From the Department of Oncology-Pathology, Cancer Centrum Karolinska
Karolinska Institutet, Stockholm, Sweden

EPSTEIN-BARR VIRUS ENCODED DECONJUGASES

AND

AMOTL2 IN CONTROL OF CELL TOPOLOGY

Sebastian Hildebrand

Stockholm 2013

Cover: MDCK cysts, stained with actin (red), ezrin (green), nuclei (blue)

Published by Karolinska Institutet.

ISBN 978-91-7549-446-3

Printed by: US-AB Stockholm
Drottning Kristinas väg 53B
SE 10044 Stockholm

"The Way is the Goal." *Mahatma Gandhi*

ABSTRACT - *EPSTEIN-BARR VIRUS ENCODED DECONJUGASES*

The post-translational conjugation or deconjugation of proteins by ubiquitin (Ub) or ubiquitin-like molecules (UbLs: e.g. SUMO, NEDD8, ISG15) has emerged as a major regulatory mechanism of various cellular activities. Viruses have developed mechanisms to exploit those pathways for their own benefit. RNA and DNA viruses express their own E3 ligases or manipulate cellular E3 ligases. Also several viral encoded DUBs or ULPs have been described, e.g. facilitating the suppression of ubiquitination and ISGylation mediated antiviral effects.

Epstein-Barr virus (EBV) is a large double-stranded DNA tumor virus encoding ~100 open reading frames (ORFs). EBV is associated with a variety of malignancies of lymphoid cells, like Burkitt´s lymphoma and Hodgkin´s lymphoma as also of epithelial cells, like nasopharyngeal carcinoma and gastric carcinoma.

The overall aim of this study was to identify and functional characterize EBV encoded deconjugases. We screened an EBV-ORFeome library for their activity against Ub-, NEDD8-, SUMO-1,-2,-3 and ISG15-GFP reporter. As a result we discovered that the BSLF1- and BXLF1-ORF comprised deubiquitinating activity. We could also detect that the large tegumental protein BPLF1-N cleaves the NEDD8-GFP reporter with similar efficiency as the Ub-GFP reporter. Following this observation we could show that BPLF1-N was able to process Ub- and NEDD8-linked functional probes with similar efficiency suggesting equal affinities towards ubiquitinated and neddylated substrates. We could show that BPLF1-N binds to and deneddylates cullins, which are assembled in cullin-RING ligases (CRLs). This CRL deneddylation facilitated the stabilization of their substrates involved in cell cycle regulation. Those accumulated BPLF1-N controlled CRL substrates were essential for an S-phase like cellular environment and endoreduplication in BPLF1-N expressing cells. We further demonstrated that the impact of BPLF1-N expression on viral genome replication was dependent on stabilization of the DNA licensing factor CDT1.

ABSTRACT - *AMOTL2 IN CONTROL OF CELL TOPOLOGY*

During developmental morphogenesis, cells migrate, differentiate and organize into multicellular structures. As a distinct step in organ formation, epithelial cells join together via cell-cell junctions to form sheets of cells that separate cellular compartments from each other. Endogenous forces, generated by contractile actin, are transmitted over cell-layers in part by the connection to adhesion junctions and E-cadherin. These forces affect cellular geometry (cell size and shape) and topology (connectivity among cells in a tissue). Exactly how E-cadherin connects to the actomyosin network has been less clear.

The Angiomotin protein family of scaffold proteins comprises three members Amot, AmotL1 and AmotL2, were each is expressed as two isoforms in mammalians. These proteins act as scaffolds in that they contain interaction sites enabling the formation of protein complexes.

In our work, we show that AmotL2 p100 binds to the adherens junction components E-cadherin and MAGI1 and associates to contractile actin fibers which connect cells over multiple layers. Silencing of AmotL2 in epithelial cells *in vitro* and in zebrafish keratinocytes *in vivo* resulted in loss of actin filaments perpendicular to cellular junctions and dramatic changes in cellular geometry. As a consequence, the packing of epithelial cells in the typical hexagonal patterns was severely perturbed and the ability to form 3-D structures was lost. Cells depleted of AmotL2 also showed increased fluidity and elasticity when subjected to mechanical force. We propose that AmotL2 is a critical component in the adhesion junctions that controls intracellular contractility as well as relaying forces between cells.

LIST OF PUBLICATIONS

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

I Sompallae R, Gastaldello S, **Hildebrand S**, Zinin N, Hassink G, Lindsten K, Haas J, Persson B, Masucci MG. Epstein-barr virus encodes three bona fide ubiquitin-specific proteases. Journal of Virology 2008 Nov;82(21):10477-86.

II Gastaldello S, **Hildebrand S**, Faridani O, Callegari S, Palmkvist M, Di Guglielmo C, Masucci MG. A deneddylase encoded by Epstein-Barr virus promotes viral DNA replication by regulating the activity of cullin-RING ligases. Nature Cell Biology 2010 Apr;12(4):351-61.

III **Hildebrand S**, Hultin S, Cao X, Majumdar A, Johansson S, Zheng Y, Holmgren L. AmotL2 links E-Cadherin to contractile actin filaments and controls cell topology. Manuscript.

ABBREVIATIONS

AJ	Adherens junctions
Amot	angiomotin
AmotL1	Angiomotin like 1
AmotL2	Angiomotin like 2
CDT	Chromatin licensing and DNA replication factor
CRL	Cullin-ring ligase
Cul	Cullin
DUB	Deubiquitinating enzyme
E1	Ubiquitin activating enzyme
E2	Ubiquitin conjugating enzyme
E3	Ubiquitin ligase
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EPLIN	Epithelial protein lost in neoplasm
HCMV	Human cytomegalovirus
HHV	Human herpes virus
HMM	Hidden Markov Model
HPV	Human papilloma virus
HSV	Herpes simplex virus
ISG15	Interferon stimulated gene 15
JAMM	Jab1/MPN domain metalloenzyme
KSHV	Kaposis sarcoma herpes virus
LMP	Latent membrane protein
MAGI-1	membrane-associated guanylate kinase with inverted domain structure-1
MCMV	Murine cytomegalovirus
MJD	Machado-Joseph disease proteases
NEDD8	Neuronal-precursor cell-expressed developmentally down-regulated protein 8
ORF	Open reading frame
RING	Really interesting new gene
SCF	Skp, Cullin, F-box containing complex
SUMO	Small Ubiquitin-like Modifier
TJ	Tight junctions
Ub	Ubiquitin
UbL	Ubiquitin like modifier
Ub-AMC	Ubiquitin aminomethylcoumarin
Ub-VME	Ubiquitin vinyl methyl ester
Ub-VS	Ubiquitin vinylsulfon
UCH	Ubiquitin carboxy-terminal hydrolase
ULP	Ubiquitin like specific protease
UPS	Ubiquitin poteasome system
USP	Ubiquitin specific protease

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1.1 INTRODUCTION - *EPSTEIN-BARR VIRUS ENCODED DECONJUGASES*

1.1.1 The ubiquitin and ubiquitin-like modification system

The post-translational modification of proteins by Ub (ubiquitin) or UbLs (ubiquitin-like molecules: e.g. SUMO, NEDD8, ISG15) has emerged as a major regulatory mechanism of various cellular activities. Those controlled processes include protein transcription, translation, trafficking and degradation, signal transduction, replication and apoptosis (1, 2, 3, 4, 5). So far 13 UbLs are described in human. All of them have a size between 8-18 kDa and they all show a high secondary structure homology to ubiquitin (2).

Conjugation of Ub and UbLs to their targets requires the sequential action of three enzymes: a modifier-activating enzyme (E1), one of several modifier conjugating enzymes (E2s), and one member of a large and diverse group of modifier-target ligases (E3s) that mainly determines target specificity (1). This enzyme cascade mediates the conjugation of the C-terminal glycine of Ub or UbL to the epsilon-NH2 group of a lysine residue (K) of the target protein. Ub and UbLs are produced as precursor proteins (Ub also as a polypeptide), and carboxy-terminal processing by specific proteases is required to generate an active modifier. Those specific deconjugating enzymes, called DUBs (deubiquitinating enzymes) and ULPs (Ubl-specific proteases), also cleave the modifier from its target protein by hydrolyzing the covalent bondage between substrate and modifier. The purpose of this reaction is to establish a sufficient pool of free Ub/Ubls and to determine certain signaling or functional alteration caused by the modification (6).

An overview of the ubiquitin and ubiquitin-like modification system with examples for the non-proteolytic and proteolytic outcomes are visualized in figure 1.

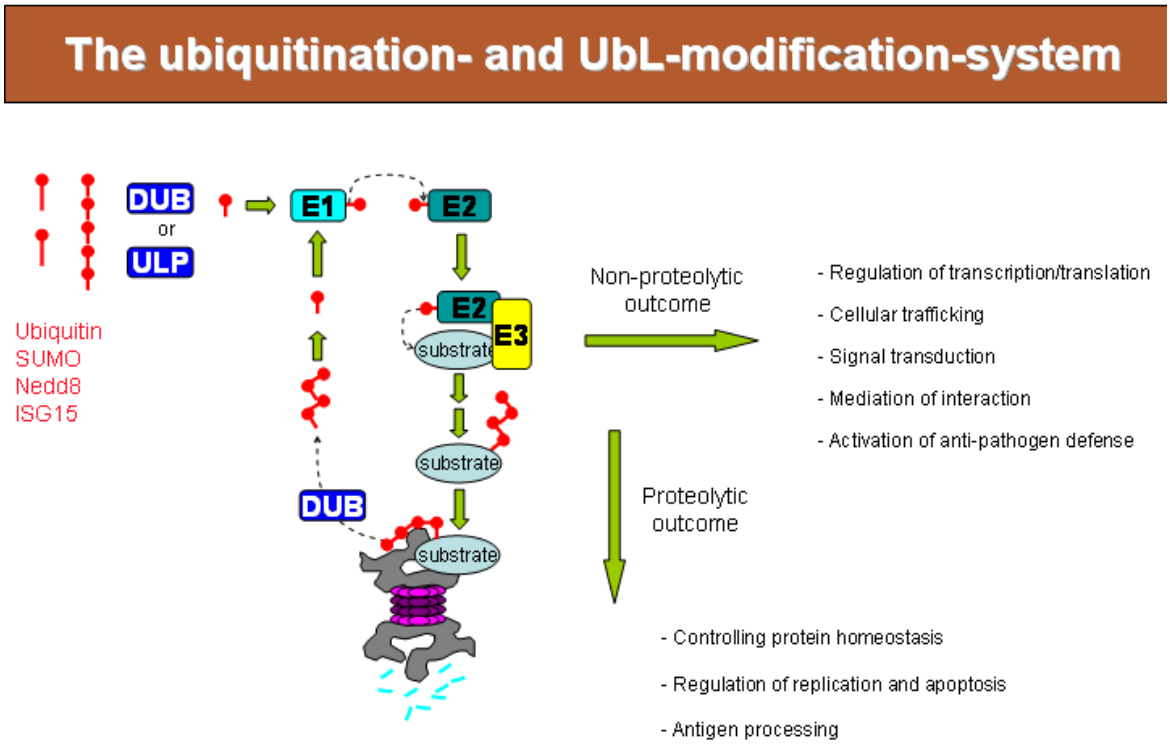

Figure 1. Ub polypeptides or UbL pro-peptides need to be processed from DUBs or ULPs to make their terminal Glycin accessible for conjugation. Via an enzymatic cascade of activating enzyme (E1), conjugating enzyme (E2) and substrate ligase (E3) the modifier gets covalently linked to its substrate. This modification can either have a non-proteolytic or a proteolytic outcome. After signaling the modifier gets deconjugated from its substrate by DUBs or ULPs for possible recycling into the system.

1.1.2 Ubiquitin modification

Ubiquitin, a small globular protein composed of 76 amino acids, is highly conserved during evolution. The first discovered function of ubiquitin is its involvement in the UPS (ubiquitin-proteasome system). This pathway is a major intracellular system for protein degradation. The connection of at least four K48 linked Ub molecules to a protein serves as localization signal to the proteasome where the labeled protein gets degraded (1). This pathway plays a crucial role in a wide variety of cellular function, including degradation of damaged or unneeded proteins, cellular trafficking, antigen processing, cell cycle regulation and apoptosis (7).

Apart from the described K48 Ub chain mediated protein degradation several other Ub mediated function have been described. Ubiquitin contains seven lysine residues K6, K11, K27, K29, K33, K48, and K63. Formation of the ubiquitin chain can occur by linking the

ubiquitin polymer to any of these lysine residues in homogeneous or mixed form. The length and the linkage construction of the created Ub chain determine the faith of the modified protein. Apart from modification via K48 Ub chains, monoubiquitination and K63 Ub chain conjugation are the best characterised Ub modifications. Many membrane receptors are monoubiquitinated, which serves in most cases as endocytosis signal and in many circumstances as signal for lysosomal degradation. Also histones are monoubiquitinated, which serves as signal for histone-mediated transcriptional regulation. Monoubiquitination has also been described to alter sub-cellular location or determine cellular interaction of the modified substrate (8, 9).

For instance PCNA (proliferating cell nuclear antigen) is a homotrimeric replication sliding clamp that encircles DNA and provides a platform to recruit other proteins involved in DNA replication and repair. Monoubiquitinated PCNA recruits TLS (translation synthesis) polymerases, which display low processivity and reduced fidelity allowing DNA lesion bypass, to replace the high-fidelity polymerases. The monoubiquitination of PCNA can be further extended to K63-linked polyubiquitin chains. This polyubiquitination activates an alternative lesion bypass pathway called template switch, which recruits the undamaged strand of the DNA duplex as a template to direct DNA synthesis (10).

Further described protein-protein interactions mediated via K63-linked polyubiquitination are important for kinase signalling activation (e.g. leading to NF-κB activation), receptor endocytosis and protein trafficking (11). The best characterized UbL- modifier in human are SUMO, NEDD8 and ISG15.

1.1.3 SUMO modification

The mammalian SUMO (Small ubiquitin-like modifier) family is comprised of three members with described physiological function. SUMOs have a size of about 95 amino acids and constitute a highly conserved protein family found in all eukaryotes. SUMO-1 share ~50% sequence identity with SUMO-2 and -3. SUMO-2 and SUMO-3 share ~95% sequence identity. SUMO-2 and -3 are able to form monomeric and heteromeric poly-SUMO chains like Ubiquitin. SUMO-1 in contrast, is not able to form poly-SUMO chains but may bind covalently to SUMO-2 and -3 conjugated chains and terminate them. Most SUMO-modified proteins, which have been characterized in human, are involved in transcription. The transcriptional effects of SUMO can be divided into two groups, those that involve PML

nuclear bodies and those that involve sumoylated transcription factors bound to DNA promoters (12).

PML nuclear bodies are matrix-associated domains that recruit an ever-growing variety of proteins. The PML-protein is the key organizer of these domains, which up on sumoylation facilitates the formation of PML nuclear bodies, recruiting among others transcription factors, which get activated via this association. Sumoylation occurring direct on the transcription factor facilitates in most cases transcriptional repression. This occurs either by altering the stability of a DNA bound transcription factor or by the recruitment of a transcriptional repressor. In general most sumoylated substrates are localized in the nucleus and many of those substrates require their NLS (nuclear localization sequence) for sumoylation (12).

The most abundant SUMO-1 conjugate in vertebrate cells is RanGAP1. This protein is the GTPase activating protein for the small GTPase Ran, which plays a central role in nuclear transport. SUMO-1 modification of RanGAP1 facilitates binding of the modified protein to the nuclear pore complex. This tightly bound RanGAP1 was shown to be crucial in nuclear import assays *in vitro* and soluble RanGAP1 could not substitute for it. One way SUMO affects the function of its substrates is sumoylation on otherwise ubiquitination specific lysine residues. PCNA or IκBα are examples for this phenomena. In the case of PCNA sumoylation and ubiquitination compete for the same site whereas sumoylation was shown to alter PCNAs function by inhibiting ubiquitination dependent post-replication DNA repair. Also the sumoylation of ubiquitination sites of particular high molecular weight proteins after various cellular stress conditions with SUMO-2 and-3 chains was reported. This sumoylation pattern was reversed after normalization of the cellular stress stimuli, suggesting a role of sumoylation to inhibit those sites for ubiquitination mediated proteasomal degradation (12).

1.1.4 NEDD8 modification

The UbL-modifier NEDD8 (Neuronal-precursor cell-expressed developmentally down-regulated protein 8) was originally discovered as a down regulated protein in neural precursor cells during the development of murine brain. The 76 amino acid big modifier is very similar to Ubiquitin in size, structure and charge distribution. Both modifiers share an almost 60% sequence identity. The most prominent neddylated substrates are the cullin family, which comprise in human six members (Cul-1,-2,-3,-4a,-4b,-5). Cullins serve as scaffold/bridge proteins in multi subunit ubiquitin ligase complexes also called CRLs (cullin-RING ligases).

The C-terminus of a cullin integrated in such a complex serves as binding site for Roc1 orRoc2, which are E2 binding adaptors. Roc1 or Roc2 attract and bind an E2 conjugating enzyme, which brings ubiquitin to the complex required for CRL-substrate ubiquitination. The N- terminus of a CRL integrated cullin serves in most cases as binding site for a variable adaptor protein. On this adaptor protein (or direct to the cullin N-terminus) binds a substrate-targeting module, which determines the pool of substrates connecting to the specific CRLs. Via the different composition of mainly those substrate recognition modules, ~400 different CRLs have been described in human. It is believed, that ~20% of all proteins targeted for proteasomal degradation are polyubiquitinated by CRLs. The C-terminal neddylation of cullins assembled in CRLs was shown to enable a sufficient polyubiquitination of the CRL substrate protein (13, 14).

Also other neddylated substrates have been described. The E3 ligase MDM2, which ubiquitinates p53, may also neddylate p53, which inhibits its transcriptional activity (15).

The VHL (von Hippel–Lindau) tumour suppressor protein is the cause of the familial VHL disease (cancer syndrome) and most sporadic RCC (renal clear-cell) carcinomas. Needylation of VHL has been reported to be important for VHL binding to fibronectin and to promote its matrix assembly. A non-neddylateable VHL mutant failed to bind to and promote the assembly of fibronectin. Deregulated ECM, as well as abnormal cell-matrix interactions are hallmarks of solid tumors. Alterations in the fibronectin component of the ECM have been correlated with cellular transformation, like VHL-associated tumorigenesis (16).

Also BCA3 (breast cancer associated protein 3) was shown to be a NEDD8 substrate and to function as a tumor suppressor upon modification. When NEDD8 is removed from BCA3, oncogenes are no longer suppressed, resulting in resistance to apoptosis and excessive cell proliferation. Neddylation of BCA3 suppresses NFκB-dependent transcription through its ability to bind to p65 and the cyclin D1 promoter (17).

The EGFR (epidermal growth factor receptor) functions as a tyrosine kinase upon activation by its ligands, which triggers DNA synthesis and cell proliferation. It was shown, that EGFR neddylation enhances its subsequent ubiquitination, resulting in its endocytosis and sorting for lysosomal degradation (18).

1.1.5 ISG15 modification

Human ISG15 (IFN-stimulated gene 15) has a size of 157 amino acids and consist of two domains, where each domain is structurally similar to ubiquitin. ISG15, like other ISGs, is an interferon-inducible gene product, which is strongly upregulated upon viral or bacterial infection. It was further described to be upregulated after LPS (Lipopolysaccharide) and double stranded RNA stimuli. The first discovered substrate of this modifier was Spi2a (serin protease inhibitor 2a), which regulates intracellular proteases in antigen-presenting cells. In general ISG15 is considered as a broad-spectrum inhibitor of virus production upon viral infection. Antiviral activity associated with viral or host protein ISGylation has been reported for DNA and RNA viruses, like HCMV (human cytomegalovirus), HSV (herpes simplex virus), SV (Sindbis virus) and HCV (hepatitis C virus). Mass spectrometric analysis has led to the identification of at least 200 putative ISG15 target proteins. Many of them have crucial functions in the type I IFN response, including JAK1 and STAT1. ISGylation of those signalling proteins was shown to increase their activity to enhance the cellular response to interferons (19). This was achieved by e.g. induction of the antiviral effector enzymes PKR and RNase L. Those protein activations trigger a global inhibition of protein synthesis and virus replication. This is achieved through the phosphorylation of eIF-2α (for PKR) and breakdown of RNA (for RNase L). It was also shown that up on activation of PKR and RNase L an upregulation of ISG15 mRNA levels was achieved (20, 21).

Upon viral infection, ISG induction occurs in two intervals: first an IFN-independent induction of a subset of ISGs is induced and second an IFN-dependent induction via the production of IFN-α/β follows. In many viral infections, IFN-independent ISG induction is mediated by IRF-3 phosphorylation, homodimerization, and nuclear translocation. Activated IRF3, in turn, induces the expression of type I IFN genes, whose products trigger strong induction of a subsets of ISGs, including IFN-β which after its release and ligand-binding to its receptor initiates IFN-dependent ISG induction via the IFN receptor and JAK/STAT signalling pathways. ISG15 has been reported in HBV (hepatitis B virus) to prevent virus-mediated degradation of IRF3 (interferon regulatory factor 3), thereby increasing the induction of IFNβ expression (22).

ISG15 can also be secreted from stimulated cells by a yet not described mechanism and is believed to function as a cytokine to modulate the immune response. In this manner ISG15 was shown to stimulate interferon γ secretion by monocytes and macrophages, proliferation of natural killer cells, and chemotactic responses in neutrophils (23).

Some viruses have developed specific strategies to counteract the activity of ISGs. The influenza B virus protein NS1B is able to bind to ISG15 and by doing so inhibits protein ISGylation (24).

ISG15 overexpression in cell culture was shown to hinder efficient budding of Ebola VP40 virus like particles. The Ebola virus matrix protein VP40 is a major viral structural protein and plays a central role in virus assembly and budding at the plasma membrane of infected cells. VP40 needs to be ubiquitinated to facilitate efficient viral budding. It has been reported, that the cellular E3 ligase Nedd4, which ubiquitinates VP40, gets ISGylated, which inhibits its enzymatic activity hindering efficient Ebola VP40 virus like particle budding (25).

1.1.6 Human DUBs and ULPs and examples of their function

All DUBs and ULPs are Cystein proteases, with the exception of the JAMM metalloproteases, which cleave the modifier from the substrates in a Zn^{2+}- and ATP-dependent manner. So far approx. 100 human DUBs have been identified, and based on the sequence similarities, they have been classified into five distinct subfamilies: USPs (Ubiquitin-specific proteases), UCHLs (Ubiquitin carboxyl-terminal hydrolases), OTUs (Otubain proteases), MJDs (Machado-Joseph disease proteases) and JAMMs (Jab1/MPN domain metalloenzymes) (6). The human ULPs are represented by the SENP (sentrin specific peptidase) family (12).

1.1.7 USPs

USPs (Ubiquitin-specific proteases) belong to the largest and most diverse DUB family. Those enzymes have specific substrates and can regulate distinct signaling pathways at various levels. Examples include USP7, which stabilizes p53 and MDM2 (E3 ligase of p53) in cells (26). CYLD is a K63-linked Ub chain specific USP in the NF-κB pathway that inhibits the activation of the IKK kinase complex (27). USP28 stabilizes the transcription factor c-myc (28), while USP8/UBPY affects endosomal trafficking (29). USP14 is associated with the proteasome and is required for ubiquitin recycling by removing K48-linked Ub chains from proteasomal substrates (30).

Beside the main characteristic of USPs, to cleave ubiquitin linkages, members of this family

have also been described to have dual activities towards UbL-modifier. For example USP2, USP5 and USP14 are able to hydrolyze ISG15 conjugates. Those activities were previously elucidated by the ability of those USPs to bind to the suicidal probe ISG15-VS (31). Those chemical probes are widely used to characterize catalytic active cysteine deconjugases. Those probes contain besides Ub or a specific UbL a reactive compound, which binds and inhibits in most cases the catalytic site of the targeted enzyme (32).

Several USPs have been described to comprise a dual specificity to Ub and NEDD8 (33). For instance USP21 has been described to recognize NEDD8 and to be able to deneddylate cellular substrates (34). Several USPs have been implicated in malignant transformation including for example the product of the cylindromatosis or turban tumor syndrome gene CYLD, which was identified as a regulator of the NF-kB pathway (2). Some USPs exert distinct growth regulatory activities by acting as oncoproteins or tumor suppressor proteins (3).

The size of USPs ranges between 330 and 3500 amino acids and their catalytic domain comprising conserved cysteine and histidine boxes has a size between 300 and 850 amino acids. The N- and C-terminal extension domains of USPs determine their substrate specificity and are involved in protein-protein interaction and determination of cellular localization (35). Structural studies have defined the USP domain fold, and 5 crystal structures of mammalian USP domains have been published (36-40). USP domains share a common fold, which is conserved in almost all USPs. This structure was defined from the crystal structure of USP7. This USP domain resembles a three-domain architecture comparable to an open hand formation containing Thumb, Palm and Fingers. The Thumb consists of eight α helices, while the Palm contains eight central β strands and two helices. The fingers are comprised of four β strands in the center and two at the tip. The catalytic triad residues are located between the Thumb (Cys) and Palm subdomains (His/Asp) (37).

1.1.8 UCHLs

The UCHL (Ubiquitin carboxyl-terminal hydrolase) family is comprised of 4 members (UCHL1, UCHL3, UCHL37 and Bab1), which have generally small sizes (20-30 kDa). Their sequences are conserved among species with approximately 40% homology. UCHL1 is highly expressed in neurons and reduced levels have been observed in various neurodegenerative diseases. Mutations in the UCHL1 gene have been reported to be linked to

Parkinson's disease and expression of UCHL1 was shown to rescue synaptic dysfunction in Alzheimer's disease model mice (41, 42). Abnormal overexpression of UCHL1 has been reported to relate to several forms of cancer (43). UCHL3 is expressed in various tissues and cleaves beside Ub also neddylated substrates. Studies using UCHL3 deficient mice have suggested a role in growth and cell survival (44, 45). UCHL37 specifically cleaves K48-linked Ub chains and is associated with the 19S regulatory subunit of the 26S proteasome (46).

1.1.9 OTUs

OTUs (Otubain proteases) are a recently identified DUB family with so far approximately 15 members. They belong to the ovarian tumor superfamily of proteins with more then 100 members. OTU homologues in different species show a high degree of homology. Also their enzymatic cores are highly conserved but their substrate specificities appear to be quite diverse. Otu1 (otubain 1) has been shown *in vitro* to cleave K48-linked Ub chains and to decrease global Ub conjugate level after overexpression *in vivo* (47), (48). Otu2 (otubain 2) was shown to be inactive *in vitro* against ubiquitin peptide/isopeptide-linked substrates (49), but was able to cleave the flurogenic probe Ub-AMC (50).

Other human OTU proteins known to have DUB activity are A20, Cezanne, TRABID, DUBA and VCIP135. A20 is a dual active enzyme comprising DUB and E3 ligase activities, which are required for termination of TLR (Toll-like receptor) signalling resulting in NF-κB activation and inactivation of TNF (tumour necrosis factor) induced cytotoxicity and apoptosis (51, 52). Similar to A20, Cezanne has been shown to inhibit NF-κB through modulation of the ubiquitination state of two of its positive regulators, TRAF6 and RIP-1 (53, 54). Cezanne was shown accumulate in acute lymphoblastic leukemia and Burkitt lymphoma (55). While the precise role of Cezanne in cancer is unknown, its inhibition of NF-κB suggests that Cezanne may act as a tumor suppressor. VCIP135´s DUB function was described to be required for Golgi membrane fusion (56) and DUBA1 was reported to deubiquitinate the E3 ligase TRAF3 resulting in negative regulation of IFN-1 signalling and down regulation of the innate immune response (57).

1.1.10 MJDs

MJDs (Machado-Joseph disease proteases) are conserved cysteine proteases that include four human DUBs (Ataxin-3, ATXN3L, Josephin-1 and Josephin-2). MJDs are conserved throughout eukaryotes and share a common cysteine protease domain (Josephin domain) of approximately 180 amino acids. The first discovered and best characterized MJD is Ataxin-3. Insertion of a polyglutamine repeat in Ataxin-3 was shown to cause the neurodegenerative Machado-Joseph disease giving this DUB class its name. In contrast to ataxin-3, much less is known about the other three human MJDs (58). NMR and crystallization based structural analysis has been published for Ataxin-3 but for no other MJDs (59). ATXN3L, Josephin-1 and -2 have been shown to possess DUB activity but no substrates are described so far (58).

1.1.11 JAMMs

JAMMs (Jab1/MPN domain metalloenzymes) comprise 5 members with described DUB activity. With a big exception to all other DUBs, JAMMs are lacking a catalytic cysteine. Instead, they are metalloproteases, which coordinate a catalytically essential zinc ion within their active sites. The three best known JAMMs are Poh1, Jab1 and Brcc36. Poh1 is a subunit of the 19 S subcomplex which releases K48-linked polyUb chains from substrates targeted to the proteasome for degradation. It has been suggested that Poh1 functions as a proteasomal "proofreading" device that determines the fate of incoming substrates as to whether they will be rescued or degraded (60). Jab1 is a component of the CSN (COP9 signalosome), a multisubunit complex very similar to the 19S proteasome lid complex, which cleaves NEDD8 of the cullins assembled in CRLs (61). Brcc36 was shown to recognize and cleave particular K63-linked ubiquitin chains. Among others Brcc36 is a component of the BRCA1-A complex, which specifically recognizes K63-linked ubiquitinated histones H2A and H2AX at DNA lesions sites, leading to target the BRCA1-BARD1 heterodimer to sites of DNA damage at double-strand breaks (62).

1.1.12 ULPs

Human ULPs (Ubl-specific proteases) comprised the SENP (sentrin specific peptidase) family, which consist of eight members. They are all homologues of the earlier discovered yeast ULP1 homologue. ULP1 and SENPs are classified as a large group of cysteine proteases, which catalyze cleavage of the peptide bond after the C-terminal glycine to activate the modifier as well as deconjugate them from their substrate. ULP1 and SENPs are comprised of a ~200 amino acid long catalytic domain containing the active residue triad Cys, His and Asp. Their N- terminal domain has a variable length and sequence and is responsible for localisation, target interaction and specificity. One member, SENP8, also called NEDP1 or DEN1, is specific for NEDD8 activation and substrate deconjugation. SENPs have been shown to have distinct sub-cellular localizations controlled by their non-conserved N-terminal regions. SENP1 and SENP3 are predominantly localized into the nucleus. SENP2 isoforms are found in the cytoplasm, binding to the nucleoplasmic side of the nuclear pore complex ore localize to PML nuclear bodies. SENP6 is localized in the cytoplasm (12).

1.1.13 Human virus encoded DUBs and their described function

Nairoviruses and Arteriviruses, two unrelated RNA virus families, have been shown to express OTU domain-containing proteases. The L-protein of Nairoviruses, e.g. from the highly pathogenic CCHFV (Crimean-Congo Haemorrhagic Fever Virus) has been shown to process Ub and ISG15 conjugates and pro-ISG15 and pro-NEDD8 *in vitro*. CCHFV was also able to cleave Ub and ISG15 conjugates in cells. ISGylation and ubiquitination mediate antiviral effects. The NF-κB immune pathway is regulated by ubiquitination. CCHFV-L was described to inhibit NF-κB activation by the inhibition of endogenous p65 nuclear translocation upon TNFα stimulation (63).

The SARS corona virus encoded PLpro protein was described to show deubiqitinase and isgylase activity *in vitro*. It is an enzyme with dual function first described to carry out N-terminal processing of the viral replicase polyprotein and then later also to cleave K48-linked polyubiquitin chains and ISG15 pro-peptides *in vitro*. Crystallization of PLpro revealed that it belongs to the USP family but its function as a DUB in the course of infection has not been demonstrated (64). Also the human corona virus NL63 was reported to encode the PLP2 protein, an enzyme with dual function. PLP2 was also first described to be involved in viral

replicase polyprotein processing and later on found to posses DUB activity. PLP2 was able to cleave K48-linked Ub chains and bind to the Ub-VS suicidal probe *in vitro* (65).

Also the adenovirus encoded protease adenain has been described as an enzyme with dual function. Adenain is a nuclear and cytosolic protein. It was first described to be involved in virion maturation by cleaving e.g. viral capsid precursor and to be involved in cell lysis and release of the virions by cleaving cytoskeletal proteins (66). Adenain was described to cleave K48-linked Ub chains and ISG15 pro-peptides *in vitro*. During adenoviral infection the overall levels of ubiquitinated proteins (especially in the nucleus) decreased with increased adenain expression levels. The involvement of the adenain comprised DUB activity in the viral life cycle is not elucidated and no substrates are described yet (67).

The HHV (human herpesvirus) family comprises eight members, which are subcategorized in α-, β- and γ- herpesviruses according to their site of latency. All human herpesviruses encode for a large tegumental protein. The N-termini of all of these proteins have been shown to contain a conserved region with comprised DUB activity (68). The first discovered member was UL36 of HSV1, which was detected by usage of the suicidal probe Ub-VME in HSV-1 infected cell lysates. The crystal structure of a mouse herpesvirus homologue, MCMV (murine cytomegalovirus) suggests that the herpesviral DUBs represent a new family of deubiquitinating enzymes, which is called "herpesvirus tegument USPs"(69).

Among the best characterized UL36 homologues in human herpes viruses are BPLF1 from Epstein-Barr virus, UL48 from human cytomegalovirus and ORF64 from Karposi Sarcoma virus. UL36 was shown in further investigation to cleave K48-linked Ub-chains *in vitro* (69). In earlier work a null mutant was generated in the UL36 gene to elucidate its role in the virus life cycle. Absence of UL36 lead to failure in targeting capsids to the correct maturation pathway resulting in accumulation of DNA-filled capsids in the cytosol not maturing into enveloped viruses (70). It remains unclear if this detected phenotype is due to elimination of UL36´s DUB activity because the UL36 null mutation, containing an internal deletion of amino acid 362-1555, does not eliminate the active site residues but may interfere with folding and enzymatic activity. Also UL48 was shown to bind to the DUB probe Ub-VME and cleave K48-linked Ub-chains *in vitro* as described for UL36. Its catalytic active Cys- and His- residues were characterized via mutation analysis (71). Orf64 from KSHV was shown to be capable of deubiquitinating cellular proteins *in vitro* and *in vivo*. Orf64´s DUB activity was detectable against K48- and K63-linked Ub chains *in vitro*. Also the catalytic active cysteine was characterized via mutation analysis. Cell fractionation studies revealed that Orf64 is localized in the cytosol and nucleus. To test the function of ORF64, siRNA (short interfering

RNA) knockdown studies on latently infected cell, which were induced into lytic replication, were conducted. The depletion of Orf64 by siRNA resulted in decreased lytic viral transcription and decreased lytic protein expression (72).

1.1.14 The role of Ubiquitin and UbL-modifiers in the life cycle of human viruses

Given the central role of Ub- and UbL-modifications it is not surprising that many pathogens have devoted a considerable part of their genetic information to the production of proteins that mimic, block or redirect the activities of their host conjugating/deconjugating machineries. Modulation of those pathways is involved in virtually every step of the virus life cycle including virus entry, replication and assembly of new virus particles, immunity avoidance and virus exit.

1.1.15 Viral entry

Viral entry can be broadly defined as all events, leading to the arrival of the uncoated virus genome into the nucleus. HSV (Herpes simplex virus) entry into cells is a multistep process that engages the host cell machinery. In experiments, were the proteasome was blocked by usage of proteasomeal inhibitors, viral entry was disabled at an early step, after capsid penetration into the cytosol but prior to capsid arrival at the nuclear periphery. In addition, HSV successfully entered cells in the absence of a functional host ubiquitin-activating enzyme, suggesting that viral entry is ubiquitin independent. It was proposed, that proteasomal degradation of virion and/or host proteins is required for efficient delivery of incoming HSV capsids to the nucleus. The candidate proteins remain to be elucidated (73).

1.1.16 Viral replication

In the case of host replication interference, generally DNA tumor viruses modulate the cell cycle to enhance their own replication. A common mechanism shared by these viruses, is to target cell cycle regulator proteins for degradation, which often results in cell transformation. A classical example is the viral mediated down regulation of the tumor suppressor p53. The

HPV (human papillomavirus) -16 and -18, E6 proteins redirect the cellular single protein E3 ligase E6AP to mediate proteasomal degradation of p53, which contributes to their oncogenicity by allowing uncontrolled cellular proliferation without induced apoptosis (74).

A more common mechanism to control the turn over of particular cellular proteins is the manipulation of CRLs (cullin-RING-ligases). Examples for viral targeting of CRLs are the HPV-16 and -18, E7 proteins, which function as a substrate-binding module in a cullin 2 based CRL. This assembled complex targets the Retinoblastoma protein (pRb), a tumor suppressor controlling G1-S-phase transition, for proteasomal degradation, shutting of this cell cycle arrest point (75).

The Adenovirus proteins E1B55K and E4orf6 form a dimer, which functions as a substrate binding module in a cullin 5 based CRL, targeting the tumor suppressor p53 and the MRN-(Mre11, Rad50, Nbs1) complex, which is involve in DNA double strand break repair, for proteasomal degradation (76). E1B55K also contains a consensus site for sumoylation responsible for its nuclear localization. A sumoylation site mutant (K104R) showed altered localization and reduced ability to bind to p53, which also resulted in reduced cell transformation rates (77).

The adenovirus E4orf4 and the HPV encoded E2 proteins were shown to inhibit the cell cycle by interfering with the APC (anaphase-promoting complex). APC is a cellular E3 ubiquitin ligase, which is essential for progression through the M-phase. Both viral proteins were additional described to inhibit the degradation of cyclin B resulting in a G2/M phase arrest (78).

SV40 (Simian-Virus 40) expresses its LT (large T antigen), which binds to the SCF (Skp1, Cullin1, F-box)-complex (cullin1 based CRL) by inhibiting the substrate binding module F-box protein Fbw7. Most of the Fbw7 targeted substrates are potential cell cycle regulator or oncogenes like cyclin E, c-Myc, c-Jun and Notch, which up on inhibition of Fbw7 get stabilized (79).

The HPV encoded E2 protein and the adenovirus E4orf4 were shown to inhibit the cell cycle by interfering with the APC. Both viral proteins were additional shown to inhibit the degradation of cyclin B resulting to a G2/M phase arrest (80).

Cytidine is a component of RNA, which is formed when cytosine is attached to a ribose ring. After infection of RNA retroviruses, host cells are trying to diminish cytidine to disturb viral replication. The cellular APOBEC- (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like) family are mRNA editing enzymes, which edit cytosine to uracil essential for RNA synthesis. It has been shown that APOBEC3G was able to diminish the non-coding

strand of the HIV genome by converting deoxycytosin to deoxyuracil resulting in guanin to adenin hypermutations in the viral coding strand, restricting its propagation. To prohibit this pathway HIV encodes for the Vif protein, which functions as a substrate-targeting module in two described Cul5 based CRLs that target APOBEC3G for polyubiquitination and proteasomal degradation (81, 82).

Instead of hijacking cellular E3 ubiquitin ligases several viruses have been described to encode their own E3 ubiquitin ligases within their genome. HSV-1 encodes for the ICP0 (infected cell protein 0), an E3 ubiquitin ligase, which has been shown to induce polyubiquitination and degradation of a variety of proteins, including the PML protein, G1/S-phase specific cyclin D3, p53 and the DUB USP7. ICP0 contains two E3 ligase sites. One of those sites recruits the cellular E2 ubiquitin conjugating enzyme UbcH5a as well as the DUB USP7. ICP0 has also been shown to undergo autoubiquitination, which was reversed by the recruited USP7. ICP0 was also shown to facilitate polyubiquitination and proteasomal degradation of USP4. When cells were infected with an ICP0 null mutant an enhanced viral replication was detected. Among all known targeted proteins of ICP0, the polyubiquitination and degradation of PML was suggested for the detected replication phenotype (83).

Also KSHV (Kaposi sarcoma herpesvirus) was shown to encode a protein with E3 ubiquitin ligase function, called RTA (replication and transcription activator). RTA was earlier described to serves as transactivator protein, which is essential for activation of viral DNA replication upon initial infection and reactivation from latency. This viral multifunctional protein was shown to target several RTA repressor, like K-RBP (KSHV-RTA binding protein) and Hey1, a cellular transcriptional repressor, for polyubiquitination and proteasomal degradation. This RTA mediated repression of those two transcriptional repressors was shown to be essential for the reactivation of lytic DNA replication (84, 85)

1.1.17 Immune response

In addition to altering the cell cycle, viruses manipulate CRLs to interfere with the immune response of the host cells. HIV-1 (Human immunodeficiency virus 1) encodes the Vpu-protein, which functions as a substrate-binding module in the SCF complex. The Vpu modified CRL induces the polyubiquitination of the CD4 receptor in the ER-membrane, which causes its cytosolic translocation and proteasomal degradation. CD4 functions as a co-receptor for the TCR (T-cell receptor). Both receptors together mediate adaptive immunity by

binding to MHC class I (86).

Viruses often activate the antiviral interferon response during infection. The JAK-STAT pathway is an interferon induced cytokine signaling pathway in mammals and a key player in antiviral defense. STAT (signal transducer and activator of transcription) proteins comprise a family of transcription factors important in cell growth, survival and differentiation, which get activated after pathway induction. The rubulavirus encoded V protein serves as a substrate-targeting module in a Cul4a based CRL. It also binds directly to STAT2 (for human parainfluenza virus type 2) or STAT2 can be used to recruit STAT1 (for simian virus 5 and mumps virus). STAT2 or the heterodimer STAT1/STAT2 get connected to the CRL via the V protein and in following polyubiquitinated as signal for proteasomal degradation (87).

The mumps virus encoded V protein was shown to target STAT3 directly via this above described composition, also as mechanism to diminish the antiviral response (88).

There are also viral proteins known, which dislocate unwanted proteins from the ER. The HCMV (human cytomegalovirus) encoded US11 and US2 proteins dislocate MHC-I from the ER to the cytosol, were it becomes polyubiquitinated and proteasomal degraded (89).

Also the KSHV (Kaposi's sarcoma herpes virus) encoded proteins K3 and K5 have been shown to down-regulate MHC-I mainly on the cell surface. K3 and K5 are membrane proteins containing an N-terminal located E3-ligase domain, which adds K63- linked Ub chains to MHC-I, mediating its endosomal sorting and lysosomal degradation (90). The destruction of MHC-I decreases antigen presentation to T cells, avoiding immune recognition. The CD4 receptor is a co-receptor, which assists the TCR (T cell receptor) of helper T-cells by antigen-presenting cell recognition essential for innate immunity activation. After cellular CD4 synthesis, the protein gets located within the ER-membrane and sorted to the cell outer membrane. The HIV encoded Vpu protein is a transmembrane protein, which is also integrated into the ER-membrane. It binds to CD4 and βTRCP, a substrate targeting module in the Cul1 based SCF complex. This CRL interaction modulates CD4 polyubiquitination and proteasomal degradation, resulting in inefficient antigen-presenting cell recognition of helper T-cells (91).

As described above, induction of ISG15 expression upon viral infection appears to be a broad-spectrum inhibitor of virus production. Influenza B virus infection causes strong induction of ISG15 expression. Its viral encoded NS1 protein was found to bind to ISG15, preventing it from binding to its E1 activating enzyme UBE1L, causing inhibition of cellular ISGylation (92).

1.1.18 Viral budding

RNA viruses, like HIV-1 and RSV (Rous sarcoma virus) complete their replication cycle by forming vesicles that bud from the plasma membrane. The Gag polyprotein has been reported to be necessary and sufficient for the assembly and budding of virus-like particle. A central located Gag protein domain, called L (late) domain, was reported to bind to cellular proteins involved in the host VPS (vacuolar protein sorting) pathway, a cellular budding process that formats multivesicular bodies, very similar to virus budding. Among those recruited cellular proteins are Nedd4-family E3 ligases, which have been shown to facilitate ubiquitination of the viral Gag-protein. Gag ubiquitination is correlated with successful virus release, also facilitating its own sorting into viral vesicles as they bud from the plasma membrane (93).
Tsg101 (tumor supressor gene 101) is involved in vascular protein sorting and multivesicular body biogenesis by binding to monoubiquitinated proteins. Also Tsg101 was shown to bind to the ubiquitinated Gag protein by binding to Gag and ubiquitin. This interaction was shown to be essential for viral budding and release of HIV and Ebola virus (94).
These above described findings emphasize the pivotal role of Ub/UbL conjugation/deconjugation in modulating critical aspects of the virus-host cell interaction.

1.1.19 Introduction to EBV

EBV is a 172 kb long double-stranded DNA tumor virus, which establishes latent infections in 90% of the human population worldwide. Infection occurs in most cases during childhood with no or mild symptoms. Infection occurring in adulthood is known to cause a benign lymphoproliferative disease called “infectious mononucleosis”. This viral illness is characterized by moderate symptoms like fever, sore throat and swollen lymph glands. EBV is associated with a variety of malignancies of lymphoid cells, like Burkitt´s lymphoma, Hodgkin´s lymphoma and NK- and T-cell- lymphomas as also of epithelial cells, like nasopharyngeal carcinoma and gastric carcinoma. Like other herpesviruses EBV has a latent and a lytic life cycle. Epithelial cells of the oropharynx are the site of primary infection and are also believed to be the major site for viral replication. B-lymphocytes have been shown to serve as sites for the viral latent cycle and life long persistence. The EBV-genome does not normally integrate into the cellular DNA but persists as circular episome in the host cell. The viral latency comprehends the expression of a subset of latent proteins and an array of

transcribed non-coding RNAs (EBERs) and microRNAs depending of the type of latency (Type I, II, III). Those nine latent proteins are comprised of six "EBV nuclear antigens", called EBNA-1, -2, -3A, -3B, -3C and LP and three "latent membrane proteins", called LMP-1, -2, and -3. The viral latency is well studied and understood to a higher degree then the regulation of the viral lytic phase (95, 96).

Upon induction of the lytic phase the first detectable expressed proteins are the two immediate-early gene products BZLF1 and BRLF1. Both proteins transactivate viral promoters, which leads to an ordered cascade of viral early and late gene expression. Early gene products include proteins involved in viral DNA replication and DNA metabolism. The lytic phase of DNA replication is dependent on seven viral replication proteins: BZLF1, binding to the oriLyt (origin of lytic phase); BALF5, a DNA polymerase; BMRF1, a polymerase processivity factor; BALF2, a single-stranded DNA-binding protein and BSLF1, BBLF4, BBLF2/3 proteins comprising a primase-helicase complex. Viral lytic replication occurs in discrete nuclear sites, named replication compartments, in which viral replication proteins are assembled. Induction of the EBV lytic program results in inhibition of cellular DNA replication as well as explosive replication of viral DNA (97).

1.1.20 The role of ubiquitin and UbL-modifiers in the EBV life cycle

Nedd4 ubiquitin ligase family members have been reported to have a function in the maintenance of EBV latency in B lymphocytes. The BCR (B cell receptor) is known to mediate reactivation of the lytic cycle from the latent state. This is achieved by BCR cross-linking resulting in activation of signaling pathways, which initiates the viral transactivator BZLF1. Among others Lyn and Syk are two cytoplasmic tyrosine kinases, which upon binding to activated BCR mediate activation of BZLF1. The viral latent protein LMP2A contains two PPPY motifs, which were shown to recruit Nedd4 family members. After LMP2A binding to Lyn and Syc the recruited Nedd4 E3 ligases facilitate the polyubiquitination of those two tyrosine kinases, which causes their proteasomal degradation. The depletion of Lyn and Syk prevents signaling of activated BCR resulting in the maintenance of the latent cycle (98).

The lytic switch gene BZLF1 was found to be modified by SUMO-1. Unconjugated SUMO-1 level in cells are quite low. It has been shown, that BZLF1 competes with the PML-protein for SUMO-1 required for their both modification. By doing so BZLF1 degreases PML-protein

sumoylation, causing the inhibition of PML body formation. Among others PML body formation is induced by type I and II interferons, induced upon viral infection, suggesting a function with an antiviral capacity. The disruption of PML bodies, which was demonstrated by all classes of herpesviruses, suggests that this function is important for efficient lytic replication. It has also been shown, that sumoylation of BZLF1 decreases its transactivating ability (99). BZLF1 was also shown to serve as a substrate binding module in a Cul2 and Cul5 based CRL. Those assembled CRLs were reported to target p53 for polyubiquitination and proteasomal degradation (100) as described already for other viruses above.

EBNA3C is a critical component for EBV immortalization of infected B lymphocytes. EBNA3C was found to be modified with SUMO-1 and upon this modification co-localized with PML nuclear bodies. This co localisation was shown to decrease the effect of PML body formation, possibly due to competition among PML and EBNA3C for limited free SUMO-1 resources. The site of sumoylation was elucidated by usage of an EBNA3C truncated form. An EBNA3CΔ343-545 was not able to undergo sumoylation and did not co localize with PML nuclear bodies. Since EBNA3C transcriptional activation is mediated by amino acids 365 to 545, which are required for binding to SUMO, EBNA3C modification with SUMO could be important for EBNA3C described transcriptional effects (101). EBNA3C was also shown to interact with the Rb protein and to facilitate its proteasomal degradation via the SCF-Skp2 complex. Overexpressed EBNA3C into EBV negative epithelial and lymphoid cell lines resulted in decreased Rb protein level. It has been shown, that the ability of EBNA3C to facilitate the degradation of Rb was down regulated by expression of a dominant negative SCF-Skp2 complex. No down regulation of Rb was detected in the absence of EBNA3C (102).

Most intracellular proteins are destroyed via polyubiquitination mediated degradation in the proteasome. Resulting peptide fragments of degraded antigens are presented at the cell surface in association with MHC-I for immune recognition (103). EBNA1 is the most prominent latent protein of EBV with the broadest array of function. It is known to be essential for the maintenance of the viral genome, transcription and translation of the viral DNA, viral host persistence and cellular transformation. It has been demonstrated, that the internal Gly-Ala repeat domain of EBNA1 was able to interfere with the proteasome resulting in its protection from degradation. As a result, no EBNA1 peptide fragments are assessable for MHC-I binding and presentation on the cell surface, impairing immune recognition (104). EBNA1 has also been shown to interact with the DUB USP7, which has also been described to deubiquitinate p53 and its E3 ligase Mdm2. EBNA1 and p53 compete for the same interaction

site on USP7. EBNA1 was shown to have a ~10 fold higher affinity to the USP7 binding site then p53, being able to control p53 binding and deubiquitination. Functional studies were able to show, that binding of EBNA1 to USP7 was able to protect cells to undergo apoptosis by lowering p53 levels (105).

Most substrates are ubiquitinated on lysine residues, although ubiquitination on Cysteine and Serine residues as the ubiquitination of N-termini has also been reported. A database search identified several lysine free viral proteins, with lysine rich cellular and viral homologues. It is believed, that the comprehension of Lysine less proteins could be advantageous to viruses to protect them from polyubiquitination mediated degradation. The EBV encoded BHRF1 protein is a lysine less protein and a Bcl2 (B-cell lymphoma 2) family member. Bcl2 as BHRF1 are responsible for the release of mitochondrial cytochrome C, controlling cellular apoptosis (106, 107). It was shown in apoptotic cells, that Bcl2 was polyubiquitinated and degraded. To elucidate the impact of the lysine residues within Bcl2 concerning its described function, all lysines were removed via site-directed mutagenesis. It was demonstrated, that this lysine less Bcl2 mutant was resistance to degradation and was able to inhibit cell apoptosis (108). It could be speculated, that the lysine less BHRF1 is also able to escape ubiquitination and degradation with resulting pro-apoptotic conditions.

LMP1 is one of the EBV gene products, which are essential for B-cell transformation. It is also the only EBV protein that has oncogenic potential in non-B cells. The family of IRFs (Interferon regulatory factors) comprise transcription factors, which are predicted to contribute to EBV oncogenesis through regulation of a spectrum of oncogenes or apoptosis related genes. IRF7 is a central regulator of type 1 IFN-mediated innate and adaptive immune responses. It has been shown that IRF7 is modified from TRAF6 with K63-linked polyubiquitin, which protects the protein from proteasomal degradation and also causes its transcriptional activation. TRAF6 is a member of the TRAF protein family, which are involved in signal transduction controlling both innate and adaptive immune responses. TRAF6 has also been shown to comprise E3 ubiquitin ligase activity. TRAF6 and its E3 ligase activity are required for LMP1 stimulated IRF7 ubiquitination. A20 is an enzyme with dual function, comprising DUB and E3 ubiquitin ligase activity in one protein. Further it has been shown that LMP1 induces A20, which upon induction negatively regulates IRF7 transcriptional activity by deubiquitinating IRF7. A DUB deficient truncation or point mutation reduced the ability of A20 to inhibit IRF7, a ligase mutant didn't show any effect. Knockdown of A20 resulted in an increase in endogenous IRF7 K63-linked polyubiquitination and transcriptional activation (109, 110).

Upon EBV infection, several cellular DUBs are known to increase in activity including USP-5, -7, -13, -15 and -22. Those DUBs recruited by EBV may stabilize β-catenin in latently infected B-cells. β-catenine is a key component of the Wnt signaling pathway that regulates growth and differentiation of cells. Wnt signaling deregulation has been implicated in cancer development and could also play a role in EBV associated cancer development (111).

An overview of all the interactions of EBV with the cellular ubiquitin and ubiquitin like-modification system introduced above, are visualized in figure 2.

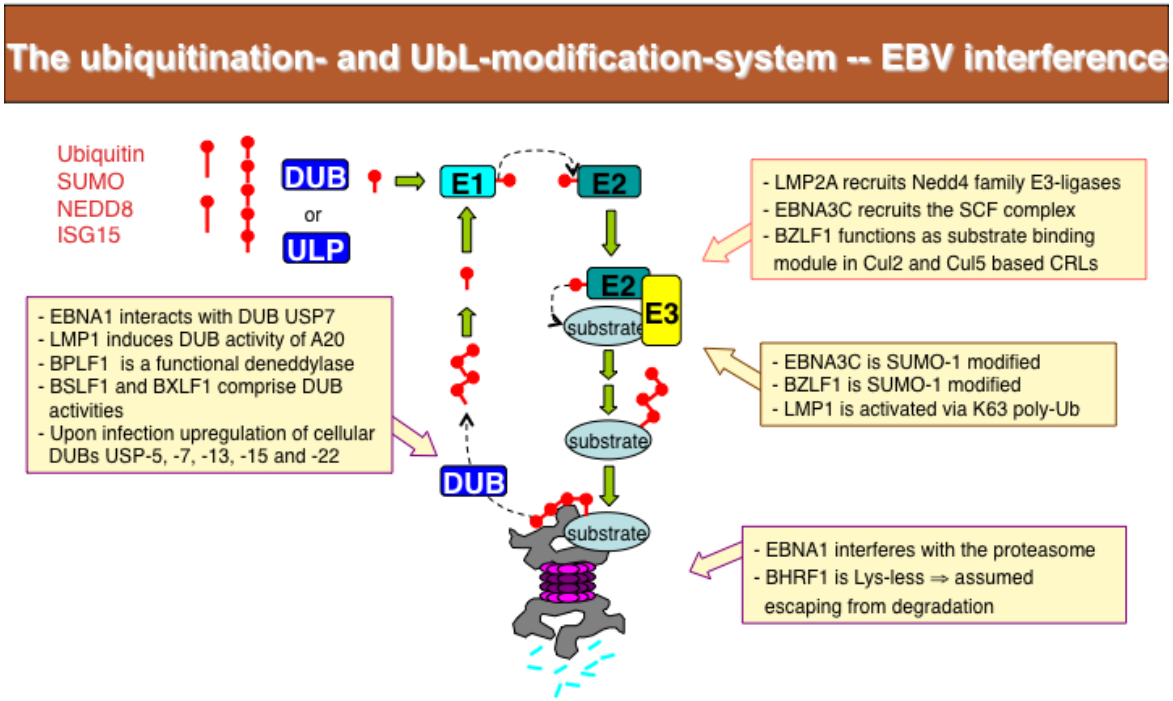

Figure 2. EBV encoded ORFs interfere with the ubiquitination and UbL-modification system at different sites.

1.2 INTRODUCTION – *AmotL2 in control of cell topology*

1.2.1 Epithelia

During embryonic development single cells join together to form functional units or organs. The Epithelium is a highly packed and dense tissue, which separates cavities and covers surfaces of newly formed organs. Epithelial tissues serve as a barrier against pathogens and support the structure of organs. The epithelium does not only protect from e.g. mechanical injury (skin) but also facilitates diffusion of oxygen and CO_2 in lungs, produces milk in glands and absorbs nutrients in the intestine. Three major groups of epithelia can be classified [1] by their number of cell layer (one layer: simple epithelium; more than one layer: stratified epithelium), [2] by the form of their cells (columnar-, cuboidal-, and squamous-epithelium) [3] and by the form of the cells at the outer layer (stratified columnar-, stratified cuboidal-, and stratified squamous-epithelium). The simple squamous epithelium which lines blood and lymphatic vessels is called endothelium. These different types of cell layers depend on the strict regulation of their cellular shapes and morphology for proper function (Cooper 2000, Guillot and Lecuit 2013).

Studies of epithelial function are of importance as they may provide clues regarding the development of novel therapies against human pathologies. For example, the majority of human cancers arise in tissues of epithelial origin and account for more than 80% of all cancer-related deaths. A strong correlation between loss of epithelial organization and malignancy has been documented for most carcinomas. The loss of tissue organization may be attributed to the deregulation of the normal mechanisms that are essential for epithelial morphology. These include the control of apical-basal polarity, cell-cell contacts and the cytoskeleton (Wodarz and Nathke 2007, Muthuswamy and Xue 2012).

In this section, I will describe the different components that are of importance for epithelial architecture and how the Angiomotin protein family integrates these pathways.

1.2.2. Cell polarity

The generation of three-dimensional epithelial structures in organs such as colon, prostate and skin depends on the establishment of cell polarity. This complex cellular mechanism is the

result of the asymmetric distribution of different polarity protein complexes. The two main complexes PAR (Cdc42/Par3/Par6/aPKC) and Crumbs (Crb/Pals1/PATJ) are e.g. involved in the establishment and maintenance of apical-basal cell polarity and also facilitate a possible depolarization. The core protein complex Scribble (Srib/Lgl/Dlg) promotes the baso-lateral membrane formation (Bryant and Mostov 2008, Roignot, Peng et al. 2013).

Polarity processes leading to a stationary epithelium are discussed below. During this development the small GTPase Cdc42 plays a central role. This protein is a member of the Rho-GTPase family and has an astonishing ability to control and coordinate several signal transduction pathways. It functions as a molecular switch, changing from its inactive state (bound to guanosine diphoshate, GDP) to its active state (bound to guanosine triphosphate, GTP). The recharging of GDP to GTP and therewith "switching on" of Cdc42 is facilitated by Guanine nucleotide exchange factors (GEFs) (Cooper 2000, Etienne-Manneville 2004).

Filopodia are cytoplasmic extensions that elongate beyond the leading edge of lamellipodia in migrating cells. Filopodia formation is accomplished by Cdc42 binding to the WASp protein, which recruits and activates the Arp2/3 complex (Magdalena, Millard et al. 2003).

In epithelial cells filopodia have been demonstrated to establish cell-cell contacts leading to a polarized morphology, by enabling cell-cell junction assembly (Vasioukhin and Fuchs 2001).

In multicellular organisms, cell polarity is initiated mainly by external stimuli. Membrane receptors, which facilitate adhesion, such as cadherins, nectins and integrins, as well as chemokine receptors (soluble ligand receptors) sense their environment in order to establish polarity (Etienne-Manneville 2004).

During the process of polarization and epithelial sheet formation, cells generate distinct cell membrane compartments, which are localized to the apical (facing the lumen), or baso-lateral (facing the extracellular matrix and neighboring cell) cell surfaces (Rojas, Ruiz et al. 2001). This process is initiated by adhesion proteins such as E-cadherin and nectin (Takai and Nakanishi 2003). Their ligation induces Cdc42 activation by e.g. the activation of the GEFs FRG and Vav2 (Fukuhara, Shimizu et al. 2004).

The establishment and composition of the two different apical and baso-lateral membrane domains is regulated by the endocytic and exocytic secretory pathway. Cdc42 has been proposed to regulate the exit of apical and baso-lateral proteins from the trans-Golgi network (Musch, Cohen et al. 2001). This process may involve the establishment of the Par6-aPKC and Lgl-scribble polarity complex components to the plasma membrane. Lgl has been shown to interact with the membrane integrated Q-SNARE protein t-SNARE syntaxis 4 and regulate baso-lateral exocytosis (Musch, Cohen et al. 2002). The activity of the apical polarity protein

Crb is needed for vesicle exocytosis at the apical surface (Myat and Andrew 2002). The Sec6/Sec8 polarity complex, which is located just below the tight junctions, is involved in the baso-lateral delivery of proteins in epithelial cells (Matern, Yeaman et al. 2001, Kreitzer, Schmoranzer et al. 2003).

The p65PAK family of serine/threonine kinases is one of the best studied Cdc42 targets. p65PAK localizes to cell-cell adhesion contacts in epithelial cells and controls polarized actin polymerization. This process is facilitated by regulating LIM kinases which phosphorylate and inactivate the actin binding protein cofilin leading to a polarized actin rearrangement (Bokoch 2003, Edwards, Sanders et al. 1999).

1.2.3 Cell-cell adhesion

Epithelial cells are attached to eachother at contact points called cellular junctions. These contact points fall into different categories, the **tight junctions**, the **adherens junctions**, the **desmosomes** and the **gap junctions,** each with individual functions (Figure 1) (Cooper 2000).

1.2.4 Tight junctions

In epithelia, tight junctions (TJ) are found at the most apical site of the lateral membrane, were they form an impermeable intramembranous barrier to fluid. This is of importance for e.g. the maintenance of the osmotic balance in tissues (Aijaz, Balda et al. 2006). Apart from facilitating cell-cell adhesion, TJ have been associated to intracellular signaling mechanisms which influence polarization, cell proliferation and differentiation (Zahraoui 2004, Shin, Fogg et al. 2006). TJ are assembled out of two different types of transmembrane proteins. Those are the tetraspan- (occluding, tricellulin and claudins) and the singlespan- (e.g. JAMs, CAR, CLMP and CRB3) transmembrane proteins (Bazzoni 2003, Aijaz, Balda et al. 2006).

Towards those mentioned transmembrane proteins binds a so called “cytoplasmic plaque”, which are adaptor-, cytoskeletal or scaffolding proteins which mainly cross links the junctional transmembrane proteins to the actin cytoskeleton. Those “plaque” proteins regulate adhesion and paracellular permeability as they transmit signals from the junctions towards the cellular inner controlling gene expression and migration. The TJ plaque also recruits an array of signaling proteins, which includes kinases and phosphatases involved in gene expression

regulation. Some of the most prominent TJ plaque proteins are ZO1, PAR3, PAR6, Pals1 and PATJ (Assemat, Bazellieres et al. 2008, Balda and Matter 2008, Guillemot, Paschoud et al. 2008, Paris, Tonutti et al. 2008).

1.2.5 Adherens junctions

Adherens junctions (AJ) are located below the tight junctions on the apical lateral side between two neighboring epithelial cells. During cell-cell contact formation AJ are the first junctions to be formed which then promotes the subsequent development of tight junctions. The main feature of AJ is to facilitate cell-cell adhesion via the establishment and maintenance of a circumferential actin belt, which connects single epithelial cells to tightly packed epithelial sheets. This actin belt, also called "adhesion belt" is involved in epithelial morphogenesis mediation as it also facilitates the transmission of subcellular force (Harris and Tepass 2010, Guillot and Lecuit 2013).

The formation of AJ is mediated by homotypic interactions of the transmembrane adhesion molecule E-cadherin between two neighboring epithelial cells. Their extracellular domain is arranged into five repetitive domains, which contain calcium-binding sites (Overduin, Harvey et al. 1995). Calcium facilitates homotypic adhesive activity, enabling a dynamic regulation of adhesive contacts (Nagar, Overduin et al. 1996).

The cytoplasmic tail of E-cadherin associates to plaque proteins like p120, α- , β- catenin and plakoglobin, which in return mediate binding to contractile actin fibers forming e.g. the adhesion belt (Baum and Georgiou 2011).

AJ serve as a start and fixing point for cell polarity. Apical polarity proteins like Crb3, PALS1, PATJ, PAR3 and aPKC are found in high concentration just above the AJ as basolateral proteins like LGL, DLG and Scrib are found just below AJ (Georgiou and Baum 2010).

The mechanisms how cadherins connect to actin have been a subject of controversy. It was long thought that cadherins bind to β-catenin by associating to α-catenin which acted as a linker between the adhesion complex and the cytoskeleton. This dogma was later revised based on the findings that α-catenin does not bind β-catenin and actin fibers simultaneously (Drees, Pokutta et al. 2005). Apart from the circumferential actin belt, a second population of contractile actin fibers was described to localize to the junctions (Zhang, Betson et al. 2005). How E-cadherin connects to contractile actin filaments that run perpendicular to the cell-cell

junctions is the topic of paper III in this thesis.

Epithelia are very dynamic and underlie steady changes of tissue organization. Constant changes due to cell tissue reorganization, differences in cell packing and apoptosis requires an ongoing forming and disassembly of AJ. This procedure is e.g. carried out by a very active internalization and recycling back of E-cadherin from and to the plasma membrane. This process has been shown to depend on clathrin-mediated endocytosis (Bryant and Stow 2004). It has also been demonstrated, that the recycling of E-cadherin requires the octameric protein exocyst complex for the delivery of the protein to the AJ (Blankenship, Fuller et al. 2007, Baum and Georgiou 2011).

The transmission of mechanical signals through AJ leading to intracellular signaling cascades will be discussed in the following chapter "mechanotransduction".

A further AJ component is the transmembrane protein family of the nectins which promote cell-cell adhesion in a calcium independent manner. Similar to E-cadherin, they undergo homophilic interactions between two neighboring cells with their extracellular regions. They connect via their cytoplasmic regions to the actin binding protein afadin, which in turn binds to actin fibers. Nectin mediated cell adhesion is involved in the induction of polarity, as it induces the formation of adherens junctions in epithelial cells, by the activation of Cdc42 and Rac small G proteins, which in turn reorganize the actin cytoskeleton. Nectins also bind to the polarity protein PAR3 with their cytoplasmic regions (Takai, Irie et al. 2003, Nakanishi and Takai 2004).

1.2.6 Desmosomes and Gap junctions

Desmosomes are intercellular junctions that connect cellular intermediate filaments to the plasma membrane. Those adherence platforms are primarily present in tissues with higher exposure to shear stress, like in epithelia, were they support stable cell-cell adhesion. The adhesive parts of desmosomes are assembled out of two different kind of transmembrane proteins, the desmogleins and desmocollins, which are calcium dependent members of the cadherin family. Towards the cytosolic tails of the adhesive proteins bind cytosolic adaptor proteins (desmosomal plaque) which are e.g. plakophilins and plakoglobin, which are members of the armadillo gene family. Those adaptor proteins bind to intermediate filament binding proteins like desmoplakin which link the desmosomal protein complex to the filaments (Delva, Tucker et al. 2009, Kowalczyk and Green 2013).

Gap junctions are clusters of intercellular channels that facilitate a direct transfer of ions, amino acids, second messengers and electrical signals between neighboring cells. Due to this direct shuttling mechanism gap junctions enable cellular key events like apoptosis, cell differentiation and tissue homeostasis (Nicholson 2003, Nielsen, Nygaard Axelsen et al. 2012). Gap junctions are found in all kind of adhesive tissue cells where they are assembled out of connexins (Cx) which are integral membrane proteins (Goodenough, Goliger et al. 1996). Each gap junction is assembled out of two hemichannels, also called connexons, whereby each hemichannel is constructed out of six connexin molecules. The major gap junction regulating mechanism is phosphorylation, which is vital for gap junction assembly, turnover and function (Warn-Cramer and Lau 2004).

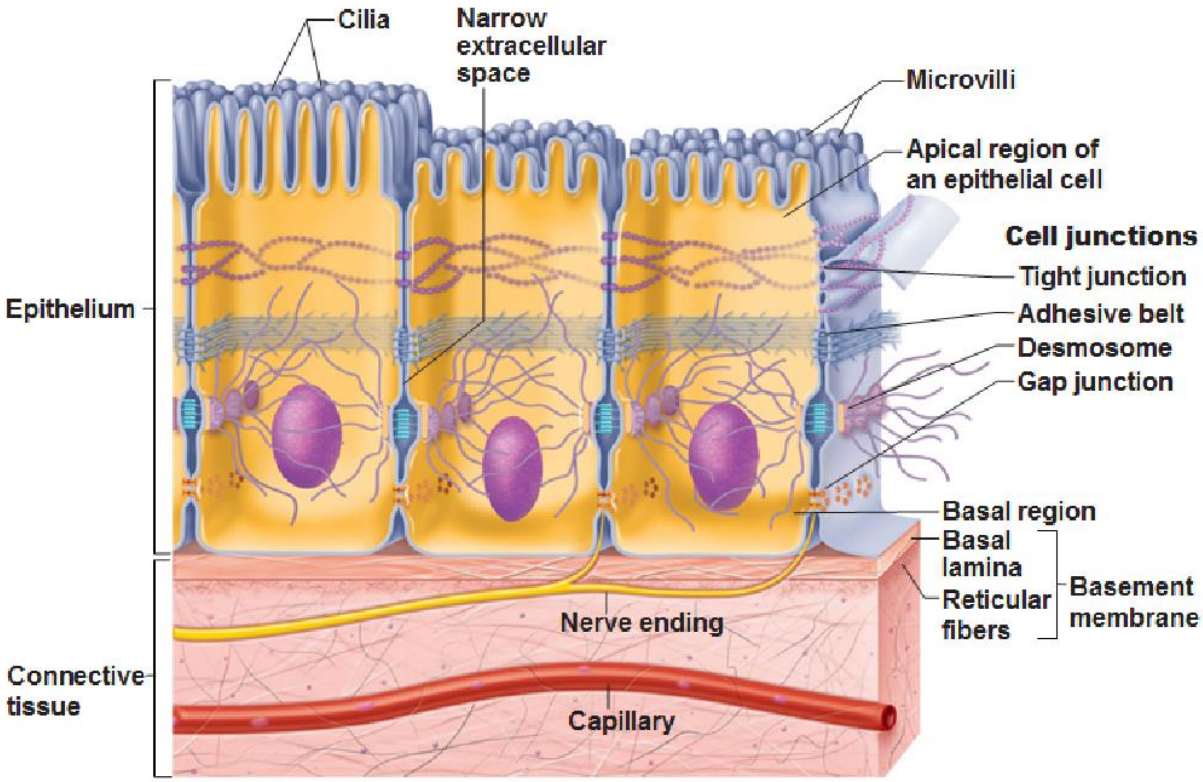

Figure1. Polarized epithelial cells of a simple epithelium visualizing cell-cell adhesion sites. Kindly provided from Nature Publishing Group.

1.2.7 Adhesion to the extra-cellular matrix

The extra cellular matrix (ECM) is a complex composition of matrix molecules (e.g. collagens, fibronectin, glycoproteins, laminins, proteoglycans) and non-matrix proteins (e.g. growth factors, kinases, phosphatases, proteases) (Naba, Hoersch et al. 2012). The main functions of the ECM are the structural support (e.g. cell migration and polarization), the regulatory function (e.g. tissue organization and differentiation) and the supply of nutrients to the attached cells. Epithelial cells bind to the ECM mainly via integrins, which are membrane adhesion proteins located on the basal cell membrane (Berrier and Yamada 2007). Integrins are heterodimers built up out of an α- and β- subunit. In mammals 18 α- and 9 β- subunits are expressed, assembling to 24 specific integrin receptors, which bind different ECM ligands (e.g. collagen, fibronectin and laminin) with various affinities (Luo, Carman et al. 2007). The main two functions of integrins are the attachment of the cell to the ECM and the signal transduction vice versa. Several integrins accumulate to clusters which are called "focal adhesions". Towards the cytosolic cell site integrins are linked mainly to the actin cytoskeleton via a variety of adaptor proteins (vinculin, talin and α-actinin) (Brakebusch and Fassler 2003). Apart of that several signaling proteins bind to the cytosolic site of integrins which are involved in various signaling cascades controlling e.g. cell migration, differentiation, gene regulation, polarity and proliferation (Harburger and Calderwood 2009). These kinds of signaling events will be discussed further in the chapter "mechanotransduction". Integrins are also involved in a process called "inside-out signaling" were intracellular signals are able to induce alterations in integrin conformation and activity leading e.g. to the loss of the cell from the ECM (Luo, Carman et al. 2007).

Epithelia also exert adhesion structures called hemidesmosomes to facilitate cellular adherence from the cellular keratin cytoskeleton to the basement membrane, which is a specialized form of the ECM (Seltmann, Roth et al. 2013). Those adherence structures are assembled within the basal cell membrane via the two transmembrane proteins $\alpha_6\beta_4$ integrin and BP180. The connection to the cellular keratin network is realized by the inner plaque proteins HD1/plectin and BP230. Apart from their function to master cellular adhesion, hemidesmosomes are also involved in mechanotransduction, by outside-inside signaling mainly via signal transduction trough the $\alpha_6\beta_4$ integrin. Those mediated signals have been demonstrated to be involved in regulating vital cellular processes like apoptosis, differentiation and proliferation (Mainiero, Pepe et al. 1995, Nievers, Schaapveld et al. 1999).

1.2.8 Cytoskeleton

The cytoskeleton is a protein fiber skeleton imbedded within the cytoplasm of all kinds of cells (prokaryotes and eukaryotes). In eukaryotes it is constructed out of three different kinds of protein filaments, the **actin filaments** (also called microfilaments), the **intermediate filaments** and the **microtubules**. All three cytoskeletal fibers are homogenous structured polymers, constructed from individual monomeric protein subunits connected via non-covalent bonds. The main function of the cytoskeleton is to physically enable a certain cell morphology and to allow morphological changes e.g. during cell division or migration. It also serves as a mediator of intracellular vesicle trafficking (Cooper 2000).

1.2.9 Actin filaments

The actin filaments are polar structures buildup of monomeric subunits called globular actin (G-actin). Those monomers are linear head-to-tail polymerized to flexible filamentous actin (F-actin) fibers, 6 to 9 nm in diameter and up to several micrometers in length. During assembly of filamentous actin, the G-actin monomers are rotated by 166^{o}, creating a fiber form of a double stranded helix. Those created actin fibers are organize into two types of structures, actin bundles or actin networks, facilitated by different actin crosslinking proteins, determining the physical properties of those structures (Giganti, Plastino et al. 2005, Winder and Ayscough 2005).

Actin bundles are parallel arrays of actin fibers tightly packed by actin crosslinking proteins. Three-dimensional actin networks are constructed out of loosely linked actin filaments obtaining the properties of semisolid gels. Actin filaments bind to the motor protein myosin, which enables them to contract. Myosin hydrolyses ATP to ADP to generate force and movement, e.g. used for cell migration or muscle contraction (Hill, Catlett et al. 1996). Contractile actin bundles, also called stress fibers, connect e.g. epithelial cells to the ECM by being linked to integrins assembled in focal adhesions. Most stress fibers are bound to myosin II motors, which enables them to contract making them to indispensable components to cell adhesion and morphogenesis (Tojkander, Gateva et al. 2012).

In a polarized epithelium actin bundles, named adhesion belt, interconnect the adherens junctions within a cell. They are linked via a subset of actin binding proteins to the transmembrane adhesion protein E-Cadherin. Their main function is to connect single

polarized epithelial cells to tightly packed epithelial sheets, contributing to establish the needed mechanical strength and enabling the transmission of subcellular force (Harris and Tepass 2010, Guillot and Lecuit 2013).

Contractile actin bundles, distinct from the adhesion belt fibers, were also described to localize to the junctions (Zhang, Betson et al. 2005).

The apical side of epithelial cells can contain protrusions which are involved in absorption of nutrients. Those surface extensions are based on actin filaments arranged in bundles or networks. An example of those surface protrusions are microvilli, which are formed on the apical surface of epithelia lining the intestine. Each of those intestine epithelial cells contains ca. 1000 microvili structures increasing the nutrient absorbing apical surface by the factor 10 to 20. Other actin based cell surface protrusions, like pseudopodia, lamellipodia or filopodia are formed on the leading edge of a moving cell and enable cell locomotion (Cooper 2000, DeRosier and Tilney 2000).

1.2.10 Intermediate filaments

Intermediate filaments (IFs) range in diameter between 10 – 12 nm and are assembled of a subset of proteins, which share common structural attributes. They are cytosolic filaments, except the lamins which form a nuclear lamina three-dimensional network (Goldman, Gruenbaum et al. 2002). Intermediate filaments are unpolar structures and assemble in most mammalian cells a network which fills out the entire cell. Keratin and vimentin based filaments connect to the nucleus, positioning and holding it in place. IFs interconnect also cell adhesion components in polarized epithelial cells. Keratin filaments bind to the desmosomes and hemidesmosomes, thus mediating cell-cell and cell-matrix adhesion and thereby facilitating mechanical stability to the epithelium. IFs even connect to the other two cytoskeletal components, actin filaments and microtubules thereby enhancing the mechanical stability of the cell. IFs have also been described to modulate signals within a cell, e.g. by binding to the Fas cell surface receptor, modulating its density and function (Cooper 2000, Coulombe and Wong 2004, Herrmann, Bar et al. 2007).

1.2.11 Microtubule

Microtubules are polar tubular structures, assembled by the globular protein tubulin. The inner diameter of a microtubule is about 12 nm and the outer diameter is about 25 nm.
Apart from giving the cell mechanical support, microtubules enable cellular mitosis, were they form the mitotic spindle, responsible for the separation of the chromosomes (Fourest-Lieuvin, Peris et al. 2006). Microtubules are able to develop directed force by assembling and disabling its own structure and by attaching to motor proteins like dynein and kinesin. Due to this characteristic microtubules are actively engaged in the cellular transport of membrane vesicles in the endocytic and secretory pathways as they also transport organelles, like Golgi apparatus, lysosomes and mitochondria within eukaryotic cells (Hirokawa 1998). Microtubule structures are able to assemble or disassemble in the shortest timeframe compared to the other cytoskeletonal structures, the actin filaments or intermediate filaments. In order to increase the stability of microtubules in certain regions of the cell, they bind to microtubule-associated proteins (MAPs). MAPs interconnect microtubule to intermediate filaments or other microtubule via a phosphorylation regulated mechanism. This microtubule stabilization at specific sites within the cell serves as an important step facilitating cell polarity and certain cell shapes (Cooper 2000, Siegrist and Doe 2007).

1.2.12 Mechanotransduction

Mechanotransduction describes the mechanisms by which cells translate mechanical cues into electrical or chemical based cell signaling events. Cells within multicellular tissues are exposed to an array of physical cues, like compression, expansion, shear stress and hydrostatic pressure. The exposed cells have to adapt to mechanical forces, resulting in e.g. changes in cell differentiation, growth, morphology and polarity (Huveneers and de Rooij 2013). The way cells respond to the exposure to physical stimuli depends on the physical properties of the individual cell type and of the physical characteristics of the tissue they are imbedded in. The transfer of the mechanical stimuli towards the cell can occur from different locations. For epithelia those loci are mainly the ECM and cell-cell adherens junctions (DuFort, Paszek et al. 2011).
Mechanical stimuli transmitted via the ECM are received mainly by integrins. They sense the nature and changes of the ECM surrounding and transmit signals via conformational changes

to cytosolic associated integrin plaque proteins like TALIN or BCAR1. Those sensor proteins paired with growth factor signalling (e.g EGF) are able to start specific cellular signalling events leading to cellular fate decisions influencing cell attachment, differentiation or migration (DuFort, Paszek et al. 2011).

Elevated tension around focal adhesion contacts e.g. increases the clustering of integrins and the phosphorylation and activation of focal adhesion kinases (FAKs). This leads to the initiation of several cellular signalling events, like activating the Rho-family GTPases RhoA, which stimulate its effector RhoKinase/ROK/ROCK resulting in the assembly of new stressfibers by connecting actin filaments with myosin motors (Arthur, Noren et al. 2002).

A further integrin mediated signalling pathway that is activated in response to mechanical force within the ECM is the MAPK–ERK pathway, which influences tissue development by regulating cell differentiation and proliferation (Yee, Weaver et al. 2008). The physical characteristics of the ECM are determined by the organization of its components and their degree of intramolecular crosslinking (DuFort, Paszek et al. 2011).

Epithelia sense via intergrins the rigidity of the ECM, which they are attached to and adjust the tension they exert themselves accordingly (Saez, Buguin et al. 2005). When epithelia attach to stiff matrices they format larger focal adhesion clusters, binding to more actin stressfibers resulting in higher stabilizing traction forces within the cell. It also results in the destabilization of cell-cell adhesion points like adherens junctions, leading to the disruption of the actin "adhesion belt" (Tsai and Kam 2009, DuFort, Paszek et al. 2011).

Cell attachment to stiff matrices also affects the microtubule rearrangement dynamics resulting in slow growing and long lasting microtubuli (Figure2b) (Myers, Applegate et al. 2011) When epithelial cells attach to soft matrices, less integrin molecules are clustered leading to fewer establishments of integrin connected stressfibers and lower established traction forces. Also cell-cell junctions like tight and adherens junctions are fully matured including the successful formation of the adhesion belt, resulting in the establishment of a functional epithelium (Figure 2a) (DuFort, Paszek et al. 2011).

Mechanosensing properties were also described at cell-cell adhesion sites. E-cadherin complexes were shown to be functional mechanosensors which transmit force between E-cadherin and F-actin. This detected intercellular force overlapped with vinculin accumulation at actin-anchored cadherin adhesions, shown in imaging experiments. Vinculin potentiates the E-cadherin mechanosensory response, which was demonstrated by using E-cadherin based cell bead adhesion assays (le Duc, Shi et al. 2010).

The motor protein myosin II is required for actomyosin contractility and for maintenance of

adherens junctions. In endothelial cells an increase of AJ size was detected after stimulation with activated myosin II and increasing tugging-force at the AJ. In those experiments myosin II level were downregulated via siRNA, which resulted in decreased traction force and AJ size. The tugging force was evaluated with a retooled version of elastomeric microneedles. Their data suggests that mechanosensitive growth is a basic mechanism for controlling AJ size and is similar to the observed growth of focal adhesions after applied force (Liu, Tan et al. 2010).

Apart from the involvement of the actin cytoskeleton in the cadherin mechanoresponse, also the plakoglobin connected intermediate filaments towards cadherin based cell-cell junctions were described to facilitate mechanoresponces. Local pulling force was applied to *Xenopus* mesendoderm cell cadherin adhesions via C-cadherin coated magnetic beads and possible changes in cell polarity or migration were analyzed. This applied tension to the cadherin based adhesions induced traction forces directed to the opposite site of the applied force. This effect was induced by start of cellular migration caused by the new formation of polarized protrusions. Cadherins are associated to actin- and intermediate- filaments (IFs). By incorporating GFP labelled keratin into the *Xenopus* mesendoderm cells, strong GFP signals were detected at the cadherin cell - beads cadherin junctions. By impairing Keratin intermediate filament assembly through usage of antisense morpholinos, the before detected protrusive activity was perturbed (Weber, Bjerke et al. 2012).

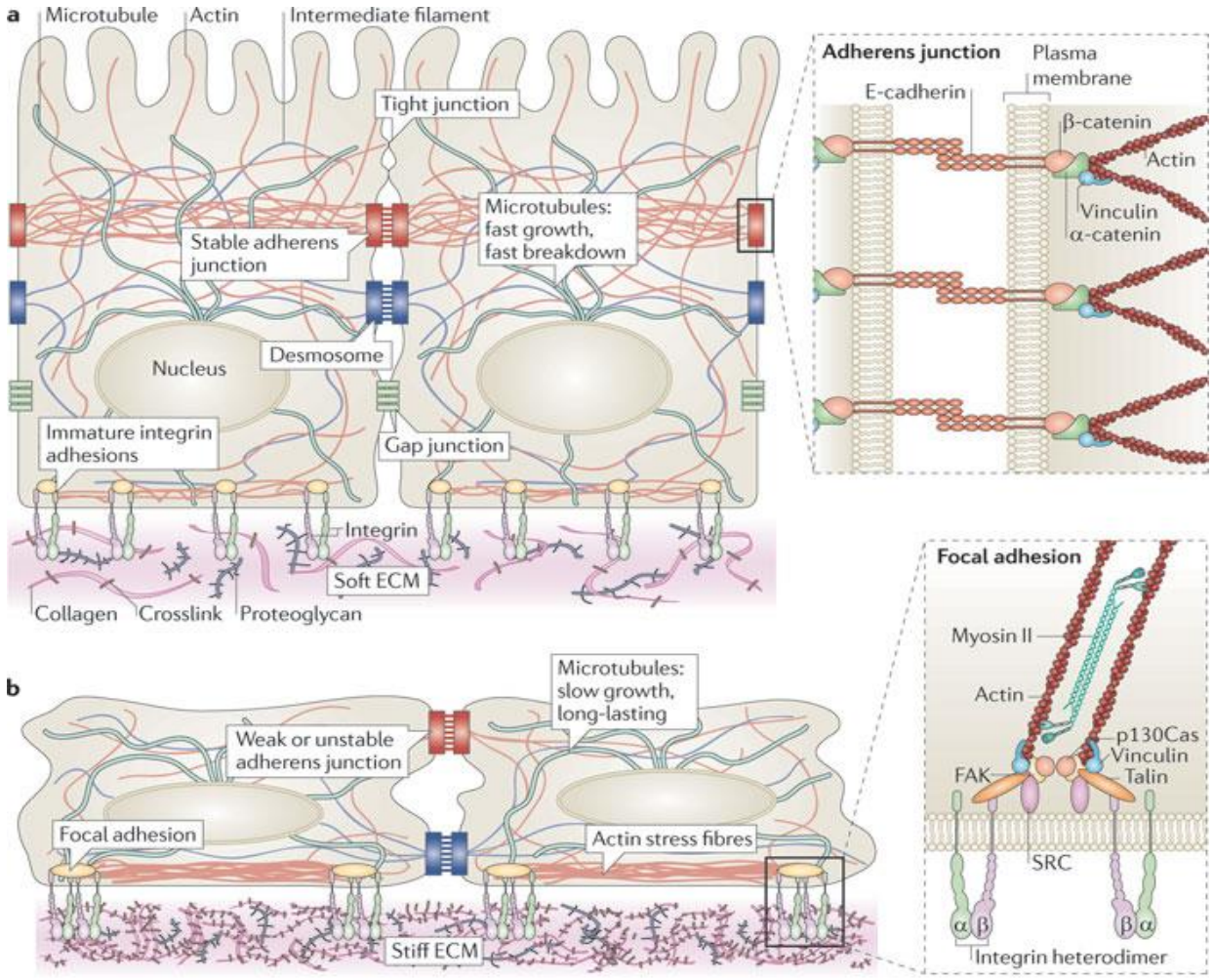

Figure 2. Physical characteristics of the extracellular matrix (ECM) control cytoskeletal and junctional properties of polarized epithelial cells. Figure adapted from DuFort, Paszek et al. 2011, Nat Rev Mol Cell Biol.

1.2.13 Angiomotin family

The Angiomotin protein family of scaffold proteins comprises three members (Bratt, Wilson et al. 2002). The founding member angiomotin (Amot) was initially identified as a receptor for the angiogenesis inhibitor angiostatin. It was first described to be essential for endothelial cell migration (Troyanovsky, Levchenko et al. 2001). Amot sequence analysis showed that this protein was not similar to any known protein at the time and was found to belong to a novel protein family including AmotL1 (or JEAP) that was identified in a screen for endothelial junction proteins and AmotL2 (or LCCP) identified by homology searches in GenBank (Bratt, Wilson et al. 2002, Nishimura, Kakizaki et al. 2002).

Angiomotin family members have been found in human, mouse and zebrafish but not in Drosophila, yeast or bacterial genomes (Bratt, Wilson et al. 2002). These proteins act as scaffolds in that they contain interaction sites enabling the formation of protein complexes. The most conserved domain is the central coiled-coil domain which serves as an oligomerization site. Amot proteins form homo-oligomers and it has also been shown that Amot and AmotL1 can associate to form hetero-oligomers (Ernkvist, Birot et al. 2008, Zheng, Vertuani et al. 2009).

Furthermore, the coiled-coil domain also allows the binding of the tumor suppressor Merlin which displaces Rich1, a small GTPase Activating Protein, and a positive regulator of Rac1, resulting in inhibition of Rac and Ras-MAPK pathways. These data indicate that Amot plays a critical role in mediating tumorigenesis caused by NF2 inactivation and could be exploited as a target in NF2-related cancers (Yi, Troutman et al. 2011).

An important role in regulating cell polarity was indicated by the findings that Amot proteins associate to the Par3 and Crb3 polarity protein complexes via a C-terminal PDZ binding motif (Wells, Fawcett et al. 2006, Ernkvist, Luna Persson et al. 2009). Deletion of this motif abrogates protein-protein interaction resulting in loss of pro-migratory activity of Amot (Levchenko, Aase et al. 2003).

The N-terminal domain of all three proteins contain conserved WW-binding motifs mediating the interactions to the tight junction protein MAGI1, the transcriptional co-activator YAP and the E3 ligase NEDD4 (Patrie 2005, Wang, Huang et al. 2011, Wang, An et al. 2012).

Furthermore Amot has been demonstrated do bind F-actin in endothelial cells controlling cellular shape and AmotL1 has been shown to bind F-actin in epithelial cells promoting actin cytoskeleton remodelling (Ernkvist, Aase et al. 2006, Gagne, Moreau et al. 2009). The interaction partners of the Amot family suggest a role in coordinating and integrating polarity, cell-cell adhesion and cytoskeletal signalling pathways, which is of course of interest in understanding morphogenic events during normal and cancer development.

Strategies to inactivate Amot in endothelial cells *in vitro*, as well as zebrafish and mouse *in vivo* have shown that Amot is essential for normal angiogenesis. The primary role appears to be the control of directional migration in response to angiogenic factors such as VEGF and bFGF. Knock-down of Amot in zebrafish results in loss of migration of inter segmental vessels which lack polarized protrusions of filopodia (Aase, Ernkvist et al. 2007).

Similar findings have been observed in conditional inactivation of the Amot gene during retinal vascularization of postnatal mice. Here, migration and the extension of filopodia are also affected (Zheng et al., unpublished data).

Furthermore it has been reported, that Amot, AmotL1 and AmotL2 bind via their C-terminal located PDZ binding motif the RhoA GTPase exchange factor Syx. This binding was essential for endothelial cell migration, as the knockdown of Amot lead to inhibition of intersegmental vessel migration during zebrafish angiogenesis. Syx is expressed in endothelial cells and is dependent on Amot for spatiotemporal targeting of RhoA to the leading edge of migrating cells (Ernkvist, Luna Persson et al. 2009).

It has been long known that tumor growth, invasion and metastasis are depending on the de-novo formation of blood vessels. Different strategies have been employed to target Amot and tumor angiogenesis. The Holmgren and Cavallo labs have shown that Angiomotin DNA vaccination could prevent breast cancer outgrowth in a transgenic breast cancer model (Holmgren, Ambrosino et al. 2006, Arigoni, Barutello et al. 2012).

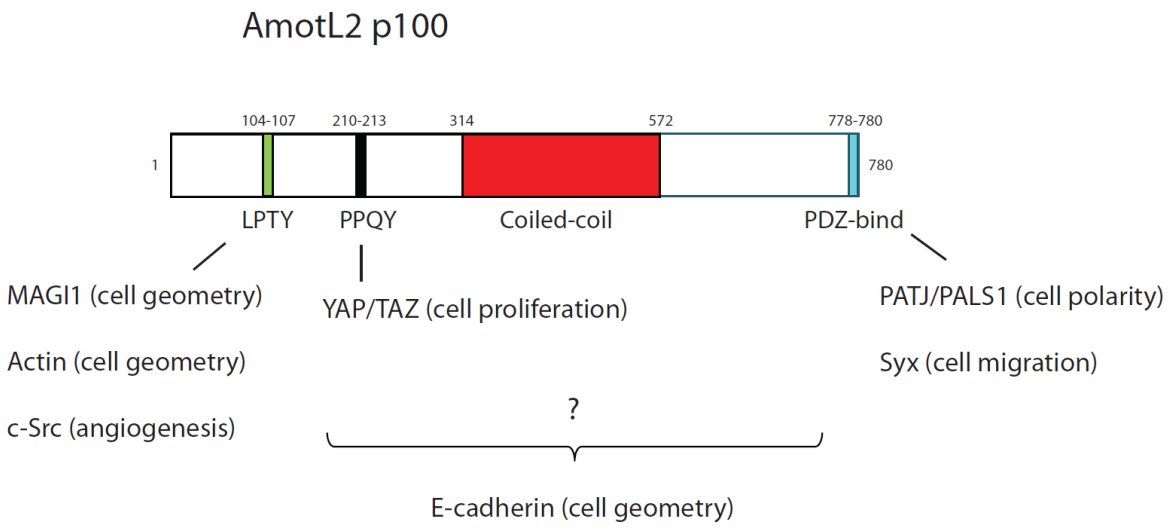

Figure 3. Primary domain structure of AmotL2 p100 included binding sites and interacting proteins.

2.1. AIMS OF THIS STUDY- *EPSTEIN-BARR VIRUS ENCODED DECONJUGASES*

The general aim of the work presented in this thesis, was to elucidate and characterize novel deubiquitinating enzymes (DUBs) or ubiquitin-like proteases (ULPs) encoded within the genome of Epstein-Barr virus (EBV).

More specifically, we wanted to:

- develop a bacterial screening assay based on usage of Ub/UbL-modifier reporter constructs, to test the EBV-ORFeome for encoded functional DUBs and ULPs

- characterize the discovered DUBs and ULPs via mutation analysis and usage of functional reporter and probes

- elucidate the functional role of EBV encoded DUBs or ULPs in the viral life cycle, including detection of interaction partners

2.2 AIMS OF THIS STUDY- *AMOTL2 IN CONTROL OF CELL TOPOLOGY*

The general aim of the work presented in this thesis, was to elucidate the function of AmotL2 p100 in epithelial cells.

More specifically, we wanted to:

- construct AmotL2 shRNA and produce AmotL2 shRNA carrying lentiviruses to produce stable transfected epithelial knockdown cell lines

- investigate changes on cell shape and polarity in stable transfected AmotL2 KD´s in MDCK and CaCo2 cells, detected in 2D- and 3D- assays, assayed by fluorescence microscopy

- investigate and detect AmotL2 p100 involved cellular pathways, using fluorescence imaging and Co-IP/pull-down strategies to detect pathway interacting proteins.

3. RESULTS AND DISCUSSION

3.1 Publication I

"Epstein-Barr Virus encodes three bona fide ubiquitin-specific proteases"

This publication was divided into a bioinformatics part and a biochemical orientated experimental part. The overall aim was, to predict *in silico* EBV encoded ORFs with distant functional homologues of human deubiquitinating enzymes (DUBs) using sequence analysis methods. Candidate ORFs were further characterized by detecting their functional ability to process an ubiquitin conjugated reporter and probe construct. In following the *in silico* detected catalytic active cysteines were confirmed via mutation analysis.

Bioinformatics approach:

EBV encodes approximately 100 ORFs were most of them remain to be functional characterized. Given the essential role of DUBs within a cell, as described in previous chapters, a bioinformatics orientated strategy was developed with the aim to predict viral DUBs with distant functional human homologues. Four search strategies based on sequence analysis were used to identify putative DUBs encoded in the EBV genome.

(i) Sequence alignment with the conserved C- and H-boxes of known DUB families. As mentioned before, all DUBs contain conserved regions within their catalytic domains, which are for most of them located around the catalytic active cysteine and histidine, also called C- and H-boxes. In the case of the JAMM DUB family, which are metalloproteases, only H-boxes are present. A data set containing the sequences of human DUBs was aligned to the EBV ORF-eome by using the program CLUSTAL W. This widely used multiple sequence alignment program starts by doing a pair wise sequence alignment. Afterwards it generates a phylogenetic tree, a branching diagram showing the evolutionary relationships among species. Thereafter the program uses the phylogenetic tree to carry out a multiple alignment. Subsequently, sequence alignment with the BLAST (Basic Local Alignment Search Tool) program was performed. BLAST belongs to the most used sequence analysis methods and enables homology detection using pair wise sequence comparison. The characteristics of this program are short running times with a minimal sacrifice to distant sequence relationships. The resulting scores from a BLAST search have a well-defined statistical interpretation, making it possible to discriminate real matches from background hits (112).

(ii) Pattern search of conserved catalytic domains is a family based method, which uses as search criteria the conserved sequence information assembled in a set of homologue proteins (113). Patterns are the occurrence of distinct amino acids, which are conserved throughout members of a certain protein family. In our study, family based search pattern were derived from the aligned C- and H-box sequences of the known human DUBs. Pattern search with those conserved boxes accomplishes a more stringed assessment of the possibility that identified domains might be true C- or H-box homologues.

(iii) Hidden Markov Model (HMM)-based searches involve two steps. At first a statistical model from the sets of aligned sequences has to be build and second that model has to be compared to query sequences. HMM is a profile-based model, which is in general more sensitive than pair wise methods like described above. HMMs are "trained" to make use of position specific scoring matrices, which represent the distribution of amino acids at each position in the conserved domains of particular protein families (114). In our studies family specific HMMs were trained from the aligned C- and H-boxes of the different human DUB families.

(iv) Identification of Cys and His residues that are conserved in homologues encoded by other members of the HHV (human herpes virus) family, followed by an HMM search was the last step of investigation. This analysis was based on the assumption that amino acid residues, which are critical for protein function, like enzymatic activity, may be conserved in the HHV-family. In our studies we were able to locate conserved Cys and His residues in identified HHV-family ORF homologues by sequence comparison. In following the patches surrounded by the detected conserved Cys and His residues were searched for known DUB, C- and H-box patterns by using the DUB family specific trained HMMs.

Via the above described search strategies several viral candidate ORFs were identified to comprehend possible DUB function. To select the most potential ORFs for following functional studies, scoring criteria were devised for each search, which were compiled in a global DUB score (see publication I for details). In total 16 EBV ORFs, which achieved the highest global DUB score, were selected for following functional studies.

Biochemical approach:

In order to evaluate the possible DUB function of the viral candidate ORFs we developed a bacterial screening assay. We cloned the candidate ORFs or ORF-domains in GST bacterial expression vectors and designed a reporter construct, which is comprised of Ubiquitin C-terminal covalently linked with the N-terminus of GFP. Afterwards the Ub-GFP reporter

construct and one of the GST-tagged EBV-ORFs at the time were transformed into E.Coli. The enzymatic activity of the candidate ORF was assayed by co-expression of the Ub-GFP reporter construct. Cleavage of the Ub-GFP reporter was detected via SDS-Page and Western blotting by usage of a specific antibody against an epitope located on the GST protein. To check the positive cleavage of the reporter during each assay, a GST fused USP19 (human DUB) was used. Via this method three EBV candidate ORFs could be identified, which were able to cleave the Ub-GFP reporter significantly above background. The N-terminal fragment of BPLF1, a large tegumental protein with earlier described DUB activity conserved amongst the HHV-family (68) was able to cleave the Ub-GFP reporter to the highest degree. The EBV ORFs BSLF1, known to function as an EBV primase (117), and BXLF1, known to function as an EBV thymidine kinase (118), were also able to cleave the reporter.

In order to further characterize their DUB activity, those three proteins were overexpressed in bacteria and in following purified. The purified proteins were assayed by detection of cleavage of the flurogenic substrate Ub-AMC. This substrate contains the flurophore AMC, which upon 380 nm wavelength excitation and cleavage of the conjugated Ubiquitin starts to emit fluorescence with a wavelength of 460 nm (115). All three EBV ORFs hydrolyzed Ub-AMC with different kinetics suggesting different affinities for the substrate.

The ability of BSLF1 and BXLF1 to cleave Ub-AMC was significantly improved when equal amounts of immunoprecipitated proteins expressed in mammalian cells were taken for the assay. This circumstance may be explained by the impact of possible posttranslational modifications in increasing the activity of BSLF1 and BXLF1, which are not achieved in bacteria.

In order to further characterize the DUB activity of BPLF1-N, BSLF1 and BXLF1, the predicted catalytic active cysteine residues were mutated to alanine residues to obtain catalytic inactive proteins. These mutations were created by PCR mediated site-directed mutagenesis. Via this method the catalytic inactive protein mutants BPLF1_C61A, BSLF1_C819A_C824A and BXLF1_C491A were generated.

According to the annotated functions of BPLF1, BSLF1 and BXLF1 those proteins are involved in DNA replication and nucleotide metabolism pathways (68, 69, 117, 118) that are known to be regulated by ubiquitination and UbL-modification (2, 8, 9, 10). The following publication (II) will discuss the essential role of BPLF1_N in virus replication. The DUB functions of BSLF1 and BXLF1 within the virus life cycle need to be investigated in further studies.

3.2 Publication II

“A deneddylase encoded by Epstein–Barr virus promotes viral DNA replication by regulating the activity of cullin-RING ligases”

The overall aim of this work was to elucidate the functional purpose of the discovered deneddylase activity comprised in the N-terminal fragment of the viral encoded BPLF1 ORF. We were able to confirm the deneddylase activity of BPLF1-N in different assays with different NEDD8 conjugated functional probes. We identified members of the cullin family as substrates of BPLF1-N´s deneddylase activity. We further elucidated the consequence of BPLF1-N binding and deneddylating cullins assembled in CRLs, which was the stabilization of CRL substrates involved in cell cycle regulation. We could show that this BPLF1-N controlled accumulation of replication regulating proteins was essential for an S-phase like cellular environment and endoreduplication in BPLF1-N expressing cells. We further could demonstrate that the impact of BPLF1-N expression on viral genome replication was controlled by one stabilized CRL substrate, the DNA licensing factor CDT1.

In order to extend the scope of the bacterial screening assay we designed new reporter constructs. Apart from the Ub-GFP reporter, we further constructed NEDD8-GFP, SUMO1-GFP, SUMO2-GFP, SUMO3-GFP and ISG15-GFP reporter by linking the C-terminus of the UbL-modifier covalently to the N-terminus of GFP. We used an EBV-ORF library, were the entire ORF or ORF-domains were cloned into GST-bacterial expression vectors. The assay was performed by expressing the particular Ub/UbL-reporter construct together with one of the GST-tagged EBV-ORFs in E.Coli bacteria. The possible cleavage of the Ub/UbL-GFP reporter was detected via SDS-PAGE and Western blotting by usage of a specific antibody against an epitope located on the GST protein. The most striking outcome of this assay was, that the N-terminal fragment of BPLF1 was able to process the NEDD8-GFP reporter with similar efficiency as the Ub-GFP reporter.
In order to characterize further the novel discovered deneedylase activity of BPLF1, we performed further functional assays. We detected, that bacterial expressed GST-BPLF1-N processed the flurogenic substrates Ub-AMC and NEDD8-AMC with equal efficiency. We compared the ability of eukaryotic expressed BPLF1-N to bind to the Ub-VS and NEDD8-VS functional probes. Those probes are used to characterize catalytic active cysteine deconjugases (32). The interaction of the probe towards the enzyme can be visualized by a

shifted enzyme protein band in a Western Blot. As a result we detected, that BPLF1-N was able to bind both probes with comparable efficiency.

Taken together all three functional assays with the functional reporter and probes Ub/NEDD8-GFP, Ub/NEDD8-AMC and Ub/NEDD8-VS suggest similar affinities for BPLF1-N towards ubiquitinated and neddylated substrates.

Next we addressed the question, if BPLF1-N was able to deneddylate cellular proteins *in vitro* and in eukaryotic cells. We expressed V5-tagged NEDD8 in eukaryotic cells and captured neddylated conjugates on anti-V5-conjugated sepharose beads. Those beads were incubated *in vitro* with purified GST-BPLF1-N, resulting in hydrolysis of cellular NEDD8 conjugates. The same experiment was repeated with the difference, that Flag-BPLF1 was co-expressed in the V5-NEDD8 expressing eukaryotic cells. After lysing the cells and detecting the V5-NEDD8 conjugates in a Western blot, we observed that BPLF1-N was able to hydrolyse the neddylated cellular conjugates. Thus, BPLF1-N appears to be a true deneddylase.

After this indication we were wondering which cellular neddylated substrates are targeted by BPLF1-N. The best characterized neddylated cellular proteins are the cullin family, which comprise in human six members: Cul-1,-2,-3,-4a,-4b,-5. The C-terminal neddylation of Cullins assembled in cullin RING ligases (CRLs) was shown to enable a sufficient polyubiquitination of the particular CRL substrate protein (13, 14). In our studies we could show, that BPLF1-N was able to deneddylate Cul1 and Cul4a. We co-expressed in eukaryotic cells Flag-BPLF1-N with V5-NEDD8 and Myc tagged Cul1 and Cul4a, which are two representative members of the cullin family. After lysing the cells and running their lysate on a SDS-PAGE, we were able to detect BPLF1-N´s ability to hydrolyze V5-NEDD8 conjugated Cul1 and Cul4a.

After showing this effect we wanted to investigate, if the enzyme also binds to those two cullins. We transfected eukaryotic cells with Flag-BPLF1-N and Myc-Cul1 or Myc-Cul4a and performed co-immunoprecipitations (Co-IPs). Cell lysates were separated and the Co-IPs were conducted in both ways, using the Flag- and Myc- antibody. Both cullins were shown to bind to BPLF1-N in both Co-IP´s (Flag- and Myc- antibody).

After showing this interaction for overexpressed Cul1 and Cul4a, we repeated this Co-IP experiment performed with endogenously expressed Cul4a. We transfected eukaryotic cells with Flag-BPLF1-N and separated again the cell lysates, for Co-IP experiments in both ways, with an anti-Cul4a and an anti-Flag antibody. Endogenous Cul4a was able to bind Flag-BPLF1-N, detected in the anti-Cul4a and anti-Flag Co-IP.

Neddylation of cullins integrated in CRLs enables the E3-ligase complex to polyubiquitinate

its specific substrate, which leads to its proteasomal degradation (13). We were wondering, if we could stabilize known CRL substrates by overexpressing BPLF-1-N. By knowing that BPLF1 is a nuclear protein expressed in the early phase of the productive virus cycle, we assumed an involvement of the protein in DNA replication. Several CRL regulated substrates are involved in cell cycle control, like the licensing factor CDT1 and the tumor suppressor p21 and p27 (120, 121). All those proteins are targeted by Cul1 and/or Cul4 based CRLs (119). Expression of those CRL substrates together with BPLF1-N lead to their stabilisation detected via Western blot. The accumulation of those cell cycle regulators was reversed in a dose dependent manner by overexpression of V5-NEDD8, whereas a mutant V5-NEDD8-VV, lacking the C-terminal Gly residue required for conjugation, and HA-ubiquitin had minor or no effects.

As described in earlier chapters, viral modulation of CRLs is a common mechanism to control pathways essential for the virus life cycle, like viral replication or hiding from the immune surveillance. Several DNA viruses, like HPV (75), Adenovirus (76), SV40 (79) and now also due to our studies EBV, have been described to manipulate CRLs to insure their undisturbed genome replication.

After overexpression of BPLF1-N in various eukaryotic cell types it could be shown, that the enzyme localises predominately to the nucleus, detected via immunofluorescence. The effect of this nuclear accumulation of BPLF1-N was an increased size of the nucleus and on prolonged expression, extensive cell death. Those detected effects were not due its overexpression but due its catalytic activity, because a catalytic inactive mutant, BPLF1-N C61A missed to establish this phenotype.

To investigate the cause of the nuclear enlargement, BPLF1-N transfected cells were stained with propidium iodide, a fluorescent agent, which binds to DNA. After this treatment the cell cycle profile and DNA content was assessed. Taken together the compiled results of those experiments indicated a deregulation of the S-phase and an ongoing re-replication of cellular DNA without mitosis (endoreduplication). Almost all BPLF1-N expressing cells died within 4-5 days. This phenotype was reversed in a dose-dependent manner by overexpression of NEDD8 but not by overexpression of ubiquitin. This result suggests that BPLF1-N´s deneddylase activity rather then its DUB activity seems to be responsible for this here described phenotype. Further BPLF1-N overexpression experiments in different eukaryotic cell lines elucidated the stabilisation of several CRL substrates or activation of certain regulator proteins, essential for DNA synthesis and cell cycle progressing. The composition of those activated or stabilised proteins favours the earlier described S-phase like

environment and endoreduplication in BPLF1-N expressing cells (see for details publication II). Among those stabilised proteins was the DNA licensing factor CDT1 (chromatin licensing and DNA replication factor 1). CDT1 functions as a regulatory protein of the pre-RC (pre-replication complex), which is essential for the initiation of DNA replication. The pre-RC binds to the oriP (origin of replication) and "licenses" the initiation of replication during the S-phase. Cdt1 is tightly regulated through ubiquitin-dependent proteolysis facilitated by Cul1 and Cul4 based CRLs (122). The involvement of the cellular DNA replication machinery in replication of the viral genome is poorly understood for the HHV-family.

To elucidate whether the detected S-phase like environment and endoreduplication in BPLF1-N expressing cells has an impact of viral replication we used the Akata-Bx1 cell line. This B-cell lymphoma cell line carries a recombinant EBV genome within, which can be induced to start its lytic cycle by crosslinking of cell surface immunoglobulins. Entrance into the viral replication state can be visualised by the expression of a viral early protein substituted with GFP (green fluorescent protein). EBV replication in those cells caused the same phenotype as in BPLF1-N overexpressed cell lines, an S-phase like environment and endoreduplication, detected via cell cycle diagram assays.

The same milieu of activated or CRL stabilised proteins essential for DNA synthesis and cell cycle progressing was detected in those EBV reactivated cells as in BPLF1-N expressing cells. Among them also the stabilised Cul1 and Cul4 based CRL target CDT1. Upon silencing BPLF1 expression in those induced Akata cells via shRNA (short hairpin RNA) against BPLF1 mRNA, the normal cell cycle diagram could be reconstituted. After overexpression of CDT1 in those BPLF1 knock downed cells, the BPLF1-N detected S-phase like phenotype could be reconstituted to a certain extend. This result demonstrates the big impact of the BPLF1-N stabilized CRL substrate CDT1. It also indicates the essential involvement of probably other stabilized CRL substrates to reconstitute the BPLF1-N overexpression phenotype.

In order to investigate the impact of BPLF1 expression towards viral genome replication, we assayed the viral DNA contend in BPLF1 knock down induced Akata cells via RTq-PCR (real time quantitative-PCR). The knock down of BPLF1 resulted in an almost complete inhibition of virus DNA replication. Thereafter, we also wanted to investigate the impact of overexpressed CDT1 in BPLF1 knock downed, induced Akata cells. Overexpression of CDT1 let to reactivation of viral replication to an almost full degree.

To summaries, this data demonstrates that stabilisation of CDT1 is critical for the introduction of an S-phase-like cellular environment, and the driving factor for viral replication.

To investigate whether BPLF1-N´s deneddylase activity and caused induction of cellular DNA re-replication are conserved among the HHV-family, we tested the BPLF1 homologues UL36 of HSV and M48 of MCMV. GST fusions of the N-termini of UL36 and M48 cleaved the Ub-GFP and NEDD8-GFP reporter constructs with comparable efficiency in the above explained bacterial screening assay. Also Flag tagged UL36-N and M48-N reacted with the Ub-VS and NEDD8-VS suicidal probes as described earlier for Flag-BPLF1-N. Overexpression of Flag-UL36-N and Flag-M48-N also introduced an S-phase cellular environment and endoreduplication, as seen after Flag-BPLF1-N expression. Taken together, those results suggest that the function of BPLF1 is conserved among members of the HHV-family.

3.3 Publication III

"AmotL2 links E-cadherin to contractile actin filaments and controls cell topology"

The general aim of this work was to elucidate the function of AmotL2 p100 in epithelial cells. In this study, we describe that the longer AmotL2 isoform, AmotL2 p100, localizes to the adherens junctions of polarized epithelial cells and controls cell geometry and topology.
We stably knocked down AmotL2 in MDCK and Caco-2 epithelial cells as in HaCaT keratinocytes by using shRNA carrying lentiviruses targeting AmotL2. After polarizing those AmotL2 shRNA cells we detected a loss of actin filaments that run perpendicular to the cell-cell junctions. This loss of actin filaments destabilized the cellular architecture so dramatically, that cells were not able to maintain their typical cuboidal architecture. Instead cells collapsed and grew flat on their substrate leading to a ~6-fold increase of cellular surface area. This effect was due to the depletion of AmotL2 level as overexpression of human AmotL2 p100 in AmotL2 shRNA MDCK cells rescued normal cell surface area.
Confluent grown polarized epithelial cells predominately adopt a hexagonal or pentagonal cell shape which is lost in AmotL2 shRNA cells. After polarizing AmotL2 depleted MDCK and Caco-2 cells their cellular shape was changed which resulted in cells bordering predominately to only four other neighbors. To confirm that the loss of contractile actin fibers was responsible for the seen effect we treated cells with the actin contractility inhibitor blebbistatin which affected epithelial cell geometry similar to the phenotype observed after AmotL2 depletion.

MDCK and Caco-2 cells form cyst-like structures when grown in soft matrix *in vitro*. Cyst formation was almost completely abrogated in AmotL2 shRNA cysts, which grew as clumps of cells with no distinguishable apical or basal compartments. The loss of the ability to form cysts may result from the lost capacity to establish normal epithelial cellular geometry. However, AmotL2 depletion in MDCK cells did not affect cell polarity, as detected by analyzing polarity markers as E-cadherin, β-catenin, Ezrin and podocalyxin.

After detecting the loss of actin fibers causing alterations of cell geometry in different AmotL2 shRNA epithelial cells *in vitro* we were able to detect the same phenotype in AmotL2 knock out zebrafish embryos *in vivo*. We targeted the translation initiation site of AmotL2 using an anti-sense morpholino (MO) approach. Skin keratinocytes of AmotL2 MO zebrafish embryos showed an almost complete removal of cytoplasmic actin filaments. The cell surface area was doubled and the cell shapes shifted from predominately hexagonal towards preferential pentagonal shaped cells in AmotL2 MO skin keratinocytes.

Co-immunoprecipitation strategies revealed that AmotL2 p100 binds to E-cadherin, MAGI1 and actin. By mutating the first PY-motif of AmotL2, (precisely the LPTY-motif) we were able to disrupt the binding from AmotL2 to MAGI1 and actin. The binding towards E-cadherin still needs to be elucidated.

As we detected a loss of contractile actin filaments that run perpendicular to the cell-cell junctions we addressed the question if AmotL2 depletion would render epithelial sheet topology. AmotL2 shRNA keratinocytes were grown on flexible silicon matrices to confluency and in following stretched. AmotL2 shRNA cells broke at the sites of junctions due lack of supporting actin fibers whereat Ctrl shRNA could withstand the mechanical stress and keep attached to each other.

The Hippo pathway is involved in organ size control by regulating cell proliferation, differentiation and apoptosis. The only downstream targets so far described are the transcriptional co-activators YAP and TAZ (Chan, Lim et al. 2013). By comparing AmotL2 and YAP/TAZ activated gene levels in various tissues of various patient samples (GeneSapiens database) we found a clear correlation between YAP target genes and AmotL2 expression across tissues.

To analyze whether AmotL2 knockdown affected proliferation rates and contact inhibition we conducted cell-doubling assays. Analysis of the resulting growth curves showed that AmotL2 shRNA cells had a slower growth rate and reached the stage of contact inhibition at a lower density than the Ctrl shRNA cells.

Upon cell-cell contact YAP1 was described to re-localize to the cytoplasm explaining why

cells stop proliferating upon cell-cell contact (von Gise, Lin et al. 2012). By investigating YAP1 subcellular localization in response to increased cell density we could show that AmotL2 shRNA cells localize YAP1 earlier to the cytoplasm than the Ctrl shRNA cells. This effect was due to the increased cell size and thereby premature cell-cell contact inhibition in AmotL2 shRNA cells. These data were consistent with our findings in zebrafish where the skin of AmotL2 morphants exhibited a lower cellular density than the control morphants.
We concluded that AmotL2 is a critical component in the adhesion junctions that controls intracellular contractility as well as relaying forces between cells.

4.1 FURTHER PERSPECTIVES - *EPSTEIN-BARR VIRUS ENCODED DECONJUGASES*

In previous experiments we could show, that BPLF1-N was able to bind to Cul1 and Cul4a. In order to address the question, if BPLF1-N was able to bind to the whole cullin family, we overexpressed all Myc-tagged cullins (Cul-1,-2,-3,-4a,-4b,-5) together with Flag-BPLF1-N in eukaryotic cells. In following we performed Co-IP experiments with Flag- and Myc-antibodies and were able to Co-IP Flag-BPLF1 with all Myc-cullins and also all Myc-cullins with Flag-BPLF1. These results indicate that CRLs comprised of the entire cullin family may be target of BPLF1-N.
We were also interested to investigate on which cullin domain BPLF1-N binding occurs and if the binding is direct or if cellular adaptors are involved. To address this question we generated different GST tagged Cul4a domains. In following pull-down assays with eukaryotic cell lysates containing overexpressed Flag-BPLF1-N, we could identify its site of interaction towards the cullin C-terminus, closely located to the neddylation site. In order to investigate, if cellular adaptors are involved in this binding, the experiment was repeated with bacterial expressed and purified His-BPLF1-N. Also this attempt resulted in binding of BPLF1-N to the same C-terminal located Cul4a domain, showing that this interaction is direct and no cellular adaptors are involved.
In order to elucidate the BPLF1-N residues involved in binding towards cullins, *in silico* sequence alignments among all human virus homologues of BPLF1_N were conducted. The alignment result revealed a conserved α-helical structure located on the enzymes surface. A high degree on conservation was detected on two charged residues Asp86 and Asp90 pointing outwards of this α-helix. After mutating those two residues into opposite charged residues

Asp86Arg and Asp90Arg the interaction of Flag-BPLF1_D86R_D90R towards Myc-Cul1 and Myc-Cul4a could be reduced to ~90%, detected via Co-IP experiments. To insure that this degrease in binding was not due possible structural changes caused by the mutation, the conserved α- helical structure containing Asp86 and Asp90 were cloned into a bacterial His-expression vector. In following the same mutations D86R_D90R were applied on this BPLF1-N helical fragment and pull down assays were conducted with GST-Cul4a. The result was showing again a ~90% reduction in binding compared to the wild type BPLF1-N helical fragment. The outcome of those binding experiments strongly suggests that the two conserved residues Asp86 and Asp90 located within the conserved α- helical structure are the essential residues involved in the interaction towards Cul1 and Cul4a and possibly to all cullins.

4.2 FURTHER PERSPECTIVES - *AMOTL2 IN CONTROL OF CELL TOPOLOGY*

In the third report of this thesis, we have identified a novel link between cell contacts and the cytoskeleton. The connection of E-cadherin to the actin filaments have been a point of great controversy (Drees, Pokutta et al. 2005). Our data shed light on this aspect as it identifies AmotL2 as a specific linker between E-cadherin and the cytoskeleton. However, a number of questions remain. For example, the exact composition of this complex is unclear. Future experiments using deletion constructs will provide evidence about which exact domains of AmotL2 mediate binding to E-cadherin and whether these are direct or indirect interactions.

Another issue is how the ternary complex of AmotL2 and E-cadherin specifically orchestrates the development of actin filaments that run perpendicular to the cell membrane. The physical coupling of MAGI1 to AmotL2 is essential for the binding to actin. MAGI1 has been shown to bind GTPase exchange factor PDZ-GEF1 which in its turn activates RAP1 (a small GTPase known to regulate the actin cytoskeleton) (Sakurai, Fukuhara et al. 2006). It is therefore conceivable that the homotypic interaction of E-cadherin leads to the activation of RAP1 in an AmotL2 dependent manner and thus regulates actin fiber formation. Future experiments will address whether AmotL2 is required for RAP1 activity during cell contact formation.
We present evidence that AmotL2 associates to actin filaments, which mechanically connect cells with each other and which are essential for maintaining epithelial geometry and topology. It is tempting to speculate that AmotL2 is part of a mechanotransduction complex

that can relay signals over multiple cell layers in epithelial sheets. Future experiments will perhaps provide evidence on whether mechanical and biochemical signals can be transduced via AmotL2. One possible effector is clearly YAP1, however, our experiments indicate that AmotL2 indirectly controls YAP1 cellular localization by regulating cell shape.

Is AmotL2 essential for normal organogenesis? Parallel studies in the lab by Hultin, Zheng et al. show that AmotL2 is required for the expansion of the dorsal aorta. This was shown in zebrafish and in mouse. Using conditional AmotL2 KO mice it will be possible to address whether AmotL2 is required for normal skin development and function. This should be also tested in models of wound healing and tumor development. Taken together, the data presented in paper 3 will perhaps provide the grounds for further studies on how epithelial cells connect mechanically and how force may be translated to biochemical signals.

We could show in our experiments that AmotL2 depleted keratinocytes (HaCaT) were able to undergo cell-cell contact inhibition leading to a termination of proliferation. AmotL2 shRNA cells undergo earlier inhibition than the Ctrl shRNA cells due to their increased cell size leading to lower cell counts per defined cell area. As a readout of proliferation termination we used the cytoplasmic localization of the transcriptional co-activator YAP1 towards the cytosol. The relocalization of YAP1 from the nucleus to the cytoplasm in confluent cells has been suggested to explain why cells stop proliferating upon cell-cell contact (von Gise, Lin et al. 2012). We would like to confirm the end of proliferation via the more established BrdU cell proliferation assay. BrdU is a chemical dye, which substitutes for thymidine in newly synthesized DNA. A later used BrdU antibody visualizes the compound, detecting cells which are still proliferating (Waldman, Dolbeare et al. 1988). We also want to confirm our data by reproducing the conducted proliferation assays with other epithelial cell lines, like AmotL2 shRNA MDCK and Caco2 cells.

We could detect via co-immunoprecipitation strategies, that AmotL2 p100 binds to the adherens junctions components E-cadherin, MAGI1 and actin. We pinpointed the site of interaction from MAGI1 and actin towards the N-terminal located LPTY-motif of AmotL2 p100 by substituting the tyrosine to alanine on that conserved binding site. To elucidate the site of interaction towards E-cadherin, we want to repeat our co-immunoprecipitation experiments by overexpressing truncated AmotL2 p100 fragments covering the whole protein length. We detected a vanishing of contractile actin fibers which run perpendicular to cell-cell junctions causing alterations of cell geometry in different AmotL2 shRNA epithelial cells *in vitro* as well as in AmotL2 KO zebrafish embryos *in vivo.* We generated AmotL2 KO mice

and want to evaluate cell geometry in mice skin to verify our data.

5. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who has supported me in accomplishing this thesis. Special thanks to:

My beloved wife Vivian, who has supported me very much during the time of my PhD. Vivian, thank you for encouraging me and staying by my side through the ups and downs!

My supervisors:
Maria Masucci and Lars Holmgren, who took me in as a graduate student and introduced me to the world of science. Maria and Lars, thank you for the time in you lab it was really an experience I would not like to miss. I admire your ambition and dedication to your work and wish you all the best! Lars, thank you for having so much fun in the lab!
Kristina Lindsten and Arindam Majumdar, who were my co-supervisor. Also to you many thanks for everything! I could always come to you, when I had a scientific question and you always took your time. Arindam, I will miss our philosophic talks!

My mentors:
Teresa Frisan and Gerald McInerney, who I could always turn to always. Teresa, we had so much fun in the lab, especially because our benches were next to each other. Gerry, thank you for being a good friend to me!

People, I worked close together with:
I would like to thank Thorsten Pfirmann, Stefano Gastaldello, Sara Hultin, Mahdi Mojallal and Yujuan Zheng. Working together with you on a project was a pleasure and increased my ability to work and think in a team. I wish you for your future all the best!

All former and present members of the group:

An old German adage says that the character of a person is formed by the interaction with other persons. That's why my friends, thank you for creating me! It was a pleasure to work with you and I will always remember you. Thank you Bavesh, Bin, Diego, Dimath, Eugenie, Gerco, Helena, Irmelie, Javier, Kelly, Lina, Linda, Mia, Micke, Nathalie, Nouman, Pino, Omid, Roberta, Riccardo, Rikard, Simone, Tanja, Ulrika, Xinsong, Yuanyuan.

Thank you also to the Nature publishing group for permitting me the reprint of Figure 1 and 2 in the thesis introduction- *AmotL2 in control of cell topology*.

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