EBV positive memory B cells, there is a complete silencing of the EBV genome. Latency I, often seen in long-lived EBV positive memory B cells as well as in Burkitt´s lymphoma, is characterized by the expression of LMP-2A alone or together with EBNA-1 [87, 88]. B cells that home to the germinal centers of lymphoid follicles and Hodgkin's Reed Sternberg (HRS) cells in Hodgkin's disease (HD) show latency II type of expression pattern, where EBNA-1 and LMP-1, -2A and –2B are expressed [89, 90]. This expression pattern seems to be important for the survival and differentiation of infected lymphoblasts into memory B cells. In immunocompromised persons or in in vitro cultures, where there is a lack of pressure from the immune system, EBV infected cells express more EBV proteins. This is called latency III, and all six EBV nuclear antigens (EBNA1-6), all three latent membrane proteins (LMP-1, -2A and -2B), as well as EBER1 and EBER2 and transcripts from the BamHI A region are being expressed. This is the expression pattern seen in in vitro immortalized lymphoblastoid cell lines (LCLs). (Reviewed in [86])

In the latent phase the closed circular EBV genome behaves as chromosomes, meaning that it replicates once during the cell cycle, and thus is transmitted to both daughter cells. EBNA-1 is the only viral protein needed for viral replication and maintenance. By binding to the viral origin of replication, OriP, EBNA-1 facilitates replication that is then largely dependent on the cellular replication machinery. Lytic replication instead occurs in several rounds of replication initiated within oriLyt. This process is dependent on several viral genes and in the end of the lytic replication cycle the EBV genome has been amplified 100- to 1000-fold. (Reviewed in [91])

EBV and the ubiquitin system

Many EBV proteins interfere with or use the UPS to avoid recognition by the immune system, to stimulate proliferation or as possible mechanisms of transforming cells.

EBNA-1 is expressed in all types of EBV infected cells. The protein is divided into a C-terminal part and an N-terminal part by a glycine-alanine (gly-ala) repeat of variable size (a sequence of 60-300 amino acids containing glycine and alanine only). The gly-ala-repeat inhibits proteasomal degradation of EBNA-1, preventing presentation on MHC class I molecules [92], and recognition by CTLs. Transfer of the gly-ala repeat into known proteasomal substrates such as p53 and IκB confirmed that the gly-ala repeat acts as an inhibitor of proteasomal degradation [93]. Despite the early findings that EBNA-1 is not processed and presented to T-cells, EBNA-1 was later demonstrated to provoke a T-cell response, mainly through MHC class II [94], but also to a certain extent via MHC class I presentation. This suggests some sort of cross talk between the pathways responsible for antigen processing and presentation to take place. It remains to be elucidated both by what mechanism EBNA-1 avoids degradation, and what mechanisms are responsible for the degradation of EBNA-1 leading to presentation of epitopes.

Transfection of EBNA-6 into EBV negative cells has been shown to lead to decreased pRb levels [95]. EBNA-6 binds pRb through a conserved motif that has been linked to the regulation of the E3 ligase SCF^{Skp2}. By serving as a link between SCF^{Skp2} and pRb, EBNA-6 mediates polyubiquitination and degradation of pRb [96]. Also, EBNA-6 mediates SCF^{Skp2} dependent degradation of $p27^{Kip1}$ [96], suggesting yet another way of this EBV protein to contribute to proliferation.

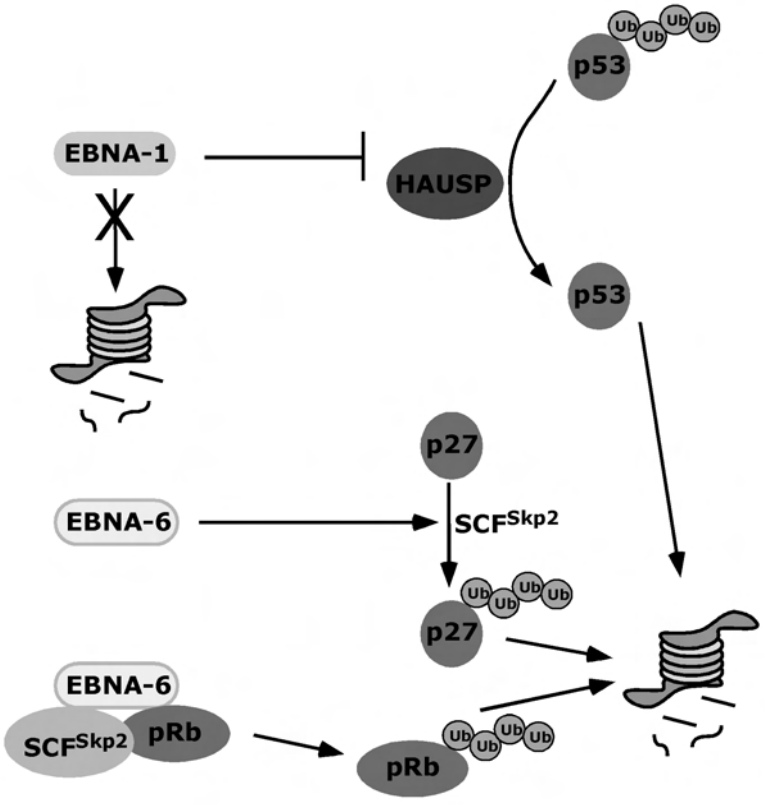

Figure 6. The EBV proteins EBNA-1 and EBNA-6 utilize the UPS to promote degradation of tumor suppressor proteins such as p53 and pRb and the cell cycle inhibitor protein $p27^{Kip1}$. EBNA-1 avoids degradation and thus presentation of epitopes to cells of the immune system, through the Gly-Ala repeat.

LMP-1 is a membrane protein with oncogenic potential. Structurally, it consists of a short amino-terminal sequence, six transmembrane-spanning regions and a long c-terminal domain. The protein clusters into lipid rafts of the infected B cell, together with LMP-2A and cellular proteins involved in B cell function and proliferation [97]. LMP-1 acts as a constitutively active tumor necrosis factor receptor (TNFR), similar to CD40, activating a number of signaling pathways in a ligand-independent manner. LMP-1 recruits TNFR associated factors (TRAFs) to two distinct C-terminal domains, resulting in the activation of the NF-κB and AP1 signaling pathways. This leads for example to the up regulation of anti-apoptotic proteins such as Bcl-2, BclxL, Mcl1 and A20. Upon CD40 activation, the recruited TRAFs normally get degraded through

the UPS leading to a transient signal. When binding to LMP-1 instead, TRAFs are activated and protected from degradation, and the signal thus remains constant. (Reviewed in [98])

A fine-tuning function in regulating the NF-κB signaling pathway has been suggested for LMP-1. LMP-1 interacts with one of the E3 ligases (SCF^{HOS}) of the NF-κB-inhibitor IκB, acting as a decoy substrate for SCF^{HOS}, reducing the levels of this ligase able to interact with IκB [99].

LMP-1 upregulates several components of the antigen presentation pathway, including the transporters associated with antigen processing (TAPs) [100, 101] and subunits of the proteasome [102], leading to altered enzymatic activity and cleavage specificity, thereby promoting antigenicity of EBV infected cells.

LMP-2A is a membrane protein that, similar to LMP-1 acts as a constitutively active, ligand-independent receptor. The protein aggregates in patches within the plasma membrane of latently infected B-lymphocytes, and provides infected cells with signals similar to those given by the B cell receptor (BCR). This way the cells avoid apoptosis even though they lack the appropriate antigenic stimulation. LMP-2A is also able to inhibit the switch from latent to lytic cycle, favoring the persistence of the virus in resting B cells (reviewed in [103]). LMP-2A has a N-terminal domain consisting of an immunoreceptor tyrosine-based activation motif (ITAM). When phosphorylated, the ITAM present in the BCR plays an essential role in mediating lymphocyte proliferation and differentiation through the recruitment of the tyrosine kinases Lyn and Syk. LMP-2A binds the effectors Lyn and Syk through its phosphorylated ITAM (reviewed in [104]). Upon binding to LMP-2A, through the interaction of LMP-2A with E3 ligases of the Nedd4 family (AIP4/Itchy, WWP2/AIP2 and KIAA0439), the effectors are ubiquitinated and Lyn gets targeted for degradation by the proteasome [105, 106]. The activation of Syk causes constitutive activation of the Syk substrate SLP-65 (SH2 domain-containing leukocyte protein-65). This induces the formation of a complex composed of the E3 ligase Cbl, C3G and the proto-oncogene CrkL [107]. Cbl-b ubiquitinates Syk, making this a potential strategy to negatively regulate BCR signaling [108]. LMP-2A thus counteracts activation of the B cells, which would lead to activation of the lytic cycle, and reveal the virus to the immune system. The ubiquitin ligases AIP4

and WWP2 ubiquitinate LMP-2A at the N-terminus. This is required for the recruitment of tyrosine kinases [109, 110], and thus probably for the modulation of BCR signaling.

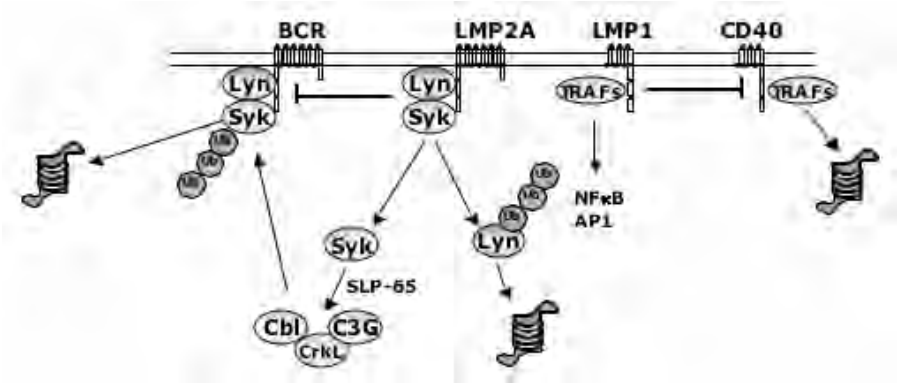

Figure 7. LMP-1 and LMP-2A highjack the signaling system of CD40 and BCR respectively. By exploiting the UPS the EBV proteins stimulate proliferation but avoids cell activation and induction of lytic cycle, which would lead to immune recognition.

BPLF1 is a homolog of the HSV-1 protein UL36 that was proven to contain a DUB-activity [82]. The proteins are expressed late in the lytic phase of herpes-viruses and are suggested to have a function in the release of viral DNA from incoming nucleocapsids, in the viral DNA synthesis, and in late gene expression. Exactly what role the DUB activity plays in these processes is not clear. As discussed previously, ubiquitination and deubiquitination are involved in the regulation of membrane protein trafficking, viruses utilize theses pathways for entry and egress.

To summarize, EBV has devoted a lot of its genomic information to regulation of the UPS in different ways, and this seem to have functions both in survival of the virus and in the EBV tumorogenic effects. It is likely that EBV also interacts with, or even encodes for, proteins with deubiquitinating activity, although not much is known so far.

EBV related cancers

Hodgkin's disease, NK/T cell lymphomas, immunoblastic lymphomas, endemic Burkitt´s lymphoma (BL), and epithelial cancers of the gastric tract such as Nasopharyngeal Carcinoma (NPC), are all cancers associated with EBV [111]. They are characterized by the presence of

multiple extra-chromosomal copies of the circular viral genome in the tumor cells, and expression of a distinct set of EBV-encoded latent proteins.

BL is the tumor in which the virus was first discovered [112]. It is common in children in equatorial Africa, and the tumor affects the jawbone, forming a huge tumor mass. Abdominal swelling, effects on the eyes, ovaries, kidneys and glandular organs such as breast, thyroid and tonsil, as well as spreading to the nervous system with damages on the nerves, may occur [84]. The tumor is defined by chromosomal translocations of the c-myc proto-oncogene into one of the immunoglobulin loci, leading to a deregulation of myc expression [98]. EBV is present in most, but not all, BL [113]. BL cells have phenotypical characteristics that suggest they are of germinal centre origin. These cells display low expression of adhesion molecules and downregulation of MHC class I, and thus act as low immunogenic cells. Some cells when put in culture, outside the context of immuno-surveilance, upregulate several viral proteins as well as cell surface antigens such as CD23, CD30, CD39, leukocyte activity-associated antigen (LFA)-1, LFA-3 and Intercellular adhesion molecule (ICAM)-1. (Reviewed in [104])

Further evidence for EBV being related to a tumorogenic outcome comes from the fact that EBV infection leads to immortalization of B cells in vitro, forming EBV-transformed LCLs that are often used as a model for EBV-related tumors. LCLs can be obtained by explantation of blood or lymphoid tissue from EBV sero-positive individuals without need for exogenous infection [114]. Alternatively, infection of PBMCs or purified B cells with EBV in vitro will lead to immortalization of the B cells. To establish LCLs it is crucial to inhibit T cells present in the culture that would otherwise recognize and kill infected B cells. Generally the EBV transformation process is established around four weeks after infection, and the early signs of transformation such as cell aggregation, increased cell size and sudden increase in proliferation are induced after one to three weeks. LCLs show a high expression level of the B-cell activation markers CD23, CD30, CD39 and CD70, and of the cellular adhesion molecules LFA-1, LFA−3 and ICAM-1 [115]. Since these markers usually are absent or expressed at low levels on resting B cells, but upregulated when these cells are activated by antigen or mitogenic stimuli, this

suggests that EBV-induced immortalization occurs through the constitutive activation of the same cellular pathways that drive physiological B-cell proliferation.

The high expression of adhesion molecules on LCLs contributes to some of the characteristics of the growth pattern of the cells. In suspension, LCLs tend to grow in big aggregates due to homotypic adhesion. BLs on the other hand, that express low levels of adhesion molecules, grow as single cells. The BLs lack signals allowing for contact inhibition, and tend to form colonies in semi solid medium, while LCLs fail to grow under the same condition. Thus, there are dramatic differences in the way these cells interact with the surrounding matrix.

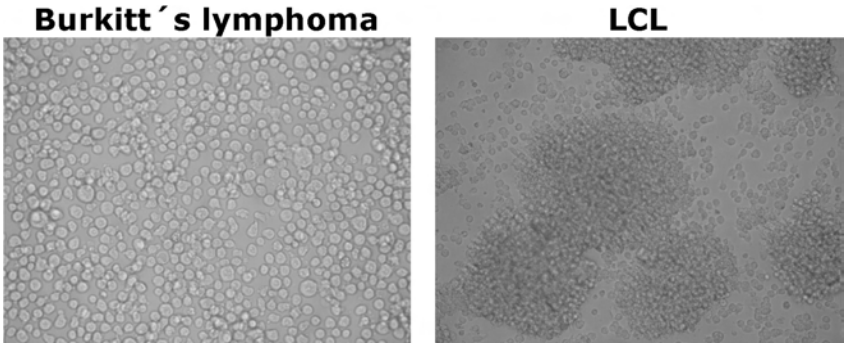

Figure 8. The growth pattern of BLs vs. LCLs is dramatically different. BL cell lines grow as single cells in suspension cultures, while LCLs express higher levels of active adhesion molecules and tend to form big clusters, adhering to each other.

The human papilloma virus

The HPVs are small viruses with a circular double-stranded DNA molecule of approximately 8000 base pairs. Strong epidemiologic, virologic and biochemical evidence indicates the high-risk subgroup of HPVs as the causative agent in the development of tumors of the anogenital tract, and in head and neck cancers. The HPV genome encodes only for eight genes, and the virus is highly dependent on host factors for proliferation. The two major HPV oncoproteins encoded by the E6 and E7 genes are necessary and sufficient for immortalization of primary cultures in vitro. The oncogenic properties of these two proteins are strongly associated with their capacities to manipulate the UPS. (Reviewed in [116])

HPV infection occurs mainly through skin-to-skin or mucosa-to-mucosa contact. HPV infects cells at a variety of anatomical sites in the basal layer of epithelium that are not differentiated and still proliferating. In these cells the virus resides in a latent state, with viral genome being produced, resulting in the formation of episomal DNA. Production of new virus particles, during lytic phase, takes place in the differentiated layers of epithelial cells that are not proliferating. HPVs have a limited coding capacity, and therefore rely largely on the cellular DNA synthesis machinery in order to replicate their genomes. The high risk HPVs infect mainly differentiated cells that have exited the cell cycle and are not producing DNA. To overcome this limitation, the high-risk oncoprotein E7 targets a number of cell cycle regulatory proteins, upregulating genes required for the G1/S transition and DNA synthesis. (Reviewed in [117])

HPV and the ubiquitin system

The E6 oncoprotein of the high risk HPVs is known to induce Ub-dependent degradation of several of its target proteins. Examples of these are the tumor suppressor protein p53 [118], the MCM7 subunit of replication licensing factor [119], the pro-apoptotic protein Bak [120, 121] and myc proteins [122], all important regulators of DNA damage response, apoptosis and proliferation. The majority of E6 targets are degraded by the UPS as a result of polyubiquitination by the human Ub ligase E6 associated protein (E6AP) of the HECT family. E6 forms a complex with E6AP, redirecting the specificity of this E3 towards p53. This

induces ubiquitination of p53 and subsequent proteasomal degradation [118]. This reduces the possibility of infected cells to undergo cell cycle arrest or apoptosis in response to DNA damage or uncontrolled proliferation. It is conceivable that the redirection of E6AP activity towards p53 by E6 might inhibit the targeting of normal substrates of E6AP. The normal targets include proteins involved in DNA repair, such as the human homolog of the yeast RAD23 protein involved in nucleotide excision repair (HHR23) [123], the src-family kinase Blk [124] and MCM7. Indeed, several studies have shown that E6 transforms cells independently of p53 [125, 126]. HPV E6 has also been shown to cause degradation of E6AP itself, probably by activating auto-ubiquitination of the E3 [127].

E6 also mediate E6AP-dependent polyubiquitination and subsequent degradation of the Rap GTPase-activating protein (RapGAP) E6 targeting protein (E6TP1) [128]. This correlates with the ability of the E6 protein to immortalize epithelial cells [129]. E6TP1 reduces the levels of GTP-bound, active, Rap. Rap is active after growth factor stimulation of cells, and constitutively active Rap has been shown to transform fibroblasts [130, 131]. In this context, the discovery of E6-mediated degradation of the Rap inhibitor E6TP1 might imply a potential role for this small GTPase in E6-induced oncogenesis.

Mutations in hScrib and hDlg, that are human homologs of Drosophila neoplastic tumor suppressors, cause loss of cell polarity and overgrowth of epithelial cells [132]. These, and several other PDZ-domain-containing proteins involved in regulation of cell signaling and cell adhesion, are targeted by E6AP in an E6 dependent manner [133-137], contributing to the oncogenic properties of E6.

In differentiated cells telomeres are shortened with age, eventually contributing to cell senescence. This is avoided in for example stem cells, by the activity of the enzyme telomerase. HPV E6 causes degradation of NFX1, a transcriptional suppressor of the telomerase component hTERT [138]. This results in increased hTERT expression, probably contributing to the increased telomerase activity seen in cervical carcinomas.

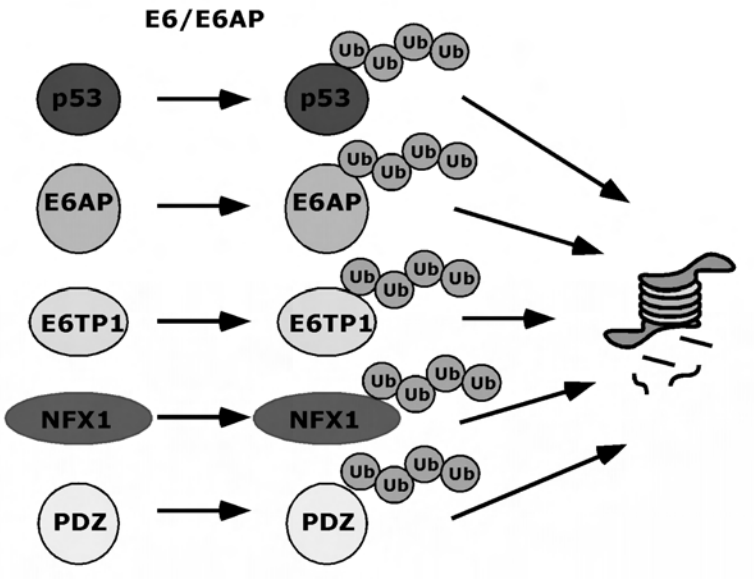

Figure 9. The HPV E6 oncoprotein forms a complex with the host Ub ligase E6AP, promoting the degradation of several proteins important for DNA damage responses, telomerase activity, regulation of cell polarity and proliferation.

The E7 protein has been shown to inactivate pRB and the related p107 and p120, as well as the cell cycle inhibitors $p21^{CIP1}$ and $p27^{Kip1}$, helping the cell to overcome the cell cycle block [139, 140]. When active, the pRB family of proteins binds to the transcriptional activator E2F, thereby interfering with its transcription activation properties, and additionally turning E2F into a transcriptional repressor. When this binding is disrupted, E2F becomes active and the following transcription promotes cell cycle progression (reviewed in [141]). E7 binds pRb in its hypophosphorylated state, i.e. when it is normally bound to E2F. This binding induces as C-terminal cleavage of pRb, by the calcium-activated enzyme calpain. This leads to a proteasomal degradation of pRb. (Reviewed in [117]) E7 has also been shown to interact with a subunit of the proteasome [142], suggesting E7 to work as a shuttle-protein targeting pRb for proteasomal degradation.

Inactivation of pRb leads to progression through S-phase. This uncontrolled proliferation would normally be sensed by the cell as inappropriate, leading to activation of the p53-depdenent pathways. However, the E6-E6AP dependent degradation of p53 prevents

accumulation of p53, cooperating with the E7 oncoprotein to establish immortalization and transformation (reviewed in [143]).

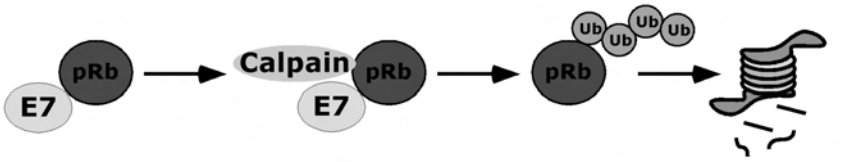

Figure 10. The HPV E7 oncoprotein stimulates calpain-dependent cleavage of pRb, leading to proteasome dependent degradation of this tumor suppressor.

In summary, several of the strategies exploiting the UPS used by HPV leads to a consequent loss of DNA damage induced cell cycle arrest and apoptosis. This probably facilitates the occurrence of chromosomal abnormalities seen in cervical carcinomas. Although these molecular events are likely to be controlled by the action of DUBs, little is known about the activity and expression profiles of these enzymes in cervical carcinomas or their regulation by HPV infection. It is plausible that E6 or E7 themselves might affect the activity of one or several of these enzymes.

HPV related cancers

Almost 200 HPVs have been identified and they all display different abilities to induce malignant lesions, resulting in symptoms ranging from the formation of benign warts to the development of cervical carcinomas, skin tumors and oral cancers (reviewed in [144]). The 30 HPVs associated with anogenital lesions can be divided into high and low risk HPVs, based on their association with clinical lesions. The high risk HPVs, such as types 16, 18, 31, 33 and 39 accounts for more than 90% of all cervical carcinomas. Cervical carcinoma arises through the cooperation of several events, allowing for a multistep carcinogenesis to occur. (Reviewed in [117, 145]) Most cervical HPV infections are cleared or suppressed by the immune system of the infected individual, but about 10% of the infections persist for several years [145]. Sustained HPV infection results in an increase of undifferentiated cells that are proliferating, suggesting that HPVs probably have means for interfering with differentiation as well as proliferation in these cells (reviewed in [116]). Cervical carcinomas

normally arise from the cervical transition zone, where the stratified squamous epithelium of the ectocervix replaces the glandular epithelium of the endocervix (reviewed in [145]). Infection with high-risk HPV is associated with cervical dysplasia or cervical intraepithelial neoplasia (CIN), and cervical cancers are believed to occur from these lesions after long persistent infection. Mild and moderate dysplasia (CIN I and CIN II) display relatively low levels of E6 and E7 expression, and the viral genome replicates episomally, while severe dysplasia or carcinoma in situ (CIN III) and invasive cancer lesions often show high levels of expression of E6 and E7, often with integration of the viral DNA into the host genome (reviewed in [117]). The role of risk factors other than HVP infection, like for example environmental factors, is unclear. A major effort has therefore been done in developing vaccines to prevent HPV infection. However, there is still a great need for more in depth knowledge on the effects of HPV infection on host cells, to find therapeutical targets for already infected individuals and for treatment of tumors.

Discussion

Paper I

Tumor development is likely to be dependent on evasion from CTL-mediated killing of the tumor cells. BL cells avoid recognition by the immune cells probably through impaired antigen processing and presentation. Low expression levels of the immunoproteasome subunits Lmp2, Lmp7 and MECL-1, and decreased levels of chymotrypsin- and trypsin-like activities have been demonstrated in BL cells [102]. The regulatory subunits of the proteasome, PA28α and PA28β, are also expressed at lower levels in BL cells as compared to other B cell tumors [20]. This is accompanied by a diverse production of epitopes, with BL cells producing low amounts of immunodominant epitopes [20]. Also, a downregulation of the TAP-1 and TAP-2 transporters, important for transport of produced peptides into the lumen of the endoplasmatic reticulum (ER) for presentation on MHC class I, might account for the reduced immunogenicity of these cells [100]. BL cells are resistant to apoptosis and do not accumulate ubiquitinated conjugates in response to otherwise toxic doses of proteasome inhibitors. This appears to be explained by increased levels of the cytosolic subtilisin-like protease tripeptidyl-peptidase-II (TPPII) and of DUB activity [146], suggesting that other proteolytic pathways may contribute to the metabolism of short-lived proteins in BL cells.

The study described in Paper I aimed to investigate which DUBs are selectively upregulated in BL cells in comparison with normal B lymphocytes and EBV transformed LCLs. To this end, we used site-directed probes that specifically target DUBs. Taking advantage of the common substrate for all DUBs, namely Ub, Borodovsky et al developed modified versions of Ub that bind covalently to the active site of these enzymes [50, 147]. Through the introduction of an electrophile at the C-terminus of Ub an irreversible trapping of this modified Ub in the enzymatic cleft of DUBs can occur. Protein extracts from cell lines or fresh tissues can this way be used for detection of active enzymes. Once bound to the modified Ub, the enzyme can be immunoprecipitated and identified through mass-spectrometry and bioinformatics. Following the identification of immunoprecipitated enzymes, the probes can be used for

labeling and detection of enzymes in regular western blot using a HA-specific antibody, where the enzymes are detected according to their migration pattern identified by MS/MS.

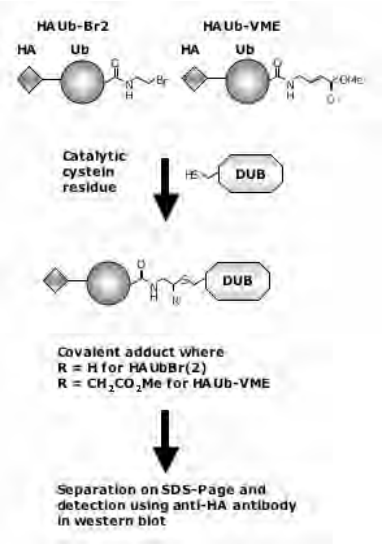

Figure 11. Labeling and identification of active DUBs. Active enzymes will bind active site-directed probes, and depending on the reactive group and the reactivity of the enzymes different enzymes will bind different probes. Labeled polypeptides are further fractionated by SDS-PAGE and can be extracted for identification through MS/MS or blotted on PVDF membranes for detection with anti-HA antibodies.

In Paper I the previously described Ub-based probes HAUb-VME and HAUb-Br_2 [50] were used. When analyzing DUB activities in extracts from a panel of cell lines of different tissue origin, tissue specific patterns of activity were detected. The most dramatic differences in activity were observed for a DUB identified as UCH-L1. In line with UCH-L1 being highly expressed in neurons, where it probably has an important function for the normal cellular functions, this DUB was highly expressed in all neuroblastoma cell lines investigated. Most cell lines of hematopoietic origin were negative for UCH-L1, with the exception of two CLL lines and a panel of LCLs and BL cell lines. Higher activity levels of UCH-L1 were seen in BLs when compare to LCLs, confirming previously published results [146].

The higher activity levels of UCH-L1 and to some extent UCH-L3 in BL cell lines suggested that this could account for the phenotypic characteristics of BL cells described above. Indeed, in freshly isolated B

cells, very low DUB activity levels could be detected in general, and no UCH-L1 and UCH-L3 activity was detected. No increase in these DUBs was seen upon mitogen stimulation, supporting that these are not merely a proliferation-marker, but that it might have important functions in BL cells.

To investigate the role of EBV infection in the expression pattern seen, freshly isolated B cell were infected with EBV. Early after infection, an increased activity was seen in UCH-L3 and USP15. USP15 is a Zn^{2+}-dependent enzyme that associates with the COP9 signalosome (CSN) [148], important for the assembly and activity of Cullin-RING E3 ligases. Ub-dependent degradation of the NFκB inhibitor IκBα is dependent on CSN that has been shown to interact with IκBα. By regulating the activity of E3s involved in degradation of this inhibitor, CSN seems to control NFκB signaling. This effect is dependent on the DUB activity of USP15 associated with CSN [149]. Interestingly, a further increase in the activity of USP15 was seen at later time points after EBV infection (Paper I).

The activity of UCH-L1, UCH37, HAUSP and USP9X was upregulated as a late event in the establishment of LCLs following EBV infection of fresh B cells. Since the upregulation is seen so late during transformation, it is not likely to be a direct effect of EBV gene expression.

HAUSP has been shown to interact directly with the tumor suppressor p53 and its role in the regulation of p53 ubiquitination and proteasome-dependent degradation is complex (reviewed in [49]). HAUSP over-expression stabilizes p53 by deubiquitination, and partial reduction of endogenous HAUSP levels by RNA interference destabilizes endogenous p53. HAUSP dependent stabilization of p53 induces p53-dependent cell growth retention and apoptosis [150]. However, complete ablation of HAUSP leads to stabilization and activation of p53 [151]. This is explained by the finding that HAUSP stabilizes Mdm2, a known E3 ligase of p53, as well as the p53 regulator Mdmx [152], providing a complex feedback loop in p53 regulation. HAUSP is required for Mdm2 and Mdmx stability in normal cells and, in the absence of HAUSP, ubiquitinated Mdm2 and Mdmx become unstable, leading to p53 activation [150, 152]. Binding of Mdm2 and p53 maps to the same domain of HAUSP, and HAUSP preferentially binds Mdm2 even in the presence of excess p53 [153]. The ATM protein kinase regulates the p53 pathway in response to DNA double

strand breaks. ATM induced phosphorylation of Mdm2 and Mdmx lowers their affinity for HAUSP, correlating with a decreased stability and activity of these proteins [152].

Interestingly, EBNA-1 has been shown to interact with the USP7 [154]. This interaction does not seem to have any effect on the stability of EBNA-1, but disruption of USP7 binding to EBNA-1 enhances the replication of an oriP containing plasmid [154], suggesting that USP7 is involved in controlling the activity of EBNA-1. EBNA-1 binds to the same pocket in USP7 as p53 but has a higher affinity for USP7. By competing with p53 EBNA1 promotes Mdm2-dependent degradation [154], suggesting a mechanism by which EBNA-1 contributes to malignant transformation.

HAUSP has also been suggested to play a role in regulating posttranslational modifications of histones, and histone H2B is deubiquitinated by HAUSP [155]. In addition, HAUSP has been shown to interact with the Forkhead box O (FOXO) transcription factors upon oxidative stress [156]. This negatively regulates FOXO transcription without affecting the stability of FOXO. HAUSP was also recently shown to interact with and stabilize the mitotic stress checkpoint Chfr, involved in regulating cell cycle progression and tumor suppression [157].

To summarize, HAUSP is a multifunctional protein stimulating proliferation and allowing for cell survival and progression through the cell cycle, possibly despite of DNA damage. The tumor suppressor p53 is a common target of inactivation in tumors, allowing for mutations to accumulate so that tumor progression may occur. Upregulating of HAUSP might therefore contribute to malignant transformation upon EBV infection.

USP9X was also upregulated after EBV infection. It interacts with several regulators of adhesion, and has been implied in the regulation of adhesion as well as migration. USP9X deubiquitinates and stabilizes β-catenin [44], a key regulator in regulation of cell adhesion as well as of the Wnt pathway and a potential oncogene regulating c-myc and cyclin D expression. This suggests that USP9X may regulate cell proliferation and adhesion. It was recently shown that EBV infected BL cells in latency III express higher levels of the proliferation promoting protein β-catenin, probably by activation of a DUB [158]. The finding that USP9X is

upregulated upon EBV infection, might suggest that this DUB is responsible for the increased β-catenin stability seen in EBV positive BL cells. This connection may suggest yet another way for the virus to induce proliferation and promote tumor formation.

USP9X also interacts with the GTPase Ras effector protein AF-6, a component of cell-cell adhesions, and prevents ubiquitination of AF-6 [46, 47]. Loss of USP9X results in inhibition of cell adhesiveness and a loss of β-catenin, while AF-6 levels remain relatively stable [159]. USP9X interacts with E-cadherin and this interaction, as well as the interaction with β-catenin, is dependent on the confluency of the cells [45], further strengthening a role for USP9X in cell-cell adhesions. USP9X could thus be involved in regulating homotypic adhesion in lymphocytes. USP9X displays higher activity levels in LCLs than in BLs, and it is upregulated upon EBV-dependent immortalization of B cells (Paper I). This suggests a possible role for this DUB in regulation of the increased clumping seen in LCLs as compared to BLs or freshly isolated B cells. USP9X was also active in T cells, and in even higher levels upon PHA-stimulation. It is tempting to speculate USP9X plays a role in the increased homotypic adhesion seen in T cells that are stimulated with PHA.

USP9X has also been reported to interact with Doublecortin (DCX), a microtubule-associated protein involved in neuronal migration [160], and although the functional significance of this interaction is unclear it points to yet another way by which USP9X might regulate cellular adhesion and migration. A role for USP9X in protein transport was suggested when one of the ligases involved in endocytosis and endocytic sorting, Itch, was shown to autoubiquinate itself and to be deubiquitinated and probably stabilized by USP9X [161].

The most prominent effect on DUB activity after EBV infection was the dramatic upregulation of UCH-L1. The role of UCH-L1 in the proliferation of cells seems to be somewhat dependent on the cell type where the protein is expressed. In normal fibroblasts UCH-L1 is highly expressed, while it is strongly downregulated in their transformed counterparts [162]. Inhibition of UCH-L1, both by chemical inhibitors and RNAi, in lung cancer cells was shown to stimulate proliferation [163], suggesting that UCH-L1 is expressed as a response to proliferation in these tumors. The level of UCH-L1 also correlated with cell size and

morphology, with cells expressing high levels of UCH-L1 displaying an increase in cell size and more extended processes. Similar results have also been reported for an acute lymphoblastic leukemia cell line [164].

In other tissues however, UCH-L1 is overexpressed in tumors and correlates with the more advanced stages of tumors. In microarray comparison between a weakly tumorogenic prostate cancer and a highly tumorogenic and metastatic cell line, the UCH-L1 gene was found highly upregulated and the upregulation correlated with mRNA and protein levels. UCH-L1 was also detected at higher levels in tumor zenografts from the more aggressive type of prostate cancer using immunohistochemistry, as compared to the less tumorogenic type [165]. Similarly, UCH-L1 expression is increased in pancreatic carcinoma as compared to normal pancreatic tissue, and UCH-L1 expression correlates with significantly shorter survival time [166]. In colorectal cancer, UCH-L1 overexpression correlates with tumor size and the extent of the tumor [167]. In a study of neuroblastoma patients, UCH-L1 was suggested as a sensitive marker for detection of minimal residual neuroblastoma cells in bone marrow and peripheral blood [168]. In these tumors, the enzyme is suggested to work as a marker for diagnosis and prognosis for tumors of different tissue origin.

Some of the studies on UCH-L1 may give a hint on the role of this DUB during transformation. It has been shown that Ub is upregulated as a response to stress conditions, and it could be that UCH-L1 is needed to process pro-Ub into free Ub in these cells. It could also be that faster proliferating cells have a higher turnover of proteins, and that this partly urges for more Ub to be accessible or for more Ub-conjugates to be unconjugated as they are being turned over. As discussed later, UCH-L1 might also regulate the levels of $p27^{Kip1}$ [74], suggesting that UCH-L1 regulates cell cycle progression.

In conclusion, Paper I provides a first insight in the tissue distribution of USPs. Activation-associated DUBs were identified, both in T and B cells after stimulation with different mitogenic agents, suggesting a role for these enzymes in the activation of lymphocytes. Interestingly, EBV infection was shown to directly induce activity of several DUBs, some of which correlate with the increased proliferation of these cells, indicating that these proteins might play an important role in transformation and

tumor development following viral infection. Together with previous reports [146], the study further suggests a correlation between UCH-L1 phenotypical characteristics of tumor cells of BL.

Paper II

The results presented in Paper I suggested that there are tissue specific, but also tumor specific activity patterns of DUBs. However, the correlation of the activity pattern seen in cell lines, adapted to culture conditions in some cases after many years in culture, may not represent the true activity pattern seen in vivo in tumor tissues. The aim of Paper II was to investigate the activity pattern of DUBs in tumor tissue. Fresh biopsies from cervical carcinomas were compared to adjacent normal tissue, to try to distinguish DUB activities that may play a role in tumor development. Biopsies of tumors classified as squamous cell carcinomas were collected from 27 cervical carcinoma patients and kept frozen. For DUB activity profiling, frozen tissues were disrupted mechanically and resuspended in labeling buffer. To preserve proteins from the tissues, the extraction procedures were done in the cold. The clarified protein extracts were snap frozen in liquid nitrogen and kept frozen until use. The expression levels of DUBs, as assessed by Western blot, corresponded to the activity levels detected by HAUb-VME cross-linking. Labeling with the functional probe thus appears to be a reliable indicator for both expression level and enzymatic activity. A significant variability between individual samples was seen. To draw firm conclusions it would therefore be critical to expand the panel of patients. Despite of this limitation, consistent differences in DUB activity between normal and malignant tissues could be seen.

No dramatic differences were seen for DUBs migrating like previously described for UCH-L1, USP9X and USP15. DUBs migrating at 38 and 45 kDa, previously identified as UCH-L3 and UCH37, displayed more consistent differences in activity. UCH-L3 was seen active in a higher percentage of tumor samples than in normal tissue. UCH37 was expressed at the same frequency in both. The activity levels of both UCH-L3 and UCH37 were dramatically increased in a majority of the tumors. UCH-L3 was detected also in most of the cervical carcinoma cell lines investigated, supporting a need of this enzyme in these tumors. A

correlation between UCH-L3 and the tumor stage could be seen, in that this DUB was upregulated in more advanced stages of the tumor. However, no correlation between the enzymatic activity and metastases was detected. UCH-L3 and UCH37 were to some extent upregulated in HPV E6/E7 transformed keratinocytes (Paper II), further strengthening a role for these enzymes in the transformation process of these cells.

Upregulation of UCH-L3 has previously been shown in breast cancer [169] and colon carcinoma patients exhibit autoantibodies to UCH-L3 [170]. The function of UCH-L3 is not clear, however. It has been associated with the maturation of sperm cells [171] and with fertilization [172], but the substrates are so far unknown. UCH-L3 has been shown to interact with both Ub and the Ub like (UBL) molecule Nedd8 [173], and this interaction seem to be preserved during evolution since the same is seen for the UCH-L3 homolog from Toxoplasma gondii and Plasmodium falciparum [174]. A murine UCH-L3 deletion mutant displays retinal degeneration, muscular degeneration and mild growth retardation. The retinal degeneration seem to be depending on a mitochondrial dysfunction leading to oxidative-stress related photoreceptor cell apoptosis [175]. UCH-L3 knock out mice do not show any changes in Ub levels, while the levels of Nedd8 and the apoptotic proteins p53, Bax and Caspase-3 are elevated [70]. This suggests a role for UCH-L3 in the maintenance of homeostatic levels of Nedd8 as well as in preventing apoptosis. UCH-L3 knock out mice also show learning deficit correlating with impaired working memory [176], pointing at a role of UCH-L3 in neuronal functions.

UCH37 interacts with the proteasome, and has been suggested to be important for overall protein degradation. In fast proliferating cells, a higher turnover of proteins by proteasomal degradation might be necessary, explaining this need for increased UCH37 levels. A role in transformation for this DUB is also suggested by its role in upregulating transforming growth factor (TGF)-receptor signaling. UCH37 has been shown to interact with the Smad transcription factors [177]. Smads are activated upon TGF-receptor signaling, and ubiquitination and degradation is a regulatory step in this cascade. Disruption of components of the TGF-β signaling cascade is commonly occurring in tumors. By deubiquitinating

and stabilizing the type I TGF-β receptor, UCH37 upregulates TGF-β-dependent signaling [177].

No significant correlation between the DUB activity pattern and HPV status or clinical stage of the tumor could be demonstrated. A study using an expanded group of patients would be needed for this kind of analysis. A correlation between UCH-L1 and tumor stage could however be seen, with lower levels of UCH-L1 expressed in tumors as compared to normal tissue, but with higher activity levels of UCH-L1 in later stages of the tumor. UCH-L1 has previously been described upregulated in tumors of several tissue types [169, 178], and even to the metastatic features of certain tumors [72]. No correlation with metastases was seen in this study however. Also, no activity of UCH-L1 was detected in cell lines of cervical carcinoma. Surprisingly, when transforming keratinocytes using HPV E6/E7 UCH-L1 was highly upregulated. This is in line with the results presented in Paper I, where EBV-induced transformation correlated with a dramatic increase in the enzymatic activity of this DUB. One possible explanation is that this enzyme is needed for transformation, or adaptation to growth in vitro. It could also be that UCH-L1 is expressed in a low percentage of cells, and that these cells gain a growth advantage in the culture allowing the eventual detection of this DUB after accumulation of UCH-L1 expressing cells.

The probes used in Paper I and Paper II only detect a small percentage of all potential DUBs encoded by the human genome. However, a rather broad range of enzymes can still be detected from a small amount of sample, making this a useful method to fill the knowledge gap on the tissue and tumor specific patterns of these enzymes. Drawing firm conclusions on the activity patterns of DUBs in tumor cells in complicated by the fact that tumors have a heterogeneous cellular composition, containing also stroma and infiltrating immunological cells. The study represents a first attempt to profile DUB activity in fresh biopsies from tissues of tumor origin and compare them with normal tissue. Further studies are needed to draw conclusion of the enzymes exact role in tumorogenesis.

Paper III

In Paper III, the importance of UCH-L1 in the tumorogenic phenotype of Burkitt´s lymphoma was investigated, and a role for UCH-L1 in the regulation of LFA-1 dependent homotypic cell adhesion was discovered. Previous studies suggest a role for UCH-L1 in EBV-induced immortalization of B cells (Paper I), and an upregulation of UCH-L1 in BL cells has been suggested to associate with the the increased resistance to apoptosis induced by proteasome inhibitors seen in BL [146].

In order to further elucidate the role of UCH-L1 in B-cell lymphomas, we used RNA interference (RNAi) to inhibit expression of UCH-L1 in BL cell lines. Surprisingly, an almost complete knock down of UCH-L1 correlated with increased homotypic adhesion of the BL cells. Normally, BL cell lines grow in single cell suspension or form small clumps through cell-cell adhesions. After knock down of UCH-L1 a dramatic effect on the growth pattern was seen, in that almost none of the cells in culture were found as single cells, and that big clusters of cells were formed. Expression of UCH-L1 in a normally UCH-L1-negative LCL lead to a loosening of homotypic adhesions seen as less dense cell aggregates in the suspension cultures. Previous studies suggest that the surface levels of LFA-1 and its ligand ICAM-1 are lower on BL cells than on LCLs and that this contributes to the lower immunogenicity of BL cells [179, 180]. The increase of cell-cell adhesion seen in Paper III after UCH-L1 inhibition was dependent on the LFA-1, as adding antibodies specific to this adhesion molecule to the cultures inhibited homotypic adhesion. Our results confirmed the observation that BL cells express lower levels of LFA-1 and ICAM-1 compared to LCLs. However, the levels were unaffected by the levels of UCH-L1, suggesting that UCH-L1 affects other aspects of the LFA-1 dependent adhesion.

Integrins control many functions important for leukocytes. Development, maturation, circulation and response to inflammatory signals are processes regulated by integrins. LFA-1 is an integrin expressed on all leukocytes. It consists of two subunits, αL and β2. The molecule is normally expressed on the cell surface of circulating, resting lymphocytes in an inactive state with low affinity to its ligands. By stimulation LFA-1 goes through conformational changes, leading to increased affinity to its ligands. The high affinity state of LFA-1 also

correlates with an increased avidity, through clustering of LFA-1 into membrane micro domains called lipid rafts. Inactive integrins are restrained by the cytoskeleton, and release triggers the integrin mobility on the cell membrane required for clustering. (Reviewed in [181])

For most leukocytes, a change in adhesion occurs as a response to chemoattractants. A conformational change starts at the cytoplasmic domain of the integrin and is transmitted to the extracellular ligand-binding part. This is referred to as inside-out signaling. (Reviewed in [182]) These inside-out signals can be initiated by G-protein coupled receptors (GPCRs). Signaling through these receptors activates small GTPases such as Rap1 and Rac1. Rap1 activates LFA-1 [183] through the interaction with several effector molecules. Rap1 forms a complex with Regulator of adhesion and cell polarization enriched in lymphoid tissues (RAPL). (Reviewed in [184]) This complex alters LFA-1 distribution and activation. A leukocyte that adhered to the vascular endothelium undergoes polarization to allow for migration. This polarization leads to the formation of a front (leading edge) and a trailing edge (the uropod). Integrins on a polarized leukocyte generate highly dynamic adhesions, clustering at the leading edge and the uropod. The high and intermediate affinity integrins localize primarily to the leading edge. Integrins are constantly internalized and recycled to the membrane. RAPL interacts with the kinase Mst1. The RAPL/Mst1 complex is involved in the transport of LFA-1 in vesicles to the leading edge, along the microtubular system. (Reviewed in [184]) GPCRs also activate DOCK2 that in turn activates Rac1. This small GTPase is important for the formation of the leading edges through rearrangements of the cytoskeleton. Through outside-in signaling when integrins bind to their ligand, Rac1 activation may occur, acting as a positive feedback loop further stimulating firm integrin adhesion. (Reviewed in [185]).

In order for integrins to form firm adhesions with their ligands, there is probably a need for the integrins to bind to the cytoskeleton. Rap1 interaction with Rap-interacting adapter molecule (RIAM) recruits talin to integrins. Talin links LFA-1 to the cytoskeleton and thereby assists its activation. Talin cleavage by calpain has been suggested to lead to the release of the Talin head domain, that through its interaction with the

cytoplasmic part of LFA-1 contributes to its conformational change (reviewed in [185]).

The activation and relocalization of LFA-1 into lipid rafts associated with low UCH-L1 levels, seen in Paper III, correlated with a constant activation of Rap1 and Rac1 that was reverted by reconstitution of UCH-L1 into a UCH-L1 negative LCL. UCH-L1 could therefore be important in negatively regulating small GTPases, allowing for integrins to be kept in an inactive state. UCH-L1 further affects the capacity of LFA-1 bind to its ligand ICAM-1 upon stimulation. Rap1 is needed for divalent cation-dependent stimulation of LFA-1/ICAM-1 binding [186]. This strengthens the hypothesis that UCH-L1 is important for keeping LFA-1 in an inactive state, and that this occurs via the inactivation of Rap1.

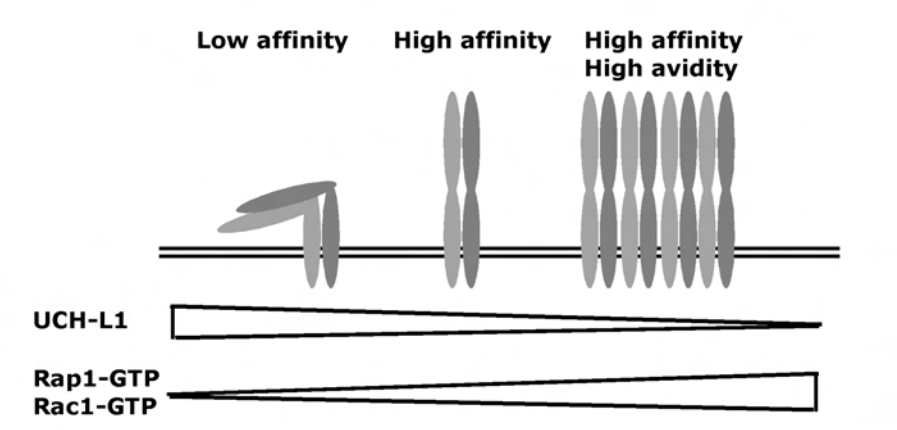

Figure 12. The affinity and avidity state of LFA-1 is affected by the UCH-L1 levels. In low affinity, the ligand binding part of LFA-1 is facing the cell surface. Due to conformational changes, LFA-1 increases the affinity to it ligand. The binding capacity can increase further through clustering, which leads to higher avidity. The levels of UCH-L1 seem to correlate inversely with the affinity and avidity state of LFA-1 and the activity of its regulators Rap1 and Rac1.

Many questions on how UCH-L1 mediates this anti-adhesive function still remain. The activity of small GTPases is regulated by several guanine exchange factors (GEFs) responsible for their activation. Several GTPase activating proteins (GAPs) stimulate the GTPase activity, thus contributing to the inactivation of small GTPases. UCH-L1 could interfere with the GTP-loading, by inhibiting one or several of the GEFs responsible for Rap1 and Rac1 activation. It is also possible that UCH-L1 stimulates

the GAPs, thus allowing for constant GTP-hydrolysis and consequent inactivation of the GTPases. Interestingly, the activation of LFA-1 by Rap1 requires the lysine residues at positions 1097 and 1099 in the cytoplasmic domain of LFA-1 [187]. So far ubiquitination of LFA-1 has not been demonstrated, but it is tempting to speculate that the lysine residues are targets of ubiquitination. This could directly link UCH-L1 to the regulation of LFA-1.

UCH-L1 has been shown to cleave peptide- and isopeptide-bonds to Ub, but to be unable to cleave K48-linked chains [188]. Therefore a function in generating free Ub from pro-Ub, i.e. from Ub head-to-tail fusion proteins or Ub-ribosomal protein conjugates coded by the Ub genes, was suggested for UCH-L1. At high concentrations however, UCH-L1 forms homodimers that have been shown to display E3 ligase activity [66]. This leaves the room for speculation open. UCH-L1 could act as an isopeptidase, specific for other Ub modifications than K48-linked chains, or it could in fact act as a ligase.

Homotypic adhesion has been shown to hinder lymphocyte proliferation [189]. In line with this, a dramatic decrease in proliferation rate was seen upon UCH-L1 knock down. This correlated with retention of the cells in the G1 phase of the cell cycle and a specific increase of the cell cycle inhibitor $p27^{Kip1}$. The upregulation of $p27^{Kip1}$ could be explained by the finding that integrins regulate cell cycle progression through the regulation of $p27^{Kip1}$ [189]. Also, the Rap1 GTPase has been suggested to oppose the role of the oncoprotein Ras, possibly by competing for downstream effectors of Ras, but also possibly through its ability to activate specific inhibitory signals [190-192]. Genetic analysis has shown that the Drosophila dacapo gene product is a target of Rap1 [193]. Since dacapo is a cyclin-dependent kinase (cdk) inhibitor similar to $p27^{Kip1}$ and blocks cell cycle progression during Drosophila eye development, this could suggest as specific rout by which UCH-L1 affects the proliferation of B-lymphocytes.

In a yeast-2-hybrid screen, UCH-L1 was shown to interact with the SUMO E2 conjugase Ubc9, the multifunctional protein RanBPM and Jab1, a component of the COP9 signalosome that acts as a Cullin de-Neddylase [74]. Jab1 is also a Jun activation domain binding protein that binds $p27^{Kip1}$ and is involved in the cytoplasmic transport of $p27^{Kip1}$ for its

degradation [194]. UCH-L1 co-localizes with Jab1 in the perinuclear cytoplasm of contact inhibited cells. Upon serum-stimulation, both UCH-L1 and Jab1 relocalize into the nucleus, correlating with reduced $p27^{Kip1}$ levels in the nucleus [74]. It was therefore suggested that UCH-L1 somehow contributes to the relocalization and consequent degradation of $p27^{Kip1}$. In human myeloma cells, UCH-L1 was upregulated as a consequence of growth-factor starvation, correlating with upregulation of $p27^{Kip1}$ [195]. This effect could be reversed by restimulation of the cells with serum, and suggests that UCH-L1 through the regulation of $p27^{Kip1}$ nuclear import and/or export regulates the levels of the cell cycle inhibitor $p27^{Kip1}$. The G1 arrest and $p27^{Kip1}$ upregulation seen upon UCH-L1 knockdown in Paper III could therefore possibly be explained by a direct interaction of UCH-L1 with Jab1 and $p27^{Kip1}$, affecting the degradation of $p27^{Kip1}$.

Few studies suggest mechanisms by which UCH-L1 might act on different signaling pathways, possibly regulating cell proliferation. Overexpression has been reported to correlate with the tumor suppressor Von Hippel Lindau (VHL), and UCH-L1 has been suggested to oppose the VHL-dependent ubiquitination of HIF-1α [72]. A recent study further supports a role for UCH-L1 in regulating de novo synthesis or remodeling of blood vessels. UCH-L1 was suggested to inhibit NFκB activity, correlating with decreased levels of several NFκB-driven genes important for vascular remodeling [73].

Paper III suggests an important role of UCH-L1 in regulating the LFA-1 dependent homotypic adhesion of B-lymphocytes. By keeping LFA-1 in an inactive state, UCH-L1 might be important in the regulation of lymphocytic proliferation, suggesting this enzyme as a potential target for future drug development.

Concluding remarks and future perspectives

Deubiquitinating enzymes play major roles in regulating processes involving ubiquitination. This thesis presents results showing that in vitro transformation by EBV infection or expression of the HPV oncoproteins E6/E7 changes the activity of several DUBs. These DUBs are involved in regulating processes such as DNA damage responses and cell cycle progression. A DUB activity has been demonstrated for several viral proteins [80-83]. The results presented in this thesis suggest that viruses may also alter the activity of DUBs expressed by the host cell. This may be a way for the viruses to interfere with the normal functions of the infected cells, contributing to the transforming capacity of these viruses.

The thesis further presents a first attempt to correlate the activity levels of DUBs to tumor progression using fresh tissue biopsies. Several DUBs were found to be upregulated in tumor tissues as compared to normal adjacent tissue. This may suggest a role for some of these DUBs in the development of cervical carcinoma.

A novel function for the DUB UCH-L1 was further suggested (Paper III). UCH-L1 seems to be important for keeping the integrin LFA-1 in an inactive state, which promotes the proliferation of B-lymphocytes. It is clear that UCH-L1 plays an important role in the growth characteristict of B cells, probably both during in vitro immortalization of B cells through EBV infection and in LCLs as well as in BLs. To better understand the mechanism behind this observation, it would be crucial to identify interacting proteins and possible substrate(s) of UCH-L1. It would also be interesting to explore the function of UCH-L1 in adherent cells where it is likely to play a very different role on the adhesiveness and proliferation capacity of the cells. Interestingly, UCH-L1 was demonstrated in Paper III to regulate the growth capacity of B cells both in suspension, and in semi solid medium. This identifies UCH-L1 as a potentially interesting target for future drug development.

Acknowledgement

The work presented in this thesis would not have been conducted by me if it was not for the inspiration and motivation given to me by persons I met. I would like to thank all of you who in one way or another thought me important things in life. Those of you who are not mentioned here, you still know that I appreciate your friendship a lot!

My supervisor Maria G Masucci. You have been a great source of inspiration and support. You presented me to many different ways of thinking, different personalities and cultures. Thank you for taking me under your wings, supporting me through though times. Thank you for sending me, and bringing me, around the world, introducing me to exciting ideas and projects. This has been an experience I value very high and will never forget.

My co-supervisor, Riccardo Gavioli. For having fun in your lab during the Ferrara-period, for helping me see things with perspectives, convincing me not to give up. And for many nice dinners, drinks, bars and clubs! I will be forever grateful for your support and friendship.

All my co-authors, who made these studies possible.

Past and present members of the lab: Yvonne. You keep track of things and persons! Thank you for being "pushy" about deadlines, for being supportive in the lunchroom and for sharing your handicraft skills! **Elio** for late nights in the lab, vivid discussions and enthusiasm! My Swedish "ok results" always turned into "beautiful, wonderful results" in your Italian eyes! I have truly missed you during the last years in the lab! **Thorsten**, the true scientist. Eager to collect new knowledge, energy to solve problems and a wonderful spirit that this is great fun! **Jianjun** for the RNAi part and for solving the hard cell-culture problems! **Vanessa**, for being the positive "we will solve this together"-person during your time in the lab! **Rama** for the "never ever give up"-spirit and calmness that you bring to the writing room! And for making sure I was never alone on Sundays when I came to feed my cells. Good luck with your future work! **Bettina!** Sharing lab space, lunchtime, protocols and writing desk with you is so fun! Even when you are frustrated you keep going and you make the lab a better place to be! May all your days in the Masucci-group be filled with red nail polish! **Kristina**, **Gerry and Gerco** for a lust and enthusiasm about science that is contagious! Also for scientific and less scientific chats in the lab, at the pub or during lunchtime! Kristina: if everyone was just a bit more like you, the world would be so much nicer. **Teresa**, stay in research, I don't think the wife of the ambassador gets to do pull-downs and run Western blots! Thank you for being so super-energetic! And for bringing your nice group, **Lina, Javier and Eugenie**, to the lab! Thanks to all of you for sharing protocols, reagents, crazy stories and weekend-plans! **Marianne,** who helped everyone in the lab with organizing and keeping things working! You kept us sane! Your support was very valuable! **Helena,** for taking over and for keeping track of everyone and everything with a smile!

André, Anna B, Anna C, Elena, Micke Melon and Victoria. Everything was lauder, later, crazier and more fun while you were still in the lab. A big thank you also to **Anders B, Cinzia, Diego, Eleonora, Eliana, Florian, Jiang, Linda, Masha, Mathieu, Nico, Nicolay, Sebastian, Siamak, Stefano, Stijn, Patrick, Pino, Vaya, Victor!**

Off course everyone at MTC, for reagents and advice, PUBs and parties!

To my friends and family: The two best friends one could wish for, **Lisette and Lisa**! I know, when it comes to both of you, that no matter the problem (broken nail before a party or totally messed up experiments the last six months) you have always, always been there for me. On the phone, on the dance-floor, at the dinner table or during trips! You are strong, intelligent and beautiful women, who I admire a lot! **Sarah, Lotta, Hanna, Nina and Sara** for brunches, weddings, discussions, laughs, cinemas, drinks, coffees, lunches, and occasional gym-visits! For advice and sharing your ideas on life! **Calle, Daniel** and the gang for parties, sailing, holidays! And for serious and less serious discussions! **Sam** for standing me nagging and crying about work, MTC felt empty after you left! All of you for support and comfort!

My mother, for supporting me and supplying me with advices. Also for forcing me to be less egoistic and take time from the lab to family matters. Thank you so much for handing me the book "Duktig flicka!" on my first vacation, the second year of my PhD studies, I read it carefully! **My father**, who with your strong character have always been a role model for me. Together with **Berth** you have taught me to stand up for my self, my ideas and choices in life. **Katarina** for always being there to cheer me up, to have fun with, and for being the best sister in the world! **Thomas** for being a spice in our life, and for bringing your family into our family. I am really proud of both of you! All my grandparents: Farmor, Farfar, Elke, G-A, Gösta, Mormor and Morfar. The Rolén family and the Bjöörn family. Hopefully now you will see a bit more of me!

To all **Darlings of the Pellas-clan**. It has been wonderful to come into your family, and to feel truly welcome despite the fact that I am a "Kungsing". All of you have helped me to keep going during the last period of these studies, by giving me the extra energy needed to fulfil long days in the lab. Thanks for showing me how to find mushrooms (even though all I had to do was to go where Mats pointed), grow tomatoes (same there, I only had to put down the plants given to us by Malin), and feeding me with energy during the weekends!

Anders. You came into my life, and brought me to life! You lift me up higher than ever before! Thank you for sharing DNA with me! Love you, now and forever.

References

1. Jin, J., et al., *Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging.* Nature, 2007. **447**(7148): p. 1135-8.
2. Pelzer, C., et al., *UBE1L2, a novel E1 enzyme specific for ubiquitin.* J Biol Chem, 2007. **282**(32): p. 23010-4.
3. Ciechanover, A., *Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting.* Exp Biol Med (Maywood), 2006. **231**(7): p. 1197-211.
4. Hoeller, D., et al., *E3-independent monoubiquitination of ubiquitin-binding proteins.* Mol Cell, 2007. **26**(6): p. 891-8.
5. Ciechanover, A., *Proteolysis: from the lysosome to ubiquitin and the proteasome.* Nat Rev Mol Cell Biol, 2005. **6**(1): p. 79-87.
6. Glickman, M.H. and A. Ciechanover, *The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.* Physiol Rev, 2002. **82**(2): p. 373-428.
7. Ciechanover, A., *N-terminal ubiquitination.* Methods Mol Biol, 2005. **301**: p. 255-70.
8. Ben-Saadon, R., et al., *The tumor suppressor protein p16(INK4a) and the human papillomavirus oncoprotein-58 E7 are naturally occurring lysine-less proteins that are degraded by the ubiquitin system. Direct evidence for ubiquitination at the N-terminal residue.* J Biol Chem, 2004. **279**(40): p. 41414-21.
9. Aviel, S., et al., *Degradation of the epstein-barr virus latent membrane protein 1 (LMP1) by the ubiquitin-proteasome pathway. Targeting via ubiquitination of the N-terminal residue.* J Biol Chem, 2000. **275**(31): p. 23491-9.
10. Ikeda, M., A. Ikeda, and R. Longnecker, *Lysine-independent ubiquitination of Epstein-Barr virus LMP2A.* Virology, 2002. **300**(1): p. 153-9.
11. Cadwell, K. and L. Coscoy, *Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase.* Science, 2005. **309**(5731): p. 127-30.
12. Wang, X., et al., *Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3.* J Cell Biol, 2007. **177**(4): p. 613-24.
13. Peng, J., et al., *A proteomics approach to understanding protein ubiquitination.* Nat Biotechnol, 2003. **21**(8): p. 921-6.
14. Xu, P. and J. Peng, *Dissecting the ubiquitin pathway by mass spectrometry.* Biochim Biophys Acta, 2006. **1764**(12): p. 1940-7.
15. Ciehanover, A., Y. Hod, and A. Hershko, *A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes.* Biochem Biophys Res Commun, 1978. **81**(4): p. 1100-5.
16. Hershko, A., et al., *Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis.* Proc Natl Acad Sci U S A, 1980. **77**(4): p. 1783-6.

17. Ciechanover, A., et al., *Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes.* J Biol Chem, 1980. **255**(16): p. 7525-8.
18. Wilkinson, K.D., M.K. Urban, and A.L. Haas, *Ubiquitin is the ATP-dependent proteolysis factor I of rabbit reticulocytes.* J Biol Chem, 1980. **255**(16): p. 7529-32.
19. Ciechanover, A. and A.L. Schwartz, *The ubiquitin system: pathogenesis of human diseases and drug targeting.* Biochim Biophys Acta, 2004. **1695**(1-3): p. 3-17.
20. Frisan, T., V. Levitsky, and M.G. Masucci, *Variations in proteasome subunit composition and enzymatic activity in B-lymphoma lines and normal B cells.* Int J Cancer, 2000. **88**(6): p. 881-8.
21. Staege, M.S., et al., *MYC overexpression imposes a nonimmunogenic phenotype on Epstein-Barr virus-infected B cells.* Proc Natl Acad Sci U S A, 2002. **99**(7): p. 4550-5.
22. Nishikawa, H., et al., *Mass spectrometric and mutational analyses reveal Lys-6-linked polyubiquitin chains catalyzed by BRCA1-BARD1 ubiquitin ligase.* J Biol Chem, 2004. **279**(6): p. 3916-24.
23. Boulton, S.J., *BRCA1-mediated ubiquitylation.* Cell Cycle, 2006. **5**(14): p. 1481-6.
24. Spence, J., et al., *A ubiquitin mutant with specific defects in DNA repair and multiubiquitination.* Mol Cell Biol, 1995. **15**(3): p. 1265-73.
25. Kirkin, V. and I. Dikic, *Role of ubiquitin- and Ubl-binding proteins in cell signaling.* Curr Opin Cell Biol, 2007. **19**(2): p. 199-205.
26. Bergink, S., et al., *DNA damage triggers nucleotide excision repair-dependent monoubiquitylation of histone H2A.* Genes Dev, 2006. **20**(10): p. 1343-52.
27. Schauber, C., et al., *Rad23 links DNA repair to the ubiquitin/proteasome pathway.* Nature, 1998. **391**(6668): p. 715-8.
28. Jacquemont, C. and T. Taniguchi, *Proteasome function is required for DNA damage response and fanconi anemia pathway activation.* Cancer Res, 2007. **67**(15): p. 7395-405.
29. Laribee, R.N., S.M. Fuchs, and B.D. Strahl, *H2B ubiquitylation in transcriptional control: a FACT-finding mission.* Genes Dev, 2007. **21**(7): p. 737-43.
30. Mukhopadhyay, D. and H. Riezman, *Proteasome-independent functions of ubiquitin in endocytosis and signaling.* Science, 2007. **315**(5809): p. 201-5.
31. Schmidt, M.H. and I. Dikic, *The Cbl interactome and its functions.* Nat Rev Mol Cell Biol, 2005. **6**(12): p. 907-18.
32. Sigismund, S., et al., *Clathrin-independent endocytosis of ubiquitinated cargos.* Proc Natl Acad Sci U S A, 2005. **102**(8): p. 2760-5.
33. Polo, S. and P.P. Di Fiore, *Endocytosis conducts the cell signaling orchestra.* Cell, 2006. **124**(5): p. 897-900.
34. Hoeller, D., et al., *Regulation of ubiquitin-binding proteins by monoubiquitination.* Nat Cell Biol, 2006. **8**(2): p. 163-9.
35. Dikic, I., et al., *Targeting ubiquitin in cancers.* Eur J Cancer, 2006. **42**(18): p. 3095-102.
36. Nijman, S.M., et al., *A genomic and functional inventory of deubiquitinating enzymes.* Cell, 2005. **123**(5): p. 773-86.

37. Verma, R., et al., *Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome.* Science, 2002. **298**(5593): p. 611-5.
38. Wilkinson, K.D., *Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome.* Semin Cell Dev Biol, 2000. **11**(3): p. 141-8.
39. Baek, K.H., *Conjugation and deconjugation of ubiquitin regulating the destiny of proteins.* Exp Mol Med, 2003. **35**(1): p. 1-7.
40. Qiu, X.B., et al., *hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37.* Embo J, 2006. **25**(24): p. 5742-53.
41. Yao, T., et al., *Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1.* Nat Cell Biol, 2006. **8**(9): p. 994-1002.
42. Hamazaki, J., et al., *A novel proteasome interacting protein recruits the deubiquitinating enzyme UCH37 to 26S proteasomes.* Embo J, 2006. **25**(19): p. 4524-36.
43. Li, T., et al., *Identification of two proteins, S14 and UIP1, that interact with UCH37.* FEBS Lett, 2001. **488**(3): p. 201-5.
44. Taya, S., et al., *The deubiquitinating enzyme Fam interacts with and stabilizes beta-catenin.* Genes Cells, 1999. **4**(12): p. 757-67.
45. Murray, R.Z., L.A. Jolly, and S.A. Wood, *The FAM deubiquitylating enzyme localizes to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and beta-catenin.* Mol Biol Cell, 2004. **15**(4): p. 1591-9.
46. Taya, S., et al., *The Ras target AF-6 is a substrate of the fam deubiquitinating enzyme.* J Cell Biol, 1998. **142**(4): p. 1053-62.
47. Kanai-Azuma, M., et al., *Co-localization of FAM and AF-6, the mammalian homologues of Drosophila faf and canoe, in mouse eye development.* Mech Dev, 2000. **91**(1-2): p. 383-6.
48. Deng, S., et al., *Over-expression of genes and proteins of ubiquitin specific peptidases (USPs) and proteasome subunits (PSs) in breast cancer tissue observed by the methods of RFDD-PCR and proteomics.* Breast Cancer Res Treat, 2007. **104**(1): p. 21-30.
49. Li, M., et al., *A dynamic role of HAUSP in the p53-Mdm2 pathway.* Mol Cell, 2004. **13**(6): p. 879-86.
50. Borodovsky, A., et al., *Chemistry-based functional proteomics reveals novel members of the deubiquitinating enzyme family.* Chem Biol, 2002. **9**(10): p. 1149-59.
51. Balakirev, M.Y., et al., *Otubains: a new family of cysteine proteases in the ubiquitin pathway.* EMBO Rep, 2003. **4**(5): p. 517-22.
52. Evans, P.C., et al., *A novel type of deubiquitinating enzyme.* J Biol Chem, 2003. **278**(25): p. 23180-6.
53. Soares, L., et al., *Two isoforms of otubain 1 regulate T cell anergy via GRAIL.* Nat Immunol, 2004. **5**(1): p. 45-54.
54. Urbe, S., et al., *Control of growth factor receptor dynamics by reversible ubiquitination.* Biochem Soc Trans, 2006. **34**(Pt 5): p. 754-6.
55. Clague, M.J. and S. Urbe, *Endocytosis: the DUB version.* Trends Cell Biol, 2006. **16**(11): p. 551-9.
56. Mizuno, E., et al., *Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes.* Mol Biol Cell, 2005. **16**(11): p. 5163-74.

57. Mizuno, E., et al., *A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes.* Traffic, 2006. **7**(8): p. 1017-31.
58. Ardley, H.C., et al., *UCH-L1 aggresome formation in response to proteasome impairment indicates a role in inclusion formation in Parkinson's disease.* J Neurochem, 2004. **90**(2): p. 379-91.
59. Naze, P., et al., *Mutation analysis and association studies of the ubiquitin carboxy-terminal hydrolase L1 gene in Huntington's disease.* Neurosci Lett, 2002. **328**(1): p. 1-4.
60. Tan, E.K. and L.M. Skipper, *Pathogenic mutations in Parkinson disease.* Hum Mutat, 2007. **28**(7): p. 641-53.
61. Lowe, J., et al., *Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases.* J Pathol, 1990. **161**(2): p. 153-60.
62. Leroy, E., et al., *The ubiquitin pathway in Parkinson's disease.* Nature, 1998. **395**(6701): p. 451-2.
63. Nishikawa, K., et al., *Alterations of structure and hydrolase activity of parkinsonism-associated human ubiquitin carboxyl-terminal hydrolase L1 variants.* Biochem Biophys Res Commun, 2003. **304**(1): p. 176-83.
64. Maraganore, D.M., et al., *Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease.* Neurology, 1999. **53**(8): p. 1858-60.
65. Manago, Y., et al., *Potentiation of ATP-induced currents due to the activation of P2X receptors by ubiquitin carboxy-terminal hydrolase L1.* J Neurochem, 2005. **92**(5): p. 1061-72.
66. Liu, Y., et al., *The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility.* Cell, 2002. **111**(2): p. 209-18.
67. Saigoh, K., et al., *Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice.* Nat Genet, 1999. **23**(1): p. 47-51.
68. Bonin, M., et al., *Microarray expression analysis of gad mice implicates involvement of Parkinson's disease associated UCH-L1 in multiple metabolic pathways.* Brain Res Mol Brain Res, 2004. **126**(1): p. 88-97.
69. Harada, T., et al., *Role of ubiquitin carboxy terminal hydrolase-L1 in neural cell apoptosis induced by ischemic retinal injury in vivo.* Am J Pathol, 2004. **164**(1): p. 59-64.
70. Kwon, J., et al., *Two closely related ubiquitin C-terminal hydrolase isozymes function as reciprocal modulators of germ cell apoptosis in cryptorchid testis.* Am J Pathol, 2004. **165**(4): p. 1367-74.
71. Bifsha, P., et al., *Altered gene expression in cells from patients with lysosomal storage disorders suggests impairment of the ubiquitin pathway.* Cell Death Differ, 2007. **14**(3): p. 511-23.
72. Seliger, B., et al., *Ubiquitin COOH-terminal hydrolase 1: a biomarker of renal cell carcinoma associated with enhanced tumor cell proliferation and migration.* Clin Cancer Res, 2007. **13**(1): p. 27-37.
73. Takami, Y., et al., *Ubiquitin carboxyl-terminal hydrolase L1, a novel deubiquitinating enzyme in the vasculature, attenuates NF-kappaB*

activation. Arterioscler Thromb Vasc Biol, 2007. **27**(10): p. 2184-90.
74. Caballero, O.L., et al., *Interaction and colocalization of PGP9.5 with JAB1 and p27(Kip1).* Oncogene, 2002. **21**(19): p. 3003-10.
75. Everett, R.D., et al., *A novel ubiquitin-specific protease is dynamically associated with the PML nuclear domain and binds to a herpesvirus regulatory protein.* Embo J, 1997. **16**(3): p. 566-77.
76. Everett, R.D., M. Meredith, and A. Orr, *The ability of herpes simplex virus type 1 immediate-early protein Vmw110 to bind to a ubiquitin-specific protease contributes to its roles in the activation of gene expression and stimulation of virus replication.* J Virol, 1999. **73**(1): p. 417-26.
77. Boutell, C. and R.D. Everett, *The herpes simplex virus type 1 (HSV-1) regulatory protein ICP0 interacts with and Ubiquitinates p53.* J Biol Chem, 2003. **278**(38): p. 36596-602.
78. Canning, M., et al., *A RING finger ubiquitin ligase is protected from autocatalyzed ubiquitination and degradation by binding to ubiquitin-specific protease USP7.* J Biol Chem, 2004. **279**(37): p. 38160-8.
79. Boutell, C., et al., *Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7.* J Virol, 2005. **79**(19): p. 12342-54.
80. Lindner, H.A., et al., *The papain-like protease from the severe acute respiratory syndrome coronavirus is a deubiquitinating enzyme.* J Virol, 2005. **79**(24): p. 15199-208.
81. Balakirev, M.Y., et al., *Deubiquitinating function of adenovirus proteinase.* J Virol, 2002. **76**(12): p. 6323-31.
82. Kattenhorn, L.M., et al., *A deubiquitinating enzyme encoded by HSV-1 belongs to a family of cysteine proteases that is conserved across the family Herpesviridae.* Mol Cell, 2005. **19**(4): p. 547-57.
83. Wang, J., et al., *High-molecular-weight protein (pUL48) of human cytomegalovirus is a competent deubiquitinating protease: mutant viruses altered in its active-site cysteine or histidine are viable.* J Virol, 2006. **80**(12): p. 6003-12.
84. Young, L.S. and A.B. Rickinson, *Epstein-Barr virus: 40 years on.* Nat Rev Cancer, 2004. **4**(10): p. 757-68.
85. Babcock, G.J., E.M. Miyashita-Lin, and D.A. Thorley-Lawson, *Detection of EBV infection at the single-cell level. Precise quantitation of virus-infected cells in vivo.* Methods Mol Biol, 2001. **174**: p. 103-10.
86. Thorley-Lawson, D.A., *Epstein-Barr virus: exploiting the immune system.* Nat Rev Immunol, 2001. **1**(1): p. 75-82.
87. Miyashita, E.M., et al., *Identification of the site of Epstein-Barr virus persistence in vivo as a resting B cell.* J Virol, 1997. **71**(7): p. 4882-91.
88. Chen, F., et al., *A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1.* J Virol, 1995. **69**(6): p. 3752-8.
89. Babcock, G.J. and D.A. Thorley-Lawson, *Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-*

associated tumors. Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12250-5.
90. Babcock, G.J., D. Hochberg, and A.D. Thorley-Lawson, *The expression pattern of Epstein-Barr virus latent genes in vivo is dependent upon the differentiation stage of the infected B cell.* Immunity, 2000. **13**(4): p. 497-506.
91. Tsurumi, T., M. Fujita, and A. Kudoh, *Latent and lytic Epstein-Barr virus replication strategies.* Rev Med Virol, 2005. **15**(1): p. 3-15.
92. Levitskaya, J., et al., *Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1.* Proc Natl Acad Sci U S A, 1997. **94**(23): p. 12616-21.
93. Heessen, S., et al., *Inhibition of ubiquitin/proteasome-dependent proteolysis in Saccharomyces cerevisiae by a Gly-Ala repeat.* FEBS Lett, 2003. **555**(2): p. 397-404.
94. Paludan, C., et al., *Endogenous MHC class II processing of a viral nuclear antigen after autophagy.* Science, 2005. **307**(5709): p. 593-6.
95. Knight, J.S., N. Sharma, and E.S. Robertson, *Epstein-Barr virus latent antigen 3C can mediate the degradation of the retinoblastoma protein through an SCF cellular ubiquitin ligase.* Proc Natl Acad Sci U S A, 2005. **102**(51): p. 18562-6.
96. Knight, J.S., N. Sharma, and E.S. Robertson, *SCFSkp2 complex targeted by Epstein-Barr virus essential nuclear antigen.* Mol Cell Biol, 2005. **25**(5): p. 1749-63.
97. Rothenberger, S., et al., *Association of the Epstein-Barr virus latent membrane protein 1 with lipid rafts is mediated through its N-terminal region.* Cell Mol Life Sci, 2002. **59**(1): p. 171-80.
98. Masucci, M.G., *Epstein-Barr virus oncogenesis and the ubiquitin-proteasome system.* Oncogene, 2004. **23**(11): p. 2107-15.
99. Tang, W., et al., *Interaction of Epstein-Barr virus latent membrane protein 1 with SCFHOS/beta-TrCP E3 ubiquitin ligase regulates extent of NF-kappaB activation.* J Biol Chem, 2003. **278**(49): p. 48942-9.
100. Frisan, T., et al., *Defective presentation of MHC class I-restricted cytotoxic T-cell epitopes in Burkitt's lymphoma cells.* Int J Cancer, 1996. **68**(2): p. 251-8.
101. Rowe, M., et al., *Restoration of endogenous antigen processing in Burkitt's lymphoma cells by Epstein-Barr virus latent membrane protein-1: coordinate up-regulation of peptide transporters and HLA-class I antigen expression.* Eur J Immunol, 1995. **25**(5): p. 1374-84.
102. Frisan, T., et al., *Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines.* J Immunol, 1998. **160**(7): p. 3281-9.
103. Dolcetti, R. and J. Menezes, *Epstein-Barr virus and undifferentiated nasopharyngeal carcinoma: new immunobiological and molecular insights on a long-standing etiopathogenic association.* Adv Cancer Res, 2003. **87**: p. 127-57.
104. Young, L.S. and P.G. Murray, *Epstein-Barr virus and oncogenesis: from latent genes to tumours.* Oncogene, 2003. **22**(33): p. 5108-21.

105. Ikeda, M., et al., *The Epstein-Barr virus latent membrane protein 2A PY motif recruits WW domain-containing ubiquitin-protein ligases.* Virology, 2000. **268**(1): p. 178-91.
106. Winberg, G., et al., *Latent membrane protein 2A of Epstein-Barr virus binds WW domain E3 protein-ubiquitin ligases that ubiquitinate B-cell tyrosine kinases.* Mol Cell Biol, 2000. **20**(22): p. 8526-35.
107. Engels, N., et al., *Epstein-Barr virus latent membrane protein 2A (LMP2A) employs the SLP-65 signaling module.* J Exp Med, 2001. **194**(3): p. 255-64.
108. Sohn, H.W., H. Gu, and S.K. Pierce, *Cbl-b negatively regulates B cell antigen receptor signaling in mature B cells through ubiquitination of the tyrosine kinase Syk.* J Exp Med, 2003. **197**(11): p. 1511-24.
109. Ingham, R.J., et al., *The Epstein-Barr virus protein, latent membrane protein 2A, co-opts tyrosine kinases used by the T cell receptor.* J Biol Chem, 2005. **280**(40): p. 34133-42.
110. Ingham, R.J., et al., *WW domains provide a platform for the assembly of multiprotein networks.* Mol Cell Biol, 2005. **25**(16): p. 7092-106.
111. Dolcetti, R. and M.G. Masucci, *Epstein-Barr virus: induction and control of cell transformation.* J Cell Physiol, 2003. **196**(2): p. 207-18.
112. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus Particles in Cultured Lymphoblasts from Burkitt's Lymphoma.* Lancet, 1964. **1**: p. 702-3.
113. Gregory, C.D., M. Rowe, and A.B. Rickinson, *Different Epstein-Barr virus-B cell interactions in phenotypically distinct clones of a Burkitt's lymphoma cell line.* J Gen Virol, 1990. **71 (Pt 7)**: p. 1481-95.
114. Nilsson, K., et al., *The establishment of lymphoblastoid lines from adult and fetal human lymphoid tissue and its dependence on EBV.* Int J Cancer, 1971. **8**(3): p. 443-50.
115. Rowe, M., et al., *Differences in B cell growth phenotype reflect novel patterns of Epstein-Barr virus latent gene expression in Burkitt's lymphoma cells.* Embo J, 1987. **6**(9): p. 2743-51.
116. Scheffner, M. and N.J. Whitaker, *Human papillomavirus-induced carcinogenesis and the ubiquitin-proteasome system.* Semin Cancer Biol, 2003. **13**(1): p. 59-67.
117. Narisawa-Saito, M. and T. Kiyono, *Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: roles of E6 and E7 proteins.* Cancer Sci, 2007. **98**(10): p. 1505-11.
118. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. **63**(6): p. 1129-36.
119. Kuhne, C. and L. Banks, *E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box.* J Biol Chem, 1998. **273**(51): p. 34302-9.
120. Thomas, M. and L. Banks, *Inhibition of Bak-induced apoptosis by HPV-18 E6.* Oncogene, 1998. **17**(23): p. 2943-54.
121. Jackson, S., et al., *Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins.* Genes Dev, 2000. **14**(23): p. 3065-73.

122. Gross-Mesilaty, S., et al., *Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 8058-63.
123. Kumar, S., A.L. Talis, and P.M. Howley, *Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination.* J Biol Chem, 1999. **274**(26): p. 18785-92.
124. Oda, H., S. Kumar, and P.M. Howley, *Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination.* Proc Natl Acad Sci U S A, 1999. **96**(17): p. 9557-62.
125. Kiyono, T., et al., *Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells.* Nature, 1998. **396**(6706): p. 84-8.
126. Spitkovsky, D., et al., *p53-independent growth regulation of cervical cancer cells by the papillomavirus E6 oncogene.* Oncogene, 1996. **13**(5): p. 1027-35.
127. Kao, W.H., et al., *Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase.* J Virol, 2000. **74**(14): p. 6408-17.
128. Gao, Q., et al., *Human papillomavirus E6-induced degradation of E6TP1 is mediated by E6AP ubiquitin ligase.* Cancer Res, 2002. **62**(11): p. 3315-21.
129. Gao, Q., et al., *Human papillomavirus type 16 E6-induced degradation of E6TP1 correlates with its ability to immortalize human mammary epithelial cells.* J Virol, 2001. **75**(9): p. 4459-66.
130. Vossler, M.R., et al., *cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway.* Cell, 1997. **89**(1): p. 73-82.
131. Altschuler, D.L. and F. Ribeiro-Neto, *Mitogenic and oncogenic properties of the small G protein Rap1b.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7475-9.
132. Gardiol, D., et al., *Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression.* Int J Cancer, 2006. **119**(6): p. 1285-90.
133. Massimi, P., et al., *HPV E6 specifically targets different cellular pools of its PDZ domain-containing tumour suppressor substrates for proteasome-mediated degradation.* Oncogene, 2004. **23**(49): p. 8033-9.
134. Thomas, M., et al., *The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins.* Oncogene, 2005. **24**(41): p. 6222-30.
135. Lee, C. and L.A. Laimins, *Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31.* J Virol, 2004. **78**(22): p. 12366-77.
136. Matsumoto, Y., et al., *Involvement of a cellular ubiquitin-protein ligase E6AP in the ubiquitin-mediated degradation of extensive substrates of high-risk human papillomavirus E6.* J Med Virol, 2006. **78**(4): p. 501-7.
137. Nakagawa, S. and J.M. Huibregtse, *Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase.* Mol Cell Biol, 2000. **20**(21): p. 8244-53.

138. Gewin, L., et al., *Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex.* Genes Dev, 2004. **18**(18): p. 2269-82.
139. Dyson, N., et al., *Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins.* J Virol, 1992. **66**(12): p. 6893-902.
140. Dyson, N., et al., *The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product.* Science, 1989. **243**(4893): p. 934-7.
141. Giacinti, C. and A. Giordano, *RB and cell cycle progression.* Oncogene, 2006. **25**(38): p. 5220-7.
142. Berezutskaya, E. and S. Bagchi, *The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome.* J Biol Chem, 1997. **272**(48): p. 30135-40.
143. Huibregtse, J.M. and S.L. Beaudenon, *Mechanism of HPV E6 proteins in cellular transformation.* Semin Cancer Biol, 1996. **7**(6): p. 317-26.
144. Munger, K., et al., *Mechanisms of human papillomavirus-induced oncogenesis.* J Virol, 2004. **78**(21): p. 11451-60.
145. Schiffman, M., et al., *Human papillomavirus and cervical cancer.* Lancet, 2007. **370**(9590): p. 890-907.
146. Gavioli, R., et al., *c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells.* Nat Cell Biol, 2001. **3**(3): p. 283-8.
147. Borodovsky, A., et al., *A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14.* Embo J, 2001. **20**(18): p. 5187-96.
148. Hetfeld, B.K., et al., *The zinc finger of the CSN-associated deubiquitinating enzyme USP15 is essential to rescue the E3 ligase Rbx1.* Curr Biol, 2005. **15**(13): p. 1217-21.
149. Schweitzer, K., et al., *CSN controls NF-kappaB by deubiquitinylation of IkappaBalpha.* Embo J, 2007. **26**(6): p. 1532-41.
150. Li, M., et al., *Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization.* Nature, 2002. **416**(6881): p. 648-53.
151. Cummins, J.M., et al., *Tumour suppression: disruption of HAUSP gene stabilizes p53.* Nature, 2004. **428**(6982): p. 1 p following 486.
152. Meulmeester, E., et al., *Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2.* Mol Cell, 2005. **18**(5): p. 565-76.
153. Hu, M., et al., *Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53-MDM2 pathway.* PLoS Biol, 2006. **4**(2): p. e27.
154. Holowaty, M.N., et al., *Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7.* J Biol Chem, 2003. **278**(32): p. 29987-94.
155. van der Knaap, J.A., et al., *GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7.* Mol Cell, 2005. **17**(5): p. 695-707.

156. van der Horst, A., et al., *FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP.* Nat Cell Biol, 2006. **8**(10): p. 1064-73.
157. Oh, Y.M., S.J. Yoo, and J.H. Seol, *Deubiquitination of Chfr, a checkpoint protein, by USP7/HAUSP regulates its stability and activity.* Biochem Biophys Res Commun, 2007. **357**(3): p. 615-9.
158. Shackelford, J., C. Maier, and J.S. Pagano, *Epstein-Barr virus activates beta-catenin in type III latently infected B lymphocyte lines: association with deubiquitinating enzymes.* Proc Natl Acad Sci U S A, 2003. **100**(26): p. 15572-6.
159. Pantaleon, M., et al., *FAM deubiquitylating enzyme is essential for preimplantation mouse embryo development.* Mech Dev, 2001. **109**(2): p. 151-60.
160. Friocourt, G., et al., *Doublecortin interacts with the ubiquitin protease DFFRX, which associates with microtubules in neuronal processes.* Mol Cell Neurosci, 2005. **28**(1): p. 153-64.
161. Mouchantaf, R., et al., *The ubiquitin ligase itch is auto-ubiquitylated in vivo and in vitro but is protected from degradation by interacting with the deubiquitylating enzyme FAM/USP9X.* J Biol Chem, 2006. **281**(50): p. 38738-47.
162. Honore, B., et al., *Neuronal protein gene product 9.5 (IEF SSP 6104) is expressed in cultured human MRC-5 fibroblasts of normal origin and is strongly down-regulated in their SV40 transformed counterparts.* FEBS Lett, 1991. **280**(2): p. 235-40.
163. Liu, Y., et al., *Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line.* Chem Biol, 2003. **10**(9): p. 837-46.
164. Maki, A., et al., *Role of ubiquitin carboxyl terminal hydrolase in the differentiation of human acute lymphoblastic leukemia cell line, Reh.* Differentiation, 1996. **60**(1): p. 59-66.
165. Chen, Q., et al., *Gene expression in the LNCaP human prostate cancer progression model: progression associated expression in vitro corresponds to expression changes associated with prostate cancer progression in vivo.* Cancer Lett, 2006. **244**(2): p. 274-88.
166. Tezel, E., et al., *PGP9.5 as a prognostic factor in pancreatic cancer.* Clin Cancer Res, 2000. **6**(12): p. 4764-7.
167. Yamazaki, T., et al., *PGP9.5 as a marker for invasive colorectal cancer.* Clin Cancer Res, 2002. **8**(1): p. 192-5.
168. Yanagisawa, T.Y., et al., *Detection of the PGP9.5 and tyrosine hydroxylase mRNAs for minimal residual neuroblastoma cells in bone marrow and peripheral blood.* Tohoku J Exp Med, 1998. **184**(3): p. 229-40.
169. Miyoshi, Y., et al., *High expression of ubiquitin carboxy-terminal hydrolase-L1 and -L3 mRNA predicts early recurrence in patients with invasive breast cancer.* Cancer Sci, 2006. **97**(6): p. 523-9.
170. Nam, M.J., et al., *Molecular profiling of the immune response in colon cancer using protein microarrays: occurrence of autoantibodies to ubiquitin C-terminal hydrolase L3.* Proteomics, 2003. **3**(11): p. 2108-15.
171. Kwon, J., et al., *The region-specific functions of two ubiquitin C-terminal hydrolase isozymes along the epididymis.* Exp Anim, 2006. **55**(1): p. 35-43.

172. Yi, Y.J., et al., *Ubiquitin C-Terminal Hydrolase-Activity Is Involved in Sperm Acrosomal Function and Anti-Polyspermy Defense During Porcine Fertilization.* Biol Reprod, 2007.
173. Hemelaar, J., et al., *Specific and covalent targeting of conjugating and deconjugating enzymes of ubiquitin-like proteins.* Mol Cell Biol, 2004. **24**(1): p. 84-95.
174. Frickel, E.M., et al., *Apicomplexan UCHL3 retains dual specificity for ubiquitin and Nedd8 throughout evolution.* Cell Microbiol, 2007. **9**(6): p. 1601-10.
175. Sano, Y., et al., *Photoreceptor cell apoptosis in the retinal degeneration of Uchl3-deficient mice.* Am J Pathol, 2006. **169**(1): p. 132-41.
176. Wood, M.A., et al., *Ubiquitin C-terminal hydrolase L3 (Uchl3) is involved in working memory.* Hippocampus, 2005. **15**(5): p. 610-21.
177. Wicks, S.J., et al., *The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling.* Oncogene, 2005. **24**(54): p. 8080-4.
178. al-Katib, A.M., et al., *Induced expression of a ubiquitin COOH-terminal hydrolase in acute lymphoblastic leukemia.* Cell Growth Differ, 1995. **6**(2): p. 211-7.
179. Billaud, M., et al., *Low expression of lymphocyte function-associated antigen (LFA)-1 and LFA-3 adhesion molecules is a common trait in Burkitt's lymphoma associated with and not associated with Epstein-Barr virus.* Blood, 1990. **75**(9): p. 1827-33.
180. Gregory, C.D., et al., *Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumor cell escape from virus-specific T cell surveillance.* J Exp Med, 1988. **167**(6): p. 1811-24.
181. Hogg, N., et al., *Mechanisms contributing to the activity of integrins on leukocytes.* Immunol Rev, 2002. **186**: p. 164-71.
182. Rose, D.M., R. Alon, and M.H. Ginsberg, *Integrin modulation and signaling in leukocyte adhesion and migration.* Immunol Rev, 2007. **218**: p. 126-34.
183. Katagiri, K., et al., *Rap1 is a potent activation signal for leukocyte function-associated antigen 1 distinct from protein kinase C and phosphatidylinositol-3-OH kinase.* Mol Cell Biol, 2000. **20**(6): p. 1956-69.
184. Mor, A., M.L. Dustin, and M.R. Philips, *Small GTPases and LFA-1 reciprocally modulate adhesion and signaling.* Immunol Rev, 2007. **218**: p. 114-25.
185. Kinashi, T., *Intracellular signalling controlling integrin activation in lymphocytes.* Nat Rev Immunol, 2005. **5**(7): p. 546-59.
186. de Bruyn, K.M., et al., *The small GTPase Rap1 is required for Mn(2+)- and antibody-induced LFA-1- and VLA-4-mediated cell adhesion.* J Biol Chem, 2002. **277**(33): p. 29468-76.
187. Tohyama, Y., et al., *The critical cytoplasmic regions of the alphaL/beta2 integrin in Rap1-induced adhesion and migration.* Mol Biol Cell, 2003. **14**(6): p. 2570-82.
188. Larsen, C.N., B.A. Krantz, and K.D. Wilkinson, *Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases.* Biochemistry, 1998. **37**(10): p. 3358-68.

189. Lwin, T., et al., *Cell adhesion induces p27Kip1-associated cell-cycle arrest through down-regulation of the SCFSkp2 ubiquitin ligase pathway in mantle-cell and other non-Hodgkin B-cell lymphomas.* Blood, 2007. **110**(5): p. 1631-8.
190. Nassar, N., et al., *The 2.2 A crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue.* Nature, 1995. **375**(6532): p. 554-60.
191. Hu, C.D., et al., *Coassociation of Rap1A and Ha-Ras with Raf-1 N-terminal region interferes with ras-dependent activation of Raf-1.* J Biol Chem, 1997. **272**(18): p. 11702-5.
192. Frech, M., et al., *Inhibition of GTPase activating protein stimulation of Ras-p21 GTPase by the Krev-1 gene product.* Science, 1990. **249**(4965): p. 169-71.
193. de Nooij, J.C., M.A. Letendre, and I.K. Hariharan, *A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during Drosophila embryogenesis.* Cell, 1996. **87**(7): p. 1237-47.
194. Tomoda, K., et al., *The cytoplasmic shuttling and subsequent degradation of p27Kip1 mediated by Jab1/CSN5 and the COP9 signalosome complex.* J Biol Chem, 2002. **277**(3): p. 2302-10.
195. Otsuki, T., et al., *Expression of protein gene product 9.5 (PGP9.5)/ubiquitin-C-terminal hydrolase 1 (UCHL-1) in human myeloma cells.* Br J Haematol, 2004. **127**(3): p. 292-8.