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The role of the deubiquitinase USP24 in multiple myeloma

Laura Häusler

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Abstract

As the second most common hematologic neoplasm, multiple myeloma (MM) accounts for 1% of all cancers in Europe. Chromosomal instability, genetic heterogeneity and slow, but persistent, progression are main characteristics of this malignancy. Although MM remains an incurable disease, therapy response rates and progression free survival have significantly improved within the last decade – an achievement mainly owed the introduction of proteasome inhibitors. To warrant balanced metabolism and gene transcription as well as controlled cellular proliferation, the ubiquitin proteasome system (UPS) implies numerous highly conserved processes, which regulate stability, turnover and function of proteins. Key members of this system are the 26S proteasome, able to recognize and degrade ubiquitin-bound proteins, the ubiquitin ligases (E3), which catalyze the covalent linkage of proteins and ubiquitin molecules, and the deubiquitinating enzymes (DUBs). DUBs hydrolyze the bonds between ubiquitin molecules and proteins, to rescue their substrates from degradation or to impart them with a new function. Despite the effectiveness of proteasome inhibitors in the treatment of MM, the underlying deregulated UPS events have remained largely unknown. In search for potential candidates, analysis of comparative genomic hybridization arrays revealed the loss of the Usp24 gene locus, encoding for the orphan DUB USP24, in 25% of MM patient samples. In an unbiased mass spectrometric screen WWP2 (WW domain containing E3 ubiquitin protein ligase 2) was identified as a binding partner of USP24 – an E3 ligase, known to interact with members of the TGF-beta system. Hence, this study characterized the connection between the TGF-beta pathway and USP24, revealing SMAD3 as a critical interaction partner of this DUB. The receptor-activated SMAD3 complies an essential function as a cytoplasmic mediator of TGF-beta signaling, responsible for the initiation of transcriptional responses. Presented experiments show enhanced SMAD3 activation and thus deregulation of TGF-beta signaling in USP24 deficient cells – representative for 25% of MM patients and in accordance with the fact, that the TGF-beta system is deregulated in this cancer. Hence, this study defined USP24 as a UPS member, that is involved in the regulation of the TGF-beta signaling pathway with a potential role as a tumor suppressor.

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Abbreviations

aCGH ..... Array comparative genomic hybridization
AIP2 ... Atrophin-1-interacting protein 2
APS ..... Ammonium persulfate
ASCT..... Autologous stem cell transplantation
ATP ..... Adenosintriphosphat
BES ..... N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
BM..... Bone marrow
BMMC Bone marrow mononuclear cell
BMP ..... Bone morphogenetic protein
BMSC..... Bone marrow stromal cell
BrdU .....5-Bromo-2'-desoxyuridine
BS ..... Bovine Serum
BSA .....Albumin fraction V
$CaCl_2$..... Calcium chloride
CD .....Cluster of differentiation
cDNA.....Complementary DNA
CO_2 .....Carbon dioxide
Co-IP .....Co-immunoprecipitation
Co-SMAD ..... Collaborating SMAD
CRAB ..... Hypercalcemia, renal insufficiency, anemia, bone lesions
dH_2O ..... Aqua destillata
DMEM .....Dulbecco's Modified Eagel's Medium
DMSO .....Dimethyl sulfoxide
DNA..... Desoxyribonucleic acid
dNTP .... Deoxynucleotide triphosphate
dsDNA.....Double-stranded DNA
DTT .....Dithiothreitol
DUB..... Deubiquitinating enzyme
E1..... Ubiquitin activating enzyme
E2..... Ubiquitin carrier protein
E3..... Ubiquitin ligase
E4 ..... Ubiquitin-chain elongation factor
ECM ..... Extracellular matrix
Ecto Ectodermin/Tif1γ
EDTA. Ethylenediaminetetraacetic acid
ESI ..... Electrospray ionization
ESMO.... European society for medical oncology
eV .....Empty vector
FACS..... Fluorescence activated cell sorting
FBS ..... Fetal Bovine Serum
FBXO ..... F-box protein
Fc .....Fragment crystallizable
FISH Fluorescence in situ hybridization
FSC .....Forward scatter
fw ..... Forward
G2P .... Glycerol 2-phosphate disodium salt hydrate
GDF.. Growth and differentiation factor
GFP..... Green fluorescent protein
HCl ..... Hydrochloric acid
HECT .... Homologous to the E6-AP C-terminus
HEPES ..... 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure
HSC90..... Heat shock cognate 90
Ig ..... Immunglobulin
IGF-1 .....Insulin-like growth factor-1
IL-6 .....Interleukin-6
IMDM..... Iscove's Modified Dulbecco's Media
IMiD..... Immunomodulatory imide drug

IP ..... Immunoprecipitation
IPTG..... Isopropyl beta-D-1-thiogalactopyranoside
I-SMAD..... Inhibitory SMAD
ISS ..... International staging system
JAMM..... JABI/MPN/MOV34 metalloprotease
KCl ..... Potassium chloride
LAP ..... Latency-associated protein
LDH..... Lactat dehydrogenase
LTBP . Latent TGF-beta binding protein
LTR ..... Long terminal repeat
m/z ..... Mass-to-charge ratio
MAD Mothers against decapentaplegic
MAF... Musculoaponeuotic fibrosarcom oncogene
MALDI ..... Matrix-assisted laser desorption/ionization
MAPK ..... Mitogen-activated protein kinase
MCL-1 ..... BCL2 family apoptosis regulator
$MgCl_2$ ..... Magnesium chloride
MGUS ..... Monoclonal gammopathy of undetermined significance
MH..... MAD-homology domain
MIF ..... Müllerian inhibitory factor
miRNA..... Micro-RNA
MJD.. Machado-Josephin-domain DUB
MM ..... Multiple myeloma
MMSET ..... Multiple myeloma SET domain containing protein
mRNA..... Messenger RNA
MS..... Mass spectrometry
Myc..... Myelocytomatosis oncogene
n Number of independent experiments
Na_2HPO_4 Dinatriumhydrogenphosphat
Na_3VO_4..... Sodium orthovanadate
NaCl ..... Sodium chloride
NES..... Nuclear export signal
NF-κB ..... Nuclear factor κB
NP40 ..... Nonyl phenoxypolyethoxylethanol
OTU..... Ovarian tumor DUB
PARP-1 Poly ADP-ribose polymerase-1
PBS ..... Phosphate buffered saline
PCR..... Polymerase chain reaction
PI ..... Proteasome inhibitor
PPXY..... Proline-proline-x-tyrosine
pSMAD3..... Phosphorylated SMAD3
PTM..... Post translational modification
PVDF..... Polyvinylidenfluorid
PY ..... Proline-tyrosine
qPCR..... Quantitative real time PCR
RING ..... Really interesting new gene
RNA..... Ribonucleic acid
R-SMAD ..... Receptor-activated SMAD
RT ..... Reverse transcription
rv ..... Reverse
SARA ..... SMAD anchor for receptor activation
SBE ..... SMAD-binding-element
SCF Skp1/Cullin/F-box
SDF-1..... Stroma-derived-factor-1
SDS..... Sodium dodecylsulfate
SEM ..... Standard error of the mean
shRNA..... Short hairpin RNA

SILAC.....Stable isotope labeling by amino acids in cell culture

siRNA..... Small interfering RNA

SMM.....Smoldering multiple myeloma

SMURF ..... SMAD ubiquitination regulatory factor

SOC ..... Super optimal broth

SSC.....Side scatter

TBB .4,5,6,7-Tetrabromo benzotriazole

TBE ..... UltraPure™ 10 x Tris/Borate/EDTA

TbRI ..... TGF-beta receptor type I

TbRII ..... TGF-beta receptor type II

TBS ..... Tris buffered saline

TCA.....Trichloroacetic acid

TEMED....Tetramethylethylenediamine

TGF-beta.Transforming growth factor-beta

TLCK..... Tosyl-L-lysyl-chloromethyl ketone hydrochloride

TMA..... Tissue microarray

TNFα ..... tumor necrosis factor α

TPCK Tosyl-phenylalanyl-chloromethyl ketone

Tris ..... Tris(hydroxymethyl)aminomethane

TUM .....Technical University Munich

UBA..... Ubiquitin-associated domain

UCH .... Ubiquitin C-terminal hydrolase

UPS..... Ubiquitin proteasome system

USP..... Ubiquitin specific peptidase

VEGF ..... Vascular endothelial growth factor

VLA-4 ..... Very late antigen-4

WB ..... Washing buffer

WW ..... Double tryptophan

WWP ..... WW domain containing E3 ubiquitin protein ligase

1 Introduction

1.1 Multiple myeloma

Multiple myeloma (MM) belongs to the group of indolent Non-Hodgkin lymphoma (Kompetenznetz-Maligne-Lymphome-e.V., 2016). It is a chronic disease caused by malignant plasma cell clones, derived from post-germinal-center B cells. With an incidence of 4.5 – 6.0/100 000/year in Europe it represents 1% of all cancers and 10% of hematologic neoplasms (Moreau et al., 2013). The average age at diagnosis ranges from 64 to 70 years. MM is more common in men than in women (Moreau et al., 2013; Raab, Podar, Breitkreutz, Richardson, & Anderson, 2009). Besides male gender and older age there are other risk factors for this cancer including African American racial background and secondary risk factors like persistent, low-grade inflammation, possibly the presence of inflammatory conditions, e.g. obesity, as well as chronic immunodeficiency (Blair, Cerhan, Folsom, & Ross, 2005; L. M. Brown et al., 2001; Fairfield, Falank, Avery, & Reagan, 2016). MM remains an incurable disease with a relative 5-year survival rate of 45% (2009 – 2010) and a mortality of 4.1/100 000/year (Gerecke et al., 2016; Moreau et al., 2013). Hence further research for a better understanding of MM pathogenesis is needed, especially for developing individualized and more effective treatment strategies.

1.1.1 Genetic aberrations and molecular pathogenesis

Nearly all MM patients evolve from a bone marrow disorder named monoclonal gammopathy of undetermined significance (MGUS). At a rate of 1% per year MGUS progresses to the asymptomatic but more severe stage of smoldering multiple myeloma (SMM) to finally become an overt or symptomatic multiple myeloma (s. figure 1) (Fairfield et al., 2016; Kuehl & Bergsagel, 2002; Moreau et al., 2013). Via clonal evolution and acquisition of further genetic changes MM might later progress to a bone-marrow independent, highly aggressive disorder, referred to as plasma cell leukemia (Fairfield et al., 2016). The mechanisms of malignant transition are not completely understood and under ongoing research, but there seems to be a pathogenic link between MM risk factors, hypoxia in the bone marrow microenvironment and genetic instability (Azab et al., 2012; Lohr et al., 2014).

To differentiate into plasma cells, immature B-cells undergo various genetic changes like VDJ-recombinations, somatic mutations and isotype switching. Thus B cells are characterized by genetic instability, favoring mutations in particular at the immunoglobulin switch region on chromosome 14(q32.33) (Sirohi & Powles, 2004). Such primary genetic changes can be found in MGUS, SMM and MM. Translocations juxtapose the immunglobulin heavy chain (IgH) locus to an oncogene – most frequently to MAF (t[14;16][q32.33;23]) or MMSET (t[4;14][p16.3;q32.33]) encoding for the transcription factor c-MAF (musculoaponeurotic fibrosarcoma oncogene) and the nuclear protein MMSET (multiple myeloma SET domain containing protein) respectively (Avet-Loiseau et al., 1999; Mirabella et al., 2013). These mutations increase genetic instability and thereby facilitate accumulation of further changes in the human genome. Another important early chromosomal abnormality is hyperploidy – in particular trisomie 13 (Morgan, Walker, & Davies, 2012). Secondary mutations are implicated in disease progression and can only be detected in overt MM but not in the premalignant MGUS (Palumbo & Anderson, 2011). Analyzing bone marrow aspirates and peripheral blood samples of 203 MM patients Lohr et al. identified KRAS, NRAS, FAM46C, DIS3 and TP53 as the most commonly mutated genes in overt MM (Lohr et al., 2014). Other secondary changes involve the members of the MAPK (mitogen-activated protein kinase) or NF-κB (Nuclear factor κB) signaling pathway as well as the inactivation of tumor suppressors like CDKN2A and CDKN2C, acting as cell cycle regulators. The importance of MAPK and NF-κB signaling for MM pathogenesis is highlighted by numerous mutations of genes controlling these pathways (e.g KRAS, BRAF, TRAF3, CYLD, NFKB1 and 2, CD40, MAP3K1 and14) (Chapman et al., 2011; Keats et al., 2007; Kuehl & Bergsagel, 2002; Lohr et al., 2014). With regard to the increasing number of identified genetic changes in MM it becomes clear, that chromosomal instability is a main feature of this disease. Hence, the identification of effective therapies is limited by the high heterogeneity of MM tumors (Gado, Silva, Paloczi, Domjan, & Falus, 2001).

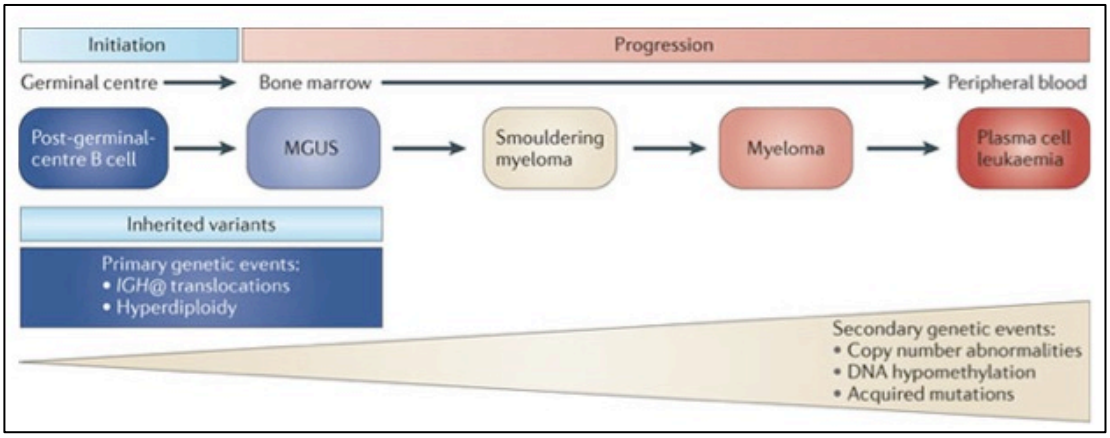


Figure 1: Gentic events in MM pathogenesis

The two upper bars (blue and red) indicate the stage of malignant transformation (initiation and progression). Beneath, the location of cells is described starting with the germinal centre, where post-germinal-centre B cells undergo primary genetic events, which are listed in the blue rectangle. After homing to the bone marrow MGUS develops to smouldering multiple myeloma through acquisition of further mutations. Latter are referred to as secondary genetic events and shown in the yellow triangle. Smouldering multiple myeloma transforms to overt multiple myeloma, which can finally progress to drug resistant plasma cell leukemia (dark red box) (adapted from (Morgan et al., 2012)).

Once differentiation to plasma cells in the lymphoid tissue is terminated, the cells remain in this stage unable to proliferate or to change their molecular characteristics; finally they localize to the bone marrow, a process called „homing“ (Manier, Sacco, Leleu, Ghobrial, & Roccaro, 2012). Just as their physiologic counterparts MM cells are attracted by the bone marrow microenvironment, where they bind to fibronectin, protecting them from apoptosis, as well as to bone-marrow stromal cells via adhesion molecules like VLA-4 (very-late antigen-4) (Sirohi & Powles, 2004). This binding induces transcription and secretion of multiple cytokines, e.g. IL-6 (interleukin-6), IGF-1 (insulin-like growth factor-1), VEGF (vascular endothelial growth factor), TNFα (tumor necrosis factor α), TGF-beta (transforming growth factor-beta) and SDF-1 (stroma-derived-factor-1), which are essential for MM progression (Anderson & Carrasco, 2011; Grivennikov, Greten, & Karin, 2010). In particular, IL-6 plays a fundamental role for the pathogenesis of MM. Being secreted from mesenchymal stem cells and MM cells this chemokine acts in a paracrine and autocrine manner. Via activation of the MAPK signaling pathway, phosphorylation of

transcription factor 3 and upregulation of antiapoptotic molecules like MCL-1 (BCL2 family apoptosis regulator) and c-MYC (myelocytomatosis oncogene) it promotes proliferation and MM-cell survival. Furthermore IL-6 induces VEGF secretion, an essential growth factor for this malignancy. Latter reinforces IL-6 production in a positive feedback loop. Both VEGF and IL-6 inhibit the antigen-presenting function of dendritic cells, thereby aggravating the immunocompromised status of MM patients. Last but not least the recent finding, that application of IL-6 siRNA (small interfering RNA) preclinically inhibits MM cell growth, emphasises the cytokine's role for the pathogenesis of this cancer (Fairfield et al., 2016; Grivennikov et al., 2010; Hodge, Hurt, & Farrar, 2005; M. Kawano et al., 1988; Teoh et al., 2016)

Osteoclasts is another important cell type involved in MM progression. Activated by numerous cytokines and stimulated by several signaling pathways in MM bone marrow microenvironment, osteoclasts destroy the physiologic bone structure. Pathologic bone lysis subsequently leads to ongoing release of calcium, growth factors and ECM (extracellular matrix) proteins, promoting MM cell growth, angiogenesis and drug resistance (e.g. to dexamethasone). By far the most important pathway for osteoclast activation is the RANK/RANKL/OPG pathway, which is deregulated in MM patients. (Kovacic, Croucher, & McDonald, 2014; Ramasamy et al., 2012; Tanaka et al., 2007)

In summary pathogenesis of MM arises not from only one genetic event, but rather from the combination of genetic instability, homing to a growth factor rich bone marrow microenvironment and conditions like low-grade inflammation or hypoxia (Fairfield et al., 2016). Equally complex and diverse is the clinical manifestation of MM, which will be introduced in the following chapter.

1.1.2 Clinical manifestation and diagnosis

MM is a disease with a slow but persistent progression. Most patients are diagnosed when becoming symptomatic with renal failure, bone lesions, infections or symptoms from hypercalcemia (Riccardi et al., 1991; Sirohi & Powles, 2004).

Dependent on the presence of an end organ damage, MM is classified in symptomatic and asymptomatic disease. End organ damage includes hypercalcemia,

renal insufficiency, anemia and bone lesions, taken together as the CRAB-criteria (Moreau et al., 2013). Bone lesions can be found in over 80% of patients at diagnosis and are due to the activation of osteoclasts and the inhibition of osteoblasts by MM cells. Besides bone lesions anemia is a frequent finding at diagnosis, caused by MM bone marrow infiltration or renal failure (Fairfield et al., 2016; Kyle et al., 2003). Renal disfunction occurs in half of the patients at diagnosis and is most likely due to the toxic effect of the excess of monoclonal light chains on the tubular cells, the formation of casts, that congest the tubuli, hypercalcemia and dehydratation as well as nephrotoxic medication (Dimopoulos, Kastritis, Rosinol, Blade, & Ludwig, 2008). A less common finding at diagnosis is hypercalcemia, caused by a pathologic bone lysis (Kyle et al., 2003). Infiltration of the bone marrow with interference of lymphocyte maturation favors infections in MM patients. In addition massive over production of dysfunctional monoclonal antibodies by MM cells contributes to this immunosuppressive status. Just as physiologic plasma cells the malignant clones secrete antibodies, which can be detected in electrophoresis as M-protein or paraprotein (Sirohi & Powles, 2004). Most MM tumors secrete monoclonal IgG, followed by IgA, light chains and in 1% IgD. In < 1% asecretory MM can be diagnosed (Sirohi & Powles, 2004). A dangerous secondary effect of this excessive gammaglobulinemia is hyperviscosity (Moreau et al., 2013; Raab et al., 2009).

For the diagnosis of symptomatic MM a bone marrow biopsie has to show ≥10% of clonal plasma cells. In addition, end-organ damage (CRAB-criteria) must be evidenced. MGUS is diagnosed in case of <10% of clonal BM plasma cells and in the absence of end-organ-damage. The interstage smouldering multiple myeloma requires ≥10% of MM cells in the bone marrow but no end-organ damage. Thus, for diagnosis of MM the following tests are required: Serum or urine electrophoresis, specification of the heavy and light chains by immunofixation, assessment of bone marrow plasma cell infiltration with the help of a bone marrow aspiration and/or a biopsy, radiological sceletal bone evaluation with X-ray or, for special indications, with CT/MRI as well as a detailed blood cell count including serum creatinine and calcium (Moreau et al., 2013). Besides these tests, physical examination and medical history are important modules (Palumbo & Anderson, 2011). Whereas in the past, when MM patients had only few treatment options, a staging system developed by Durie and Salmon was applied, nowadays the international staging system (ISS) is used for a reproducible and powerful risk assessment (Durie & Salmon, 1975; Greipp

et al., 2005; Sirohi et al., 1999). Table1 shows the 3 ISS stages and the expected median survival in months for each stage with the poorest outcome for ISS stage 3. Furthermore, genetic abnormalities detected by FISH including t(4;14), deletion(17p), chromosome 1 aberrations and t(14;16) are negative prognostic markers. Combining those cytogenetics with the ISS stage could provide a significantly better prognostic assessment (Avet-Loiseau et al., 2013; Moreau et al., 2013). A revised international staging system (R-ISS), taking into account the cytogenetics and the level of serum lactat dehydrogenase (LDH), has been suggested by Palumbo et al. (Rajkumar et al., 2014).

Concentration of components in serum		**Median survival (months)**
Stage I	beta2-microglobulin ≤3,5mg/l and albumin ≥3,5g/dl	62
Stage II	beta2-microglobulin <3,5mg/l and albumin<3,5g/dl, or beta2-microglobulin 3,5-5,5 mg/l	44
Stage III	beta2-microglobulin >5,5mg/l	29

Table 1: International staging system and median survival

The small molecule beta2-microglobulin and albumin are measured in the serum of MM patients and allow for an assessment of prognosis (adapted from (Raab et al., 2009)).

1.1.3 Current treatment

As there is no benefit from immediate treatment for patients with MGUS or asymptomatic SMM, therapy should only be started in case of overt, symptomatic MM (Moreau et al., 2013). Though it remains an incurable disease, novel pharmaceutical agents and autologous stem cell transplantation (ASCT) have substantially improved the duration and frequency of remissions in MM patients (Palumbo & Anderson, 2011). Therapy commonly consists of multiple drug combinations including chemotherapeutics, proteasome inhibitors, bisphosphonates and immunomodulatory imide drugs (IMiDs). Melphalan, vincristine, doxorubicin and liposomal doxorubicin are often used chemotherapeutics (Kyle & Rajkumar, 2004). In fit patients under the biological age of 65 years, chemotherapy is combined with ASCT, to restock stem cells after high-dose melphalan and to possibly promote BM remodelling (Hussein et al., 2016). Whereas in elderly or patients with comorbidities chemotherapy is given without ASCT (Moreau et al., 2013).

By the use of new agents like the proteasome inhibitor (PI) bortezomib in combination with dexamethasone, response rates of induction therapy were significantly increased: Harousseau et al. showed an overall response of 78,5% versus 62,8% in patients treated with bortezomib-dexamethasone compared to the classical VAD induction regime (vincristine, adriamycin and high-dose dexamethasone) (Harousseau et al., 2010). PIs like bortezomib or carfilzomib, a second-generation proteasome inhibitor, arrest the proteasome and thus lead to the accumulation of intracellular proteins with subsequent MM cell death (Fairfield et al., 2016). Furthermore, bortezomib induces apoptosis in MM cells and osteoclasts as well as osteoblast differentiation (Giuliani et al., 2007; Obeng et al., 2006; von Metzler et al., 2007). Several studies also showed a chemosensitizing effect of PIs for lenalidomide and dexamethasone as well as other therapeutic agents (Ma et al., 2003; Mitsiades et al., 2003; Popat et al., 2009). Another new aspect of MM treatment is the use of bisphosphonates. They inhibit osteoclasts and thereby decrease bone lysis. The question whether bisbhosphonates also have direct anti-tumor effects is subject of ongoing studies (Berenson et al., 1996; Levy & Roodman, 2009; Van Acker, Anguille, Willemen, Smits, & Van Tendeloo, 2016). Moreover, IMiDs like thalidomide, lenalidomide or pomalidomide nowadays play an important role in MM therapy. It was recently shown by Kawano et al., that MM cells cause distinct immunosuppression, a process that can be antagonized with IMiDs (Y. Kawano et al., 2015). These immunomodulatory drugs were shown to chemosensitize MM cells to bortezomib and dexamethason and to sustain the tumor suppressor actions of the patient's immune defense (Zhu, Kortuem, & Stewart, 2013). Last but not least monoclonal antibodies, e.g. against CD138, and histone deacetylase inhibitors start to be part of the MM therapy (de Weers et al., 2011; Laubach et al., 2014). Figure 2 gives an overview of the treatment recommendations for newly diagnosed MM patients, approved by the ESMO (european society for medical oncology) Guidlines Working Group.

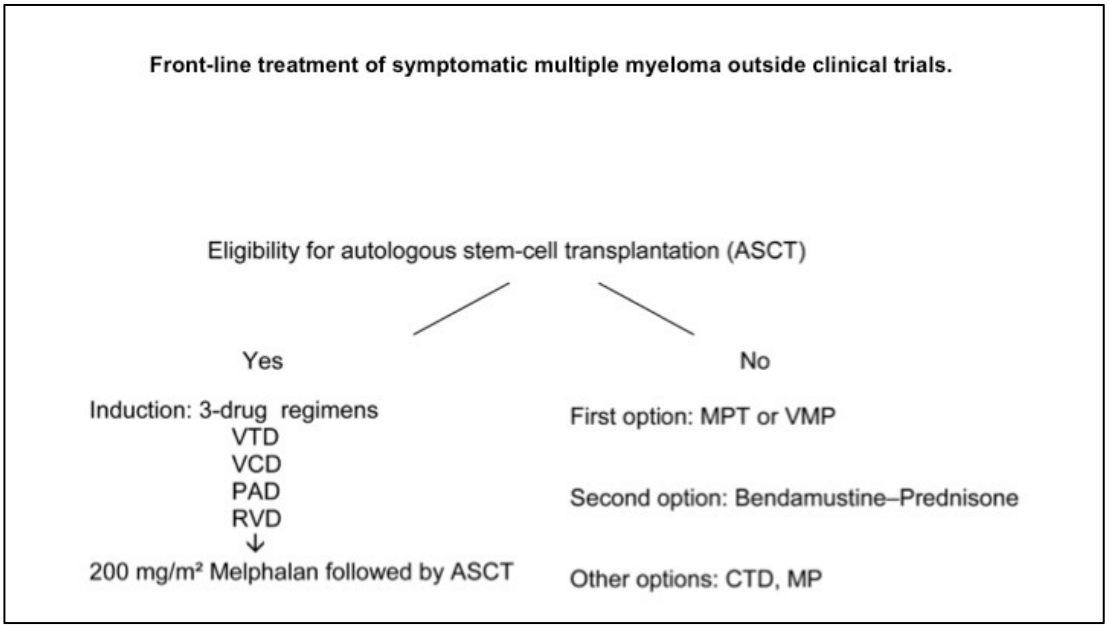


Figure 2: ESMO recommendations for the treatment of symptomatic MM

This schema shows the different treatment options for newly diagnosed symptomatic MM patients outside clinical trials. Depending on age and performance status patients are eligible for ASCT or not. On the left side the chemotherapy for ASCT-eligible patients is shown: Induction with either VTD (bortezomib, thalidomid, dexamethasone); VCD (bortezomib, cyclophosphamid, dexamethasone); PAD (bortezomib, doxorubicin, dexamethasone) or RVD (lenalidomide, bortezomib, dexamethasone) is recommended, followed by high-dose melphalan and ASCT. On the right side treatment alternatives for elderly patients are presented: MPT (melphalan, prednisone, thalidomide), VMP (bortezomib, melphalan, prednisone), Bendamustine-Prednisone, CTD (cyclophosphamid, thalidomide, dexamethasone) or MP (melphalan, prednisone) (adapted from (Moreau et al., 2013)).

In summary, MM is a highly heterogenous disease with a complex pathogenesis and clinic. Even if therapeutical strategies have been improved within the last years, it remains an incurable cancer with many open questions regarding its malignant transformation. Thus further research is required to better understand the molecular mechanisms involved in MM pathogenesis and in particular the role of the ubiquitin proteasome system (UPS) for this disease. Taking into account its good responsiveness to proteasome inhibitors, identification of UPS members, being involved in MM pathogenesis, could present an important step in direction of future therapies.

1.2 The ubiquitin proteasome system in health and disease

The effectiveness of proteasome inhibitors in MM treatment highlights the importance of the UPS for this disease. Distinct UPS members play significant roles for myelomagenesis, well known examples are F box protein 9 (FBXO9) and ubiquitin specific peptidase 9, X-linked (USP9X), both promoting MM cell survival and progression (Carrasco et al., 2006; Fernandez-Saiz et al., 2013; Munshi et al., 2004; Peterson et al., 2015). Indeed post translational ubiquitination of proteins does not only mark them for proteasomal degradation, but also plays a critical role for numerous biological processes (e.g. regulation of the cell cycle, inflammation, immune response, apoptosis, cell survival) (Ciechanover & Iwai, 2004; Karin & Ben-Neriah, 2000; Kloetzel, 2004; Murray, 2004). Consequently, deregulation of the UPS can be found in many diseases including cancer, like MM, cardiovascular and renal disease, obesity as well as diabetes (Calise & Powell, 2013; Fukasawa, 2012; Spataro, Norbury, & Harris, 1998; Wing, 2008). Thus, the UPS is of essential importance for the human organism. In the present chapter the main components of this system will be described and their functions explained.

1.2.1 Ubiquitin and the proteasome

Ubiquitin was first identified in 1975 by Goldstein et al. in a screen for polypeptides with „lymphocyte-differentiating properties“ (Goldstein et al., 1975). A few years later it’s key role in the UPS was revealed and further described (Ciechanover, 1994; Wilkinson, Urban, & Haas, 1980).

The 8.5 kDa heavy protein with 76 aminoacids, was originally named ubiquitious immunopoetic polypeptide because of its ubiquitious expression in all eukaryotic cells and its high conservation from yeast to humans (Schlesinger, Goldstein, & Niall, 1975). Key features of the protein are 7 lysine residues and its C-terminal (carboxy-terminal) glycine. Upon formation of an isopeptide bond between this glycine and the ε-amino group of a lysine in the substrate, ubiquitin is covalently bound to a protein (Hershko & Ciechanover, 1998). This post translational modfication (PTM) functions either as a signal for proteasomal degradation (e.g. K48 polyubiquitination) or for several cellular processes in case of monoubiquitination or distinct polyubiquitin chains (e.g. membrane trafficking, endocytosis, DNA damage

response) (Haglund et al., 2003; Hicke & Dunn, 2003). For polyubiquitination further ubiquitin molecules bind to one of the seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or the N-terminal methionine (M1) of the substrate-linked ubiquitin (Komander, 2009). Lysine K63 and K48-linked polyubiquitin chains are the best characterized. K63 chains have been associated with NF-κB signaling and thus with inflammation, anti-apoptotic and immune response (Deng et al., 2000). Furthermore, K63 plays a role in endocytic trafficking (Miranda & Sorkin, 2007). On the other hand K48 polyubiquitination with at least 4 ubiquitin molecules marks a protein for degradation in the 26S proteasome (Hershko & Ciechanover, 1998; Thrower, Hoffman, Rechsteiner, & Pickart, 2000).

The 26S proteasome generally recognizes target proteins, that have been conjugated to a K48-linked polyubiquitin chain (Pickart, 1997). This ATP-dependent 2.5 MDa heavy complex is composed of over 60 units forming 2 subcomplexes: the 20S core catalytic particle and the 19S regulatory particle. After substrate recognition, deubiquitination and unfolding by the 19S subcomplex, the target is translocated into the cylindrical cavity of the 20S subcomplex, where it is finally degraded (Nandi, Tahiliani, Kumar, & Chandu, 2006; Navon & Ciechanover, 2009).

1.2.2 Ubiquitination

On the way to proteasomal degradation the enzymes of the ubiquitin cascade present an important regulatory layer. Those enzymes and the enzymatic sequence, they are involved in, designated the ubiquitin cascade, were first described in 1983 by Hershko et al. (Hershko, Heller, Elias, & Ciechanover, 1983). Their function and regulation will be explained below.

1.2.2.1 The ubiquitin cascade

In a multistep process, that involves at least 3 types of enzymes (E1, E2, E3), ubiquitin is activated and transferred to the target protein (s. figure 3). The first ATP-requiring step results in the formation of a high-energy thioester bond between the C-terminal glycine of ubiquitin and a cysteine of E1, the ubiquitin activating enzyme. Without involvement of another energy donor, activated ubiquitin is subsequently transferred to the active side of E2, the ubiquitin carrier protein (Ubc). Latter specifically binds to the next enzyme E3, the ubiquitin-ligase, which is finally

responsible for the transfer of ubiquitin to the target protein (Ciechanover, 1994; Hershko et al., 1983; Weissman, 2001). Only two ubiquitin activating enzymes (E1) interact with a dozen of E2s, which can connect with hundreds of E3s. Thus it is assumed, that substrate specificity is mainly controlled by ubiquitin-ligases (E3) (de Bie & Ciechanover, 2011). Furthermore, some E2 enzymes seem to play a role for substrate specificity, showing binding preferences for particular E3 ligases (Hershko & Ciechanover, 1998). For polyubiquitination the described enzymatic cascade is repeated and the next ubiquitin molecule is connected to a lysine residue or the N-terminus of the precursor (s. chapter 1.2.1). Furthermore E4 enzymes (ubiquitin-chain elongation factors) were described. This last group is able to catalyze polyubiquitination of monoubiquitin-linked proteins and thereby mark them for proteasomal degradation or other processes (Hoppe, 2005; Koegl et al., 1999).

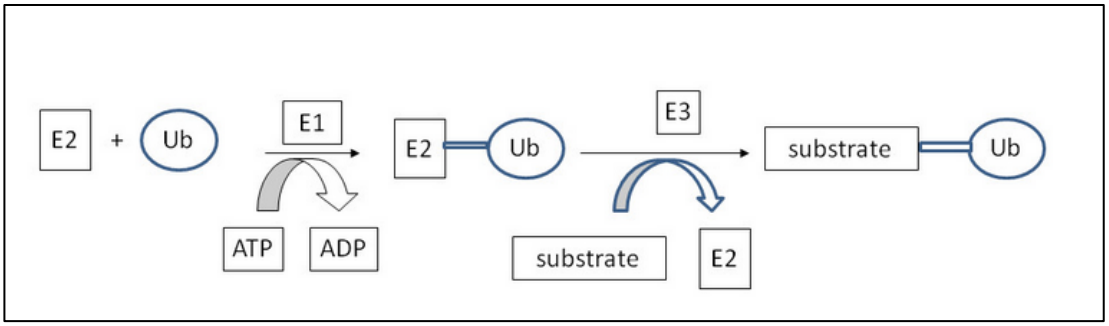


Figure 3: The ubiquitin cascade

At least 3 enzymes are involved in the ubiquitin cascade: E1, E2 and E3. In the first ATP-requiring step ubiquitin (Ub) is linked to the ubiquitin activating enzyme (E1) by formation of a high-energy thioester bond. The activated ubiquitin is then bound to the ubiquitin carrier protein (E2). With the help of an ubiquitin ligase (E3) ubiquitin is finally transferred to its substrate – a peptide bond is fomed between the C-terminus of ubiquitin and a lysine residue of the target protein (adapted from (Voutsadakis, 2012)).

1.2.2.2 Ubiquitin-ligases

As previously explained, the last step in the ubiquitin cascade, covalent binding of ubiquitin to the target protein, is catalyzed by ubiquitin-ligases (E3s). As main mediators of substrate specificity ubiquitin-ligases (E3s) are of fundamental importance for the UPS (s. chapter 1.2.2.1). Two classes of E3s were identified: The RING (really interesting new gene) domain-containing E3s, as the largest group, and the HECT (homologous to the E6-AP C-terminus) domain E3s (Ciechanover & Iwai,

2004). Both groups of ubiquitin ligases recognize specific structures in their target proteins, designated as degrons (Varshavsky, 1991). These degrons are often hidden within the target's structure to avoid constant recognition by E3s. Only in case of misfolding or stimulation (e.g. phosphorylation of the cell cycle inhibitor p27) they are exposed (Carrano, Eytan, Hershko, & Pagano, 1999). Upon identification of a substrate RING ligases mediate the direct transfer of E2-bound ubiquitin to the target via concurrent binding of E2 and the protein. Thus, RING E3s display no intrinsic ligase activity. In contrast, HECT ligases mediate thioester formation between an active cysteine residue of their C-terminus and the ubiquitin molecule, prior to its transfer to the substrate (s. figure 4) (de Bie & Ciechanover, 2011). E6-AP is a protein encoded by human papilloma virus and was found by Scheffner et al. to ubiquitinate p53. Its ubiquitin ligase domain is homologous to many ubiquitin ligases, therefore named HECT E3s, and harbours a highly conserved cysteine residue, responsible for thioester formation (Huibregtse, Scheffner, Beaudenon, & Howley, 1995; Huibregtse, Scheffner, & Howley, 1991, 1993; Pickart, 2001). Within the class of 30 HECT ligases a small subgroup designated as the Nedd4 superfamily can be distinguished. Like NEDD4 as the first ubiquitin ligase of this group, these E3s have a particular structure with up to 4 WW (double tryptophan) domains. WW domains bind to PPXY (proline-proline-x-tyrosine) motifs and are mainly responsible for substrate recognition. Important members of this E3 ligase family are SMURF1 and 2 (SMAD ubiquitination regulatory factor 1 and 2) as well as WWP2, which will be described in detail in the following chapters (Cao & Zhang, 2013; Chantry, 2011; Pirozzi et al., 1997; Sudol, 1998; Wang, Yang, & Huibregtse, 1999).

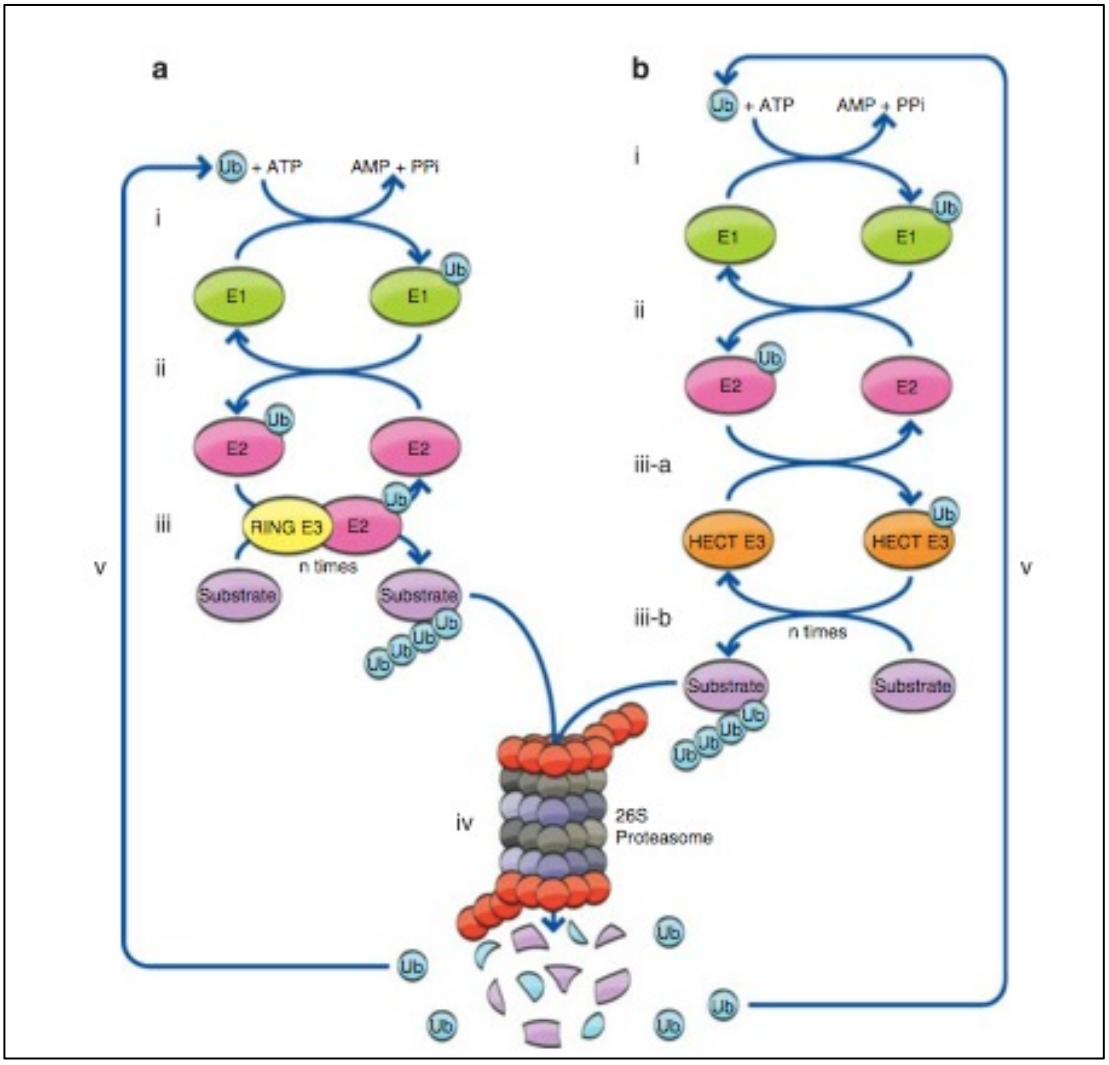


Figure 4: The functioning of RING and HECT ubiquitin ligases

(ai, bi) E1 activates ubiquitin in an ATP-requiring reaction. (aii, bii) The activated ubiquitin Is then transferred to the ubiquitin carrier protein E2. (aiii) The RING E3 ligase binds the ubiquitin linked E2 as well as the target protein and thereby serves as a scaffold for the transfer of ubiquitin to the substrate. (biiia, biiib) In the case of a HECT E3 ligase ubiquitin is covalently bound to a cysteine residue of the enzyme before its transfer to the substrate. (iv) Polyubiquitination, as it is shown in this figure, can mark proteins for proteasomal degradation. (v) Some of the ubiquitin molecules are destroyed together with the substrate, while the majority is recycled and can be reused (adapted from (de Bie & Ciechanover, 2011)).

1.2.3 Deubiquitination

Ubiquitination is a reversible protein modification, that is essential for the regulation of multiple eukaryotic processes (e.g. cellular proliferation, apoptosis, gene transcription) and plays an important role in health and disease (Hanpude, Bhattacharya, Dey, & Maiti, 2015; Komander, Clague, & Urbe, 2009). Thus, the dynamic balance of ubiquitination and deubiquitination needs to be tightly controlled by ubiquitin ligases and deubiquitinating enzymes (DUBs).

The human genome encodes over 90 DUBs, that hydrolyze the isopeptide or peptide bonds connecting ubiquitin to a substrate or to another ubiquitin molecule (Nijman et al., 2005). These enzymes can structurally be subdivided into 5 families. UCHs (ubiquitin C-terminal hydrolases), USPs (ubiquitin specific peptidases), OTU (ovarian tumor) and MJD (Machado-Josephin-domain) DUBs function as thiol proteases, relying on a nucleophilic cysteine in their active site for hydrolysis of peptide or isopeptide bonds. The fifth group of deubiquitinating enzymes, the JAMMs (JABI/MPN/MOV34 metalloproteases), instead coordinate a metal ion in the active site (Komander et al., 2009; Ronau, Beckmann, & Hochstrasser, 2016). The main tasks of DUBs can be classified into three different functional categories. (I) First of all, the generation of free ubiquitin requires DUB activity, as premature ubiquitin is transcribed from different genes in form of a linear chain or bound to ribosomal proteins (Komander et al., 2009). (II) Second, DUBs can antagonize the activity of E3 ligases, removing the post translationally-added ubiquitin. Thereby they stabilize proteins via rescue from proteasomal degradation or inhibit other ubiquitin related cellular processes. c-MYC (myelocytomatosis oncogene) stabilization by USP28, which counteracts the SCF^{Fbw7} (Skp1/Cullin/F-box)-mediated polyubiquitination of c-MYC, examplifies this second function of DUBs (Popov et al., 2007). Another important example is monoubiquitination of SMAD4, an important mediator in the TGF-beta signaling pathway, by the ubiquitin ligase Ecto (Ectodermin/Tif1γ). USP9X reverses this PTM of SMAD4, thus facilitating complex formation between SMAD4 and phospho-SMAD2 (Dupont et al., 2009). (III) Last but not least DUBs can modify ubiquitin chains and thereby influence the fate of their target (Komander et al., 2009). Figure 5 gives an overview of the most important tasks of DUBs.

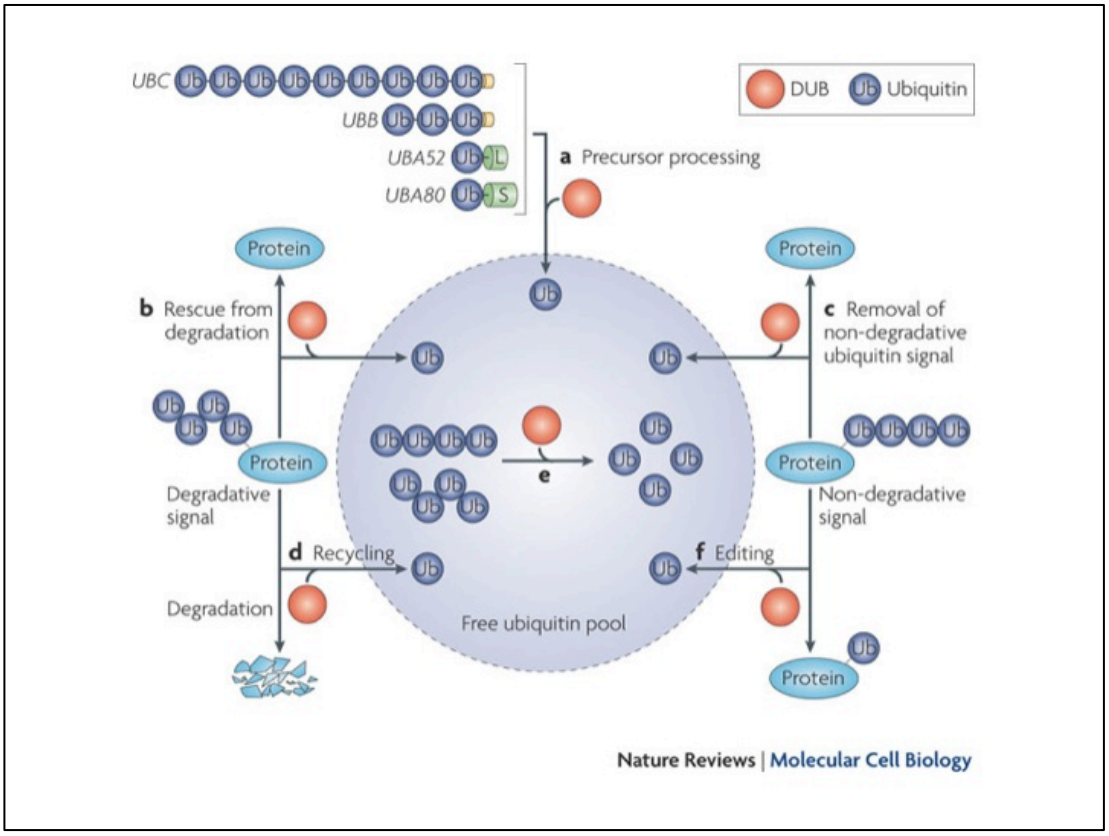


Figure 5: The main tasks of deubiquitinating enzymes (DUBs)

DUBs play an essential role in diverse biological processes and fulfil numerous functions: (a) generation of free ubiquitin molecules, (b, c) reversal of ubiquitin ligase activity and hence rescue from proteasomal degradation or removal of non degradativ ubiquitinatin chains, (d) recycling of ubiquitin, (e) disassembly of ubiquitin chains generated by en bloc removal from substrates, (f) trimming of ubiquitin chains and thereby changing one ubiquitin signal for another (adapted from (Komander et al., 2009)).

Given their diverse functions, target specificity must vary between different DUBs. This specificity depends on multiple layers of regulation, thereunder mechanisms like active site rearrangements in USP7, a regulator of p53 (Faesen et al., 2011). This DUB shows a misaligned active site, which undergoes rearrangement upon stimulation to unfold its catalytic activity. Furthermore DUBs are controlled by subcellular localization, e.g. USP30 has been localized to mitochondria and plays a role for the regulation of mitochondrial morphology (Nakamura & Hirose, 2008). Post translational modifications like phosphorylation of CYLD, a DUB which is involved in NF-κB signaling, also helps controlling DUB activity (Reiley, Zhang, Wu, Granger, & Sun, 2005). In addition, some deubiquinating enzymes show specificity for certain

ubiquitin chain linkages. The 26S proteasome associated USP14 specifically cleaves Lys48-linked ubiquitin, whereas CYLD hydrolyses Lys63-linked chains (Hu et al., 2005; Komander et al., 2008). There are many other regulatory layers besides the given examples, indicating the plasticity and complexity of DUBs. Deregulation of these precisely controlled enzymes and of the UPS in general is a common finding in hematologic cancers, making DUBs important targets for future therapeutic agents (Sahasrabuddhe & Elenitoba-Johnson, 2015). One of these deubiquitinating enzymes with a potential role in MM pathogenesis will be introduced below.

1.2.4 The Usp24 gene locus in MM

In an array comparative genomic hybridization (aCGH) study Carrasco et al. analyzed 67 MM patient samples and defined different genomic subtypes. Moreover, their research identified a handful of mutated genes, involved in the UPS. Besides different E3 ubiquitin ligases (e.g. F-box protein 3 (FBXO3), SMAD ubiquitination regulatory factor 2 (SMURF2)), the Usp24 (ubiquitin specific peptidase 24) gene locus, encoding for a deubiquitinating enzyme (DUB), seemed particularly noteworthy, as it was lost in 25% of MM patients (Carrasco et al., 2006). This gene is localized on chromosome 1p32.3 (149,009 kb); its transcription product USP24 consists of 2620 aminoacids (aa) and has a predicted molecular weight of 294 kDa (NCBI, 2016b). USP24 contains several conserved domains including a UBA (ubiquitin-associated) domain, a UBL (ubiquitin-like) domain and a USP (ubiquitin specific peptidase) domain (Komander et al., 2009). Its gene locus was first reported to play an important role in Parkinson's disease (Li et al., 2006; Oliveira et al., 2005). Furthermore, USP24 was shown to stabilize the survival protein MCL-1 with consequent induction of apoptosis in Usp24 depleted MM cell lines (Peterson et al., 2015). In addition, it is implicated in the UV damage response and recently, its interaction with the tumor suppressor p53 was evidenced (L. Zhang & Gong, 2016; L. Zhang, Lubin, Chen, Sun, & Gong, 2012; L. Zhang et al., 2015). Figure 3 shows a database search on Oncomine displaying the results from Carrasco et al. (Oncomine, 2016).The analysis of 84 MM patient samples and their comparison to physiologic leucocytes revealed a lower Usp24 DNA copy number in MM (Dickens et al., 2010). However, USP24 remains a poorly characterized protein with no clear role in cancer and in MM pathogenesis in particular. Hence, it is an interesting target for further research in the context of MM.

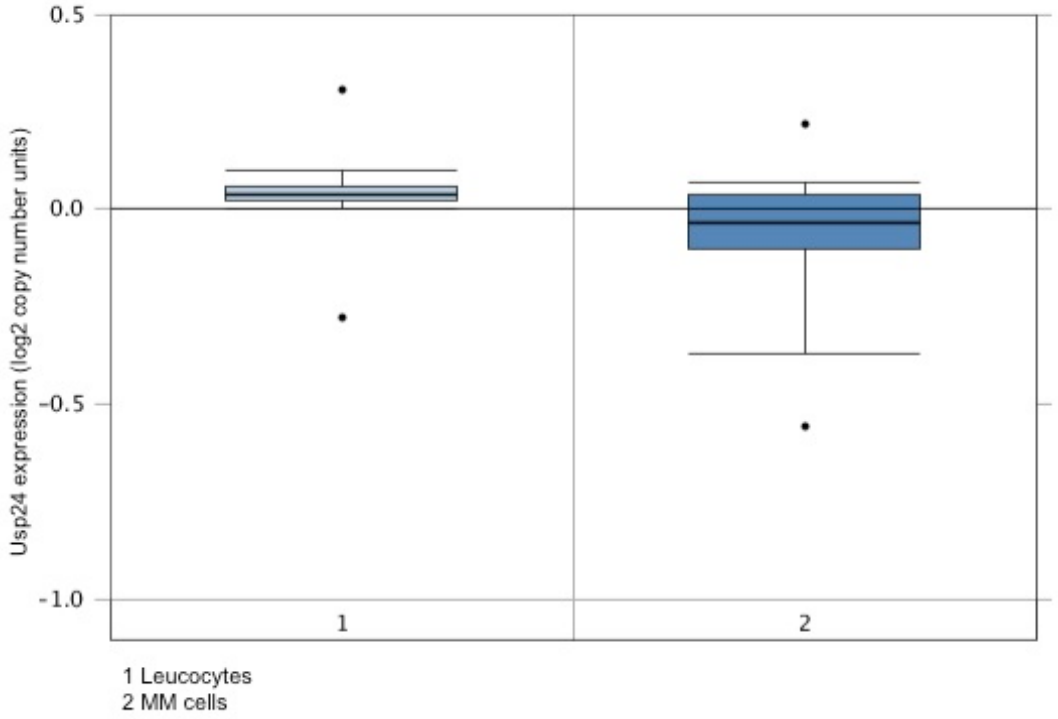


Figure 6: Loss of the Usp24 gene locus in MM cells

Analysis of 84 paired normal leucocytes and CD138^{+} purified multiple myeloma samples was performed. The obtained data was subsequently processed using AROMA.affymetrix (Bengtsson, Simpson, Bullard, & Hansen, 2008). The two box plots for leucocytes (1) and MM cells (2) show the corresponding median, maximum and minimum of the Usp24 DNA copy numbers: median (MM cells)= -0,034; median (normal leucocytes)= 0,039; p= 1,000. Values are given as log2 (copy number units). For a more detailed description of the methods, used to generate this data, please refer to Dickens et al. (retrieved from (Oncomine, 2016) adapted from (Dickens et al., 2010)).

In conclusion, the UPS plays an outstanding role within the cell and the human organism, with involvement in numerous eukaryotic mechanisms, maintaining viability, genomic stability and integrity. The disctinct signaling pathways still need to be elucidated and will certainly improve understanding and thus therapies for many diseases, including multiple myeloma.

1.3 The TGF-beta signaling pathway

The TGF-beta signaling pathway produces a vast number of biological processes including morphogenesis, stem cell differentiation, immune regulation, inflammation and wound healing (Santibanez, Quintanilla, & Bernabeu, 2011). Hence, disfunctional signaling of this highly regulated system has been associated with disorders like cancer, including MM, congenital diseases, fibrosis, pulmonary diseases and cardiovascular pathology (Ikushima & Miyazono, 2010; Morty, Konigshoff, & Eickelberg, 2009; Pannu et al., 2005; Pardali, Goumans, & ten Dijke, 2010; Pohlers et al., 2009). The following sections will give a more detailed view of TGF-beta signaling and of its tight regulation by the UPS.

1.3.1 The TGF-beta superfamily

In 1983 TGF-beta was discovered as the first member of the TGF-beta superfamily, because of its ability to „transform“ the growth of cultured fibroblasts (Frolik, Dart, Meyers, Smith, & Sporn, 1983). Depending on the corresponding signaling pathways, this superfamily, with over 40 members, is subdivided into two branches: (I) the TGF-beta/Activin branch and (II) the bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) branch. Each branch consists of different subgroups, sharing sequence similarities (Massague, 1998). TGF-beta, Activin, Inhibin, Nodal and Lefty ligands belong to the TGF-beta/Activin signaling pathway. Whereas BMP, GDF and MIF (Müllerian inhibitory factor) are part of the BMP/GDF subgroup, which regulates the transcription of several genes involved in osteogenesis, neurogenesis, male sex differentiation and many more (Santibanez et al., 2011).

TGF-beta is translated into a proprotein, that is proteolytically split into mature TGF-beta and latency-associated protein (LAP). Its bioavailability is regulated by a non covalent bond with LAP, which prevents receptor binding and signaling. Furthermore, TGF-beta bioavailability is controlled by LTBP (latent TGF-beta binding protein), to which the LAP-TGF-beta complex is bound during its secretion. To liberate bioactive TGF-beta, proteases (e.g. matrix metalloproteases, plasmin, thrombin) must cleave the LAP-TGF-beta and LTBP bonds (Annes, Munger, & Rifkin, 2003; Massague, Seoane, & Wotton, 2005; P. Xu, Liu, & Derynck, 2012). Three of six identified isoforms of TGF-beta are expressed in mammals (TGF-beta1, TGF-

beta2 and TGF-beta3). Just as other members of the TGF-beta superfamily they have a dimeric structure with a disulfide bridge and cysteine residues on each monomeric peptide, known as the „cysteine knot“ (Sun & Davies, 1995; Zi, Chapnick, & Liu, 2012).

TGF-beta and all other members of this superfamily signal through receptor serine/threonine kinases. In case of TGF-beta they are called TGF-beta type I and type II receptors (TbRI and TbRII). Bioavailable TGF-beta binds to a homodimer of TbRII on the surface of its target cell. This facilitates formation of a tetrameric complex built of the two TbRIIs and a TbRI homodimer (Massague, 1998; Shi & Massague, 2003). In this active receptor complex TbRII, a constitutively active kinase, autophosphorylates itself and catalyzes transphosphorylation of the two TbRIs, thereby activating their kinase activity. Subsequently TbRI phosphorylates effector proteins of the SMAD family, resulting in their nuclear accumulation and thus the manipulation of gene transcription (Shi & Massague, 2003). Hence, intracellular TGF-beta signaling is mediated by members of the SMAD family, which will be introduced below.

1.3.2 SMAD family-dependent TGF-beta signaling

MAD (mothers against decapentaplegic) was the first intracellular signal transducer of TGF-beta signaling, identified 1995 in Drosophilia (Sekelsky, Newfeld, Raftery, Chartoff, & Gelbart, 1995). Little time later orthologs in worm and vertebrates were discovered and named SMAD (Derynck et al., 1996; Savage et al., 1996).

A total of eight proteins of the SMAD family are encoded by the human genome, but only five of them are phosphorylated by the receptor serin/threonin kinases and hence are called R-SMAD (receptor-activated SMAD) (Massague, 1998): R-SMAD2 and R-SMAD3 are activated by TGF-beta, Activin and Nodal receptor binding, whereas R-SMAD1, 5 and 8 mediate BMP and anti-Müllerian induced signaling (Massague et al., 2005). Like these R-SMAD proteins, SMAD6 is involved in the BMP/GDF signaling branch (Goto, Kamiya, Imamura, Miyazono, & Miyazawa, 2007). The remaining SMAD4 and SMAD7 play roles in both signaling pathways (H. Hayashi et al., 1997; Lagna, Hata, Hemmati-Brivanlou, & Massague, 1996). In addition to the classification of SMAD proteins depending on the signaling

pathway, they are involved in, they can also be divided in three functional groups: R-SMAD proteins (v.s.), Co-SMAD proteins (collaborating SMAD) with SMAD4 as the only member in humans and I-SMAD proteins (inhibitory SMAD) including SMAD6 and 7 (Massague, 1998).

The R-SMAD and Co-SMAD members are characterized by two conserved structural domains: the N-terminal MH1 (MAD-homology 1) domain and the C-terminal MH2 (MAD-homology 2) domain. In between a highly variable linker region with mutiple phosphorylation sites enables specific crosstalks with other signaling pathways. This region also harbours a PY (proline-tyrosine) motif for interaction with the E3 ligases SMURF1 and 2 and in case of SMAD4 a nuclear export signal (NES). R-SMAD proteins, but not Co-SMAD4, contain a serine rich SXS (serin-x-serin) motif in their C-terminal MH2 domain that is phosphorylated by TbRI upon TGF-beta binding (Massague et al., 2005; Shi & Massague, 2003). This phosphorylation releases R-SMAD2 and 3 of the TbRI-TbRII complex and allows binding to Co-SMAD4. Furthermore, it decreases the affinity of SMAD proteins for cytoplasmic stabilization factors, at the same time increasing it for nuclear factors (Shi & Massague, 2003; L. Xu, Kang, Col, & Massague, 2002). While in unstimulated cells SMAD proteins constantly shuttle between the nucleus and the cytoplasm, the formation of this trimeric R-SMAD-SMAD4 complex inhibits their nuclear export and thus leads to R-SMAD accumultaion in the nucleus with subsequent changes in gene transcription (Nakao, Imamura, et al., 1997). However, it must be mentioned that Co-SMAD4 is not required for nuclear translocation of R-SMAD proteins, but for a sufficient activation of transcriptional responses (Fink, Mikkola, Willson, & Markowitz, 2003; Heldin & Moustakas, 2012).

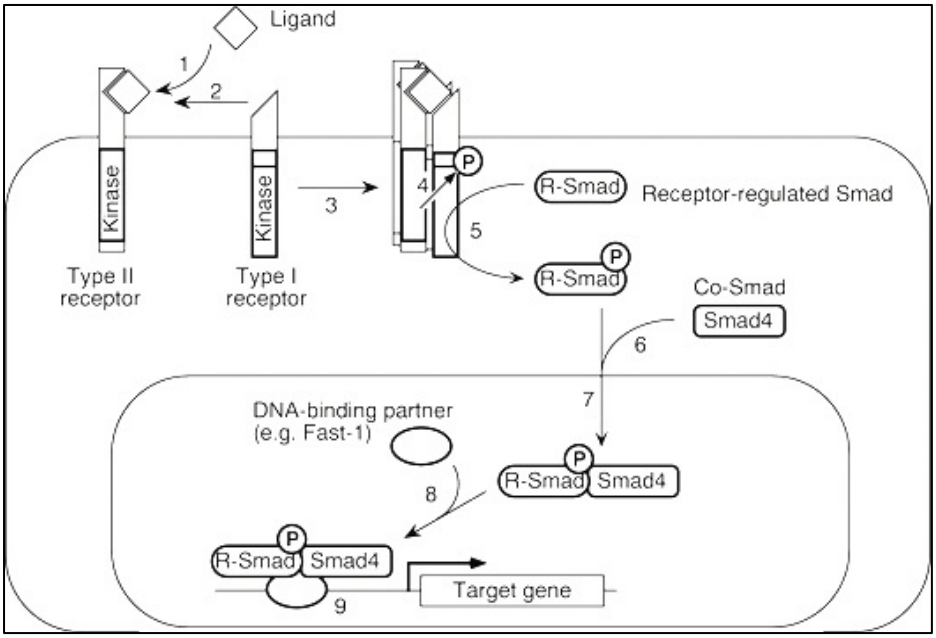


Figure 7: The transforming growth factor beta / SMAD signaling pathway

(1) TGF-beta binds to its receptor, the TGFbRII. (2,3) Subsequently a tetrameric complex of 2 TbRII and 2 TbRI is formed. (4) In this complex TbRII autophosphorylates itself and catalyzes the transphosphorylation of the TbRI homodimer, activating this receptor. (5,6) The activated TbRI kinase now phosphorylates an R-SMAD and thereby enables the formation of a heterotrimeric complex with SMAD4. (7) This complex moves into the nucleus. (8,9) Together with other DNA binding partners (e.g. Fast-1) it binds to the promoters of target genes and enhances or suppresses their transcription (adapted from (Massague, 1998)).

Nuclear R-SMAD-SMAD4 complexes target specific genes directly, with the support of coactivators or corepressors, or indirectly via DNA-binding transcription factors (Massague et al., 2005). A specific structure in the conserved MH1 domain of R-SMAD proteins, called beta-hairpin – i.e. two anti-parallel beta-strands with a linker loop in between – allows sequence-specific binding to the DNA (Massague et al., 2005). Hence, the trimeric complexes can influence the transcription of hundreds of genes via connection with SMAD-binding-elements (SBE) in their promoters (Dennler et al., 1998; Shi et al., 1998; Yingling et al., 1997). Interaction with certain transcription factors is another level of regulation of the target genes. FoxH1 (previously FAST-1) was the first transcription factor, interacting with proteins of the SMAD family, to be identified (X. Chen, Rubock, & Whitman, 1996). Another example is repression of the proliferation-stimulating c-Myc by the SMAD3-SMAD4 complex in cooperation with the transcription factors E2F4 and E2F5 (C. R. Chen, Kang, Siegel,

& Massague, 2002). Two of the R-SMAD-regulated genes are I-Smad6 and I-Smad7. In a negative feedback loop they inhibit either the formation of the trimeric transcription complex, in case of SMAD6, or prevent R-SMAD phosphorylation via binding of SMAD7 to the TGF-beta, Activin or BMP receptor type I (Derynck & Zhang, 2003; Goto et al., 2007; H. Hayashi et al., 1997; Nakao, Afrakhte, et al., 1997). Moreover, the TGF-beta signaling pathway is regulated by many other mechanisms, which will be described in the next chapter.

1.3.3 Regulation of the TGF-beta signaling pathway

While the TGF-beta signalig pathway appears to be conceptually linear and simple, it is regulated by multiple distinct mechanisms, that involve the TGF-beta protein, the serin/threonin-kinase receptors, the SMAD family and associated transcription factors (P. Xu et al., 2012). Chapter 1.3.1 describes, how bioavailability of TGF-beta is controlled. The following section will focus on the regulation of TGF-beta receptors and SMAD proteins with particular emphasis on the UPS and its role in TGF-beta signaling.

TGF-beta receptor activity is controlled on multiple levels, including receptor phosphorylation and dephosphorylation, ubiquitination as well as miRNAs (microRNAs), which specifically bind and inhibit TbRII encoding mRNA (Keklikoglou et al., 2012; Subramanyam et al., 2011; P. Xu et al., 2012). Receptor regulation via ubiquitination is examplified by SMAD7-mediated recruitment of SMURF2, an E3 ligase, to the TbRI with subsequent receptor polyubiquitination and degradation (Kavsak et al., 2000). On the other hand, USP15 can reverse this PTM and rescue TbRI from degradation. Hence, USP15 overexpression in glioblastoma, breast and ovarian cancer promotes TGF-beta signaling and thereby oncogenesis (Eichhorn et al., 2012). Besides these two, many other E3 ligases and DUBs ubiquitinate and deubiquitinate TbRI and II, thereby influencing receptor function (Komuro et al., 2004; Kuratomi et al., 2005; Wicks et al., 2005). Furthermore, the activity of TbRI and II is regulated by clathrin- or caveolin-1-mediated endocytosis. Whereas clathrin-coated endosomes are believed to play an essential role for SMAD activation, caveolin-1 and lipid raft coated pits mark TbRI and II for degradation (Y. G. Chen, 2009; Di Guglielmo, Le Roy, Goodfellow, & Wrana, 2003; Razani et al., 2001).

Like the TGF-beta receptors, SMAD proteins, as their cytoplasmic mediators, are regulated by phosphorylation, dephosphorylation and ubiquitination (P. Xu et al., 2012). C-terminal phosphorylation of the SXS motif by TbRI with subsequent R-SMAD activation, is not the only possibility to regulate SMAD activity. TGF-beta also induces slower phosphorylation of the linker region, thereby marking SMAD2 and 3 for proteasomal degradation. Consequently the half-life of R-SMAD2/3 as well as the intensity of TGF-beta signaling responses is reduced (Gao et al., 2009). MH1 dephosphorylation instead is necessary for termination of SMAD signaling. PPM1A was identified as phosphatase for SMAD2 and 3, which dephosphorylates the SXS motif and thus ends TGF-beta signaling (Lin et al., 2006). Ubiquitination, as another PTM, plays an essential role for the regulation of SMAD activity. Soond et al identified the HECT E3 ligase WWP2 and its two isoforms (N-terminal WWP2-N; C-terminal WWP2-C) as important regulators of SMAD2, SMAD3 and SMAD7. WWP2 mono- or polyubiquitinates these proteins and thus labels them for proteasomal degradation or other cellular processes (Soond & Chantry, 2011). Besides WWP2, NEDD4-2, WWP1, ROC1-SCF and SMURF1 and 2 have been shown to induce proteasomal degradation of R-SMAD proteins (Inoue & Imamura, 2008). While polyubiquitination leads to SMAD destruction, monoubiquitination has different impacts on SMAD2 and 3 signaling as well as on Co-SMAD4. Distinct monoubiquitinations on the MH1 domain of SMAD3 impact its ability to target promoters and thus to elicit transcriptional responses. USP15 reverses this PTM of SMAD3, thereby stabilizing a dynamic equilibrium of ubiquitination and deubiquitination as important control for R-SMAD-mediated transcriptional activities (Inui et al., 2011). Another E3 ligase, which monoubiquitinates SMAD3 is SMURF2: After phosphorylation of SMAD3 SMURF2 catalyzes multiple monoubiquitinations of the MH2 domain. This PTM abolishes SMAD3's ability to bind Co-SMAD4 and thus to form an efficient transcriptional unit (Tang et al., 2011). On the other hand monoubiquitination can also promote R-SMAD-SMAD4 complex formation and hence TGF-beta signaling: SMAD4 monoubiquitination at lysine 507 favors binding to activated R-SMAD proteins and thereby increases transcriptional activity (Moren et al., 2003). In addition to these examples, many other PTMs influence proteins of the SMAD family (s. figure 7), thereunder acetylation and ADP-ribosylation: In response to TGF-beta signaling the coactivator p300/CBP acetylate SMAD2 and 3, thereby promoting SMAD-mediated gene transcription (Inoue et al., 2007). Whereas ADP-ribosylation of SMAD3 by PARP-1 (poly ADP-ribose polymerase-1) results in

dissociation of SMAD3 from DNA and hence termination of SMAD-induced transcriptional activity (Lonn et al., 2010).

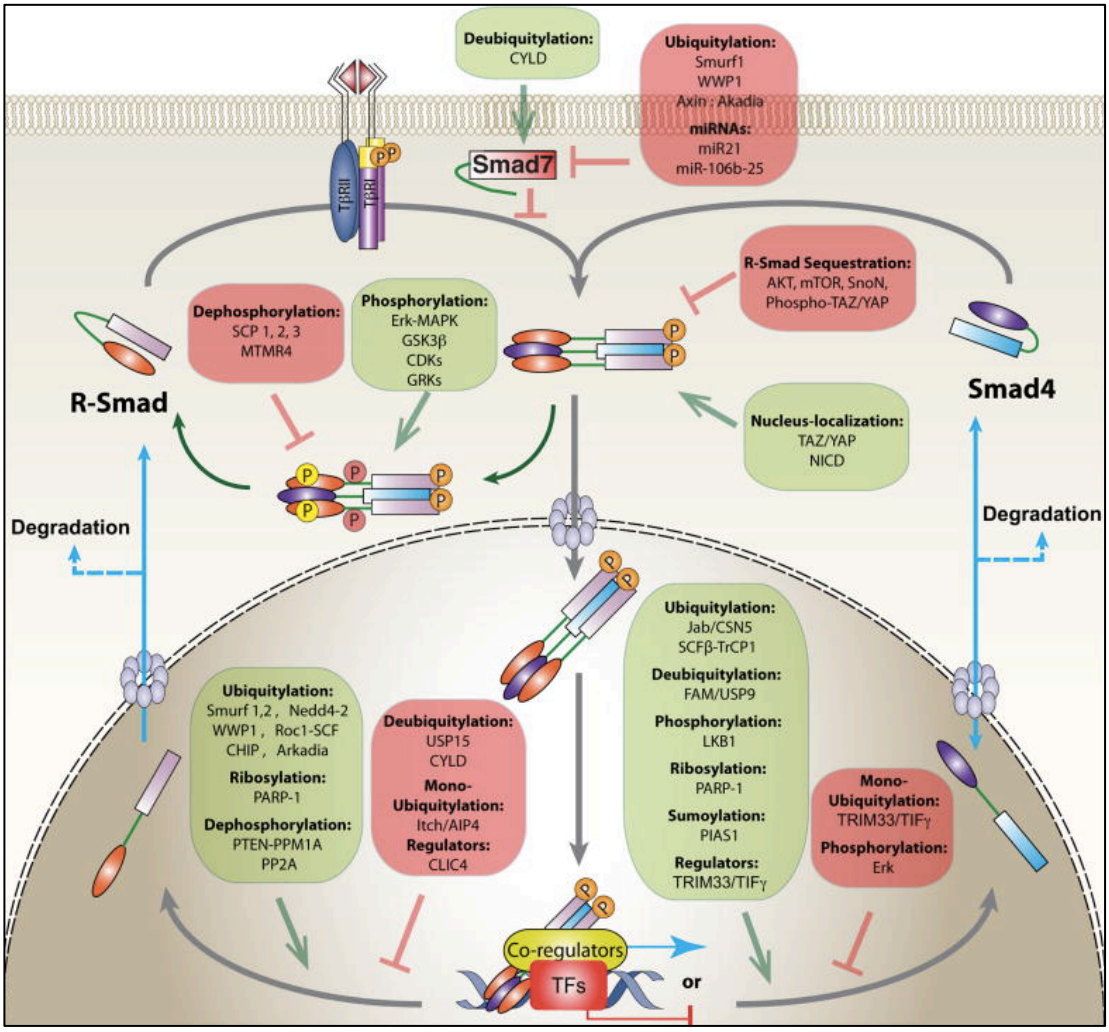


Figure 8: Post-translational regulation of SMAD proteins

Thick gray lines represent SMAD signaling, whereas solid or dashed blue lines illustrate nucleocytoplasmic shuttling or degradation of SMAD proteins, respectively. Mechanisms, which promote the indicated process, are listed in green boxes with arrows; red boxes with blunt-headed lines represent inhibitory mechanisms. In the upper part R-SMAD activation by the TGF-beta receptor complex is shown. This activation can be inhibited by SMAD7, which itself is regulated by several processes. Phosphorylation of the SXS motif in the C-terminus of R-SMAD proteins promotes nuclear translocation, while this shuttling is prevented by phosphorylation of other sites. Besides phosphorylation, ubiquitination, deubiquitination and ribosylation regulate the TGF-beta pathway. These reactions can take place in the cytoplasm or in the nucleus, limited by dashed grey lines (adapted from (P. Xu et al., 2012)).

The abundance of regulatiory mechanisms controlling every layer of the TGF-beta pathway explains the resulting versatility of specific signaling responses. Moreover, it highlights the importance of this system for various cellular processes in health and disease.

1.3.4 The TGF-beta system in MM

The bone marrow microenvironment with numerous cytokines secreted by bone marrow stromal cells (BMSC), bone marrow mononuclear cells (BMMCs) and also by malignant plasma cell clones is of essential importance for MM pathogenesis (s. chapter 1.1.1). One of these tumor growth promoting cytokines is TGF-beta (Anderson & Carrasco, 2011).

In 1996 Urashima et al. showed, that TGF-beta secretion by MM-BMMCs, MM-BMSCs and $CD38^+$ MM cells is significantly higher than in normal BMMCs, BMSCs or B cells, respectively. Furthermore, they correlated augmented TGF-beta secretion with increasing levels of IL-6 and demonstrated inhibition of IL-6 secretion upon treatment with an anti-TGF-beta-1 antibody (Urashima et al., 1996). Besides IL-6 secretion T-cell immunosenescence, as well as supression of dendritic cell maturation and hence immune dysregulation equally result from TGF-beta signaling in MM (R. Brown et al., 2004; Suen et al., 2016). Moreover, TGF-beta can promote angiogenesis in MM tumors via enhanced VEGF secretion (Anderson & Carrasco, 2011; T. Hayashi et al., 2004). It plays a role in hypoxia-related induction of myeloma cancer stem cell-like populations and thus for tumor progression and recurrence (Wen et al., 2015). Bruns et al evidenced, that impairement of hematopoiesis by haematopoietic progenitor and mesenchymal stromal cells in MM is partly caused by elevated TGF-beta leves (Bruns et al., 2012). Last but not least, TGF-beta contributes to lytic bone disease in MM patients: It inhibits late-stage osteoblast differentiation and hence bone mineralization, increases RANKL secretion and promotes osteoclast survival (Breen et al., 1994; Gingery et al., 2008; Lu et al., 2016; Ruan, Pederson, Bradley, Bamberger, & Oursler, 2010).

Taken together, TGF-beta enhances MM growth and progression in various ways: It creates a cytokine rich, susceptible bone marrow microenvironment, worsens lytic bone disease and impacts normal immune responses (T. Hayashi et

al., 2004; Isufi et al., 2007; Urashima et al., 1996). However, it remains unclear, whether the growth factor directly stimulates MM cell proliferation and survival. Moreover, no TGF-beta antagonists for the treatment of MM patients have been developed so far. Thus, further research and a better understanding of TGF-beta signaling in MM is needed. In this context the UPS, with a fundamental role in the regulation of the TGF-beta signaling pathway, could present a niche for specific and efficient therapeutic innovations in MM.

1.4 Aim of the study

Although MM remains an incurable disease, proteasome inhibitors like bortezomib or carfilzomib have significantly improved frequency and the length of remissions in MM patients (Harousseau et al., 2010; Palumbo & Anderson, 2011). This shows the essential role of the UPS for the pathogenesis and progression of MM. However, little is known about many ubiquitination and deubiquitination pathways and their deregulation in this disease. In an attempt to identify novel components of the UPS with potential involvement in MM pathogenesis, an aCGH (array comparative genomic hybridization) study was revised by Dr. Bianca-Sabrina Tragosz and revealed the DUB USP24 as an interesting target (Carrasco et al., 2006). Loss of the Usp24 gene locus on chromosome 1p32.3 was detected in 25% of the MM patient samples. Subsequently, an unbiased mass spectrometry identified WWP2, an E3 ligase, known to interact with the TGF-beta system, as a binding partner of the DUB. Based on the previous findings of Dr. Bianca-Sabrina Tragosz, this study aimed to investigate the connection between TGF-beta and the UPS and to examine, whether it is relevant for MM pathogenesis. With this objective, binding of the DUB and the E3 ligase to members of the SMAD family was characterized biochemically. *In vivo* ubiquitination assays were further performed, to possibly uncover SMAD proteins as substrates of USP24. Next experiments aimed to reveal potential consequences of the Usp24 gene locus loss in MM for TGF-beta signaling. By partly clarifying the role of USP24, this study also intended to give an additional rationale, to further investigate the corresponding pathway not only in the physiologic setting, but mainly in the context of MM pathogenesis. Future studies might identify USP24 as a prognostic marker in MM and as a potential target for therapeutic innovations.

2 Materials and Methods

2.1 Materials

In the following section the materials, used in this study, will be presented. The companies, they were purchased from, are indicated in the same row.

2.1.1 Chemicals

Reagent	Company
beta-Mercaptoethanol	Sigma-Aldrich, Taufkirchen
3x FLAG Peptide	Sigma-Aldrich, Taufkirchen
Acetic acid	Roth, Karlsruhe
Acetone	Roth, Karlsruhe
Acrylamide/Bis Solution 40%, 29:1	Bio-Rad, München
Agarose	Roth, Karlsruhe
Albumin fraction V (BSA)	Roth, Karlsruhe
Ammonium persulfate (APS)	Roth, Karlsruhe
ANTI-FLAG M2 Affinity Gel	Sigma-Aldrich, Taufkirchen
Aqua ad injectabila, sterile	Braun, Melsungen
ATX Ponceau S red staining solution	Sigma-Aldrich, Taufkirchen
Bacto agar	BD, Franklin Lakes, USA
Bacto trypton	BD, Franklin Lakes, USA
Bacto yeast extract	BD, Franklin Lakes, USA
Borate 20x Solution	Pierce, Rockford, USA
5-Bromo-2'-desoxyuridine (BrdU)	Sigma-Aldrich, Taufkirchen
Bromphenol blue	Sigma-Aldrich, Taufkirchen
Calcium chloride ($CaCl_2$)	Sigma-Aldrich, Taufkirchen
Coomassie Brilliant Blue	Roth, Karlsruhe
Cycloheximide	Sigma-Aldrich, Taufkirchen
Deoxynucleotide triphosphate (dNTP) mix (10mM)	Fermentas, St. Leon-Rot
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe
Ethanol	Merck, Darmstadt
Ethidium bromide	Roth, Karlsruhe
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, Taufkirchen
Gelatine	Sigma-Aldrich, Taufkirchen
Gluthatione Sepharose™ 4B	GE Healthcare, Louisville, USA
Glycerol	Sigma-Aldrich, Taufkirchen
Glycine	Roth, Karlsruhe
Hydrochloric acid (HCl)	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe

Isopropyl beta-D-1-thiogalactopyranoside (IPTG)	Sigma-Aldrich, Taufkirchen
L-[^{35}S]-Cysteine	Hartmann Analytics, Braunschweig
L-[^{35}S]-Methionine	Hartmann Analytics, Braunschweig
L-Glutathione	Sigma-Aldrich, Taufkirchen
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Taufkirchen
Methanol	Merck, Darmstadt
N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES) pH 7.1	Sigma-Aldrich, Taufkirchen
Nonident P40, 10% (w/v)	Roche, Penzberg
NuPAGE® MES SDS Running Buffer	Invitrogen, Karlsruhe
Polybrene (Hexdimethrine bromide)	Sigma-Aldrich, Taufkirchen
Potassium chloride	Fluka, Taufkirchen
Propidium iodide	Sigma-Aldrich, Taufkirchen
Protein A Sepharose	GE Healthcare, Louisville, USA
Protein G Agarose	Sigma-Aldrich, Taufkirchen
Protein G Sepharose	Invitrogen, Karlsruhe
Skim milk powder	Fluka, Taufkirchen
SOC Media	NEB, Frankfurt
Sodium acetate	Merck, Darmstadt
Sodium azide	Merck, Darmstadt
Sodium chloride (NaCl)	Roth, Karlsruhe
Sodium citrate	Sigma-Aldrich, Taufkirchen
Sodium dihydrogenphosphat	Merck, Darmstadt
Sodium dodecylsulfate (SDS)	Roth, Karlsruhe
Sodium fluoride (NaF)	Sigma- Aldrich, Taufkirchen
Sodium hydroxide (NaOH)	Roth, Karlsruhe
Sorbitol	Sigma-Aldrich, Taufkirchen
Strep-Tactin Superflow 50%	IBA, Göttingen
Strep-tag elution buffer (10x) with Desthiobiotin	IBA, Göttingen
SuperSignal® Chemiluminescence Substrat	Pierce, Rockford, USA
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich, Taufkirchen
Trichloroacetic acid (TCA)	Sigma-Aldrich, Taufkirchen
Tris(hydroxymethyl)aminomethane (Tris)	Roth, Karlsruhe
Tris buffered saline (TBS) (10x)	Sigma-Aldrich, Taufkirchen
Triton X-100	Fluka, Taufkirchen
Trypan Blue	Invitrogen, Karlsruhe
Tween 20	Fluka, Taufkirchen
UltraPure™ 10 x Tris/Borate/EDTA (TBE) Buffer	Invitrogen, Karlsruhe

2.1.2 Media and supplements for cell culture

Dulbecco's Modified Eagel's Medium (DMEM)	Gibco
Iscove's Modified Dulbecco's Media (IMDM)	Invitrogen, Karlsruhe
McCoy's 5A Medium Modified	Gibco
RPMI 1640	Gibco
Fetal Bovine Serum (FBS) Gold	Gibco
Bovine Serum (BS)	Gibco
Opti-MEM® I, reduced serum media	Invitrogen, Karlsruhe
Phosphate buffered saline (PBS), 10x, sterile	Gibco
Penicillin/Streptomycin (100x)	Gibco
Trypsin-EDTA (10x) solution	Gibco
Glutamine (100x)	Gibco
beta-Mercaptoethanol	Invitrogen, Karlsruhe

2.1.3 Antibiotics

Ampicillin	Roth, Karlsruhe
Kanamycin	Fluka, Taufkirchen

2.1.4 Transfection reagents

HiPerfFect	Qiagen, Hilden
Lipofectamine ®2000	Invitrogen, Karlsruhe

2.1.5 Enzymes

Benzonase	Sigma-Aldrich, Taufkirchen
DNase I	Sigma-Aldrich, Taufkirchen
Ribonuclease A	Sigma-Aldrich, Taufkirchen
Pfu Ultra DNA Polymerase	Agilent, Loveland, USA
SuperScript II Reverse Transcriptase	Invitrogen, karlsruhe
T4 DNA Ligase	Fermentas, St. Leon-Rot

Restriction enzymes: Fermentas, St- Leon-Rot

ApaI / BamHI / DpnI / EcoRI / MluI / NheI / NotI / XbaI / XhoI

All enzymes were supplied with suitable reaction buffers.

2.1.6 Inhibitors

Aprotinin	Sigma-Aldrich, Taufkirchen
Glycerol 2-phosphate disodium salt hydrate (G2P)	Sigma-Aldrich, Taufkirchen
Leupeptin	Sigma-Aldrich, Taufkirchen

Ocadaic acid	Sigma-Aldrich, Taufkirchen
Phenylmethylsulfonyl fluoride	Sigma-Aldrich, Taufkirchen
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Taufkirchen
Soybean Trypsin Inhibitor	Sigma-Aldrich, Taufkirchen
Tosyl-L-lysyl-chloromethyl ketone hydrochloride (TLCK)	Sigma-Aldrich, Taufkirchen
Tosyl-phenylalanyl-chloromethyl ketone (TPCK)	Sigma-Aldrich, Taufkirchen
4,5,6,7-Tetrabromo benzotriazole (TBB)	Sigma-Aldrich, Taufkirchen
Proteasom Inhibitor MG-132	Boston Biochem, Cambridge, USA

2.1.7 Molecular weight standards

GeneRuler 1kb DNA Ladder	Fermentas, St. Leon-Rot
GeneRuler 100bp DNA Ladder	Fermentas, St. Leon-Rot
6x DNA Loading Dye	Fermentas, St. Leon-Rot
PageRuler Plus Prestained Protein Ladder	Fermentas, St. Leon-Rot

2.1.8 Molecular biological Kits

DC™ Protein Assay	Bio-Rad, München
GeneJET™ Gel Extraction Kit	Fermentas, St. Leon-Rot
GeneJET™ PCR Purification Kit	Fermentas, St. Leon-Rot
LightCycler 480 SYBR Green I Master	Roche, Penzberg
Rapid DNA Ligation Kit	Roche, Penzberg
RNeasy Mini Kit	Qiagen, Hilden
Superscript III Reverse Transcriptase	Invitrogen, Karlsruhe
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
QIAprep Spin Miniprep Kit	Qiagen, Hilden
QIAshredder homogenizer Kit	Qiagen, Hilden

2.1.9 Buffers

If not stated differently dH_2O (aqua destillata).was used for the preparation of buffers.

Lysis Buffer:	50mM Tris (pH 7,5) 250mM NaCl 0,1% Triton X-100 1mM EDTA 50mM NaF + inhibitors
Lysis Buffer (MS/IP):	50mM Tris-HCl (pH 7,5) 150mM NaCl 0,1% NP40 1mM EDTA 5mM $MgCl_2$

	5% Glycerol 5nM Ocadaic Acid + inhibitors
Hypomolar Lysis Buffer (fractionated lysis, cytoplasmic fraction):	10mM HEPES 10mM KCl 1,5mM $MgCl_2$ 0,1% NP40 0,5mM DTT + inhibitors
BT Buffer	50mM Tris-HCl (pH7,5) 100mM NaCl 5mM EDTA 50mM $MgCl_2$
Inhibitors:	1µg/ml Aprotinin 1mM DTT 10mM G2P 1µg/ml Leupeptin 10µg/ml Soybean Trypsin Inhibitor 0,1mM PMSF 0,1mM Na_3VO_4 5µg/ml TLCK 5µg/ml TPCK
Running Buffer (10x):	250mM Tris (pH 7,5) 1,92M Glycine 1% SDS
Transfer Buffer (10x):	250mM Tris (pH 7,5) 1,5M Glycine 1% SDS for 1x: 2 vol Methanol and 7vol dH2O
Washing Buffer:	PBS (1x) 0,1% Tween 20
Blocking Buffer (Western Blot):	PBS (1x) 0,1% Tween 20 5% Milk powder
Stripping Buffer:	62,5mM Tris (pH 6,8) 0,867% β-Mercaptoethanol 2% SDS
Laemmli Buffer (5x):	300mM Tris (pH 6,8) 50% Glycerol 10% SDS 5% β-Mercaptoethanol

	0,05% Bromphenolblue
Stacking Gel Buffer:	0,5 M Tris (pH 6,8)
Separating Gel Buffer:	1,5 M Tris (pH 8,8)
Coomassie Staining:	0,25% Coomassie brilliant blue 45% Methanol 10% Acetic Acid
Comassie Destain:	45% Methanol 10% Acetic Acid
BrdU wash buffer I:	PBS (1x) 1% BSA
BrdU wash buffer II:	PBS (1x) 0,5% Tween 20 1% BSA
DNA Denaturating Buffer:	2 N HCl 0,5% Triton X-100
LB-agar plates:	1% Bacto trypton 0,5% Bacto yeast extract 1% NaCl 1,5% Bacto agar

2.1.10 Antibodies

Primary mouse antibodies

Antibody	Dilution	Company
beta-Actin (monoclonal)	1:10000	Sigma-Aldrich, Taufkirchen
BrdU Antibody, FITC (MoBU-1)	1-5µg/ 10^6cells	ThermoFisher Scientific, Waltham, USA
CUL1	1:500	Invitrogen, Karlsruhe
FLAG M2 (monoclonal)	1:3000	Sigma-Aldrich, Taufkirchen
HA.11 (monoclonal)	1:5000	Covance Princeton, USA
MYC	1:1000	Santa Cruz Biotechnologies, Santa Cruz, USA
PLK1	1:500	Invitrogen, Karlsruhe
SMAD4	1:500	Santa Cruz Biotechnologies, Santa Cruz, USA
SMAD7	1:200	Santa Cruz Biotechnologies, Santa Cruz, USA
USP32	1:1000	Santa Cruz Biotechnologies, Santa Cruz, USA
USP9X	1:1000	Novus Biologicals, Wiesbaden-Nordenstadt

Table 2: List of primary mouse antibodies

Primary rabbit antibodies

Antibody	Dilution	Company
FLAG	1:1000	Sigma-Aldrich, Taufkirchen
HA-411	1:500	Santa Cruz Biotechnologies, Santa Cruz, USA
HSP90	1:1000	Cell Signaling Technology, Danvers, USA
MYC	1:1000	Millipore, Schwalbach/Ts
Phospho-SMAD3 (monoclonal)	1:1000	Cell Signaling Technology, Danvers, USA
SMAD3 (monoclonal)	1:1000	Cell Signaling Technology, Danvers, USA
SMAD2/3 (monoclonal)	1:500	Cell Signaling Technology, Danvers, USA
USP24	1:2000	Bethyl Laboratories, Montgomery, USA
USP24	1:500	ProteinTech Group, Rosemont, USA
USP28	1:2000	Bethyl Laboratories, Montgomery, USA
USP32	1:2000	Bethyl Laboratories, Montgomery, USA
WWP2	1:2000	Bethyl Laboratories, Montgomery, USA

Table 3: List of primary rabbit antibodies

Secondary antibodies

Antibody	Dilution	Company
Alexa Fluor anti mouse 488	1:1000	Invitrogen, Karlsruhe
Alexa Fluor anti mouse 594	1:1000	Invitrogen, Karlsruhe
Alexa Fluor anti rabbit 488	1:1000	Invitrogen, Karlsruhe
Alexa Fluor anti rabbit 594	1:1000	Invitrogen, Karlsruhe
ECL anti mouse IgG horseradish peroxidase linked	1:5000	GE Healthcare, München
ECL anti rabbit IgG horseradish peroxidase linked	1:5000	GE Healthcare, München
ECL anti protein A horseradish peroxidase linked	1:5000	GE Healthcare, München

Table 4: List of secondary antibodies

2.1.11 Plasmids

For verification of the DNA sequences all plasmids were checked with Eurofins Genomics, Ebersberg.

pcDNA3.1_Flag	Cloned by Prof. Dr. F. Bassermann
pcDNA3.1_Flag_Usp9X	Cloned by Dr. K. Engel
pcDNA3.1_Flag_Wwp2	Cloned by Dr. B.-S. Tragosz
pcDNA3.1_Flag_Smad2	Cloned in this study by L. Häusler
pcDNA3.1_Ha	Cloned by Prof. Dr. F. Bassermann

pCMV5B_Flag_Smad3	Cloned in this study by L. Häusler
pCMV5B_Ha_Smad2	Cloned in this study by L. Häusler
pCMV5B_Ha_ Smad7	Cloned in this study by L. Häusler
pCMV6-AC_turboGFP_Usp24	Bought from Origene 09.10.10
pIres_puro_Flag_Usp24	Cloned by Dr. U. Baumann
pLKO.1_TRC_dsred_shscramble	Cloned by Dr. M. Heider
pLKO.1_TRC_dsred_Usp24sh	Cloned by J. Kurutz
pLKO.1_TRC_dsred_Wwp2sh	Cloned by J. Kurutz
pRibery; N-SF-TAP-pcDNA3.0	Cloned by M. Ueffing
pRibery: N_SF_TAP_pcDNA3.0_Usp24	Cloned by Dr. B.-S. Tragosz
pRibery; N_SF_TAP_pcDNA3.0 _Usp32	Cloned by Dr. B.-S. Tragosz
pRK5_HA_Ubiquitin_WT	Bought from Addgene

Table 5: List of plasmids

2.1.12 Oligonucleotides

All primers were purchased from Eurofins Genomics, Ebersberg.

Primers for cloning

Smad2_NheI_fw	5'-GGCGCTAGCTCGTCCATCTTGCCATTCACGCCG-3'
Smad2_XhoI_rv	5'-GGCCTCGAGCTATTATGACATGCTTGAGCAACG-3'
Smad3_NheI_fw	5'-CGGGCTAGCTCGTCCATCCTGCCTTTCACTCCC-3'
Smad3_XhoI_rv	5'-GGCCTCGAGCTAAGACACACTGGAACAGCG-3'
Smad7_EcoRI_fw	5'-GGCGAATTCTTCAGGACCAAACGATCTGCG-3'
Smad7_NheI_fw	5'-CCGGCTAGCATGTTCAGGACCAAACGATCTGC GCTCGTCCGG-3'
Smad7_XhoI_rv	5'-CCGCTCGAGCTACCGGCTGTTGAAGATGACCT CTAGCCAGC-3'
Usp24_ApaI_rv	5'-CCTGGGCCCACTAGGGATCAACATCATCAAGG-3'
Usp24_BamHI_fw	5'-CCGGGATCCGCCACCATGTACCCCTACGACGG CCCGACTACGCCACCACGCTGCTGTGCATGGGC-3'
Usp24_NheI_fw	5'-CCGGCTAGCACCACGCTGCTGTGCATGGGC-3'
Usp24_SalI_fw	5'-CGTGTCGACACCACGCTGCTGTGCATGGGC-3'
Usp24_SalI_rv	5'-GGCGTCGACCTAGGGATCAACATCATCAAGG-3'
Usp24_XhoI_rv	5'-GGCCTCGAGCTAGGGATCAACATCATCAAGG-3'
Wwp2_BglII_fw	5'-CGAAGATCTGCATCTGCCAGCTCTAGCCGG-3'
Wwp2_NheI_fw	5'-CGAGCTAGCGCATCTGCCAGCTCTAGCCGG-3'
Wwp2_XhoI_rv	3'-GCACTCGAGTTACTCCTGTCCAAAGCCC-3'

Table 6: List of cloning primer sequences

Target sequences for shRNA

shUsp24_AgeI_fw	5'-CCGG**TGCCACAGCTTTGTTGAATGACTCGAGT CATTCAACAAAGCTGTGGCA**TTTTTG-3'

shUsp24_EcoRI_rv	5'-AATTCAAAAA**TGCCACAGCTTTGTTGAATGACT CGAGTCATTCAACAAAGCTGTGGCA**-3'
shWwp2_AgeI_fw	5'-CCGGC**TCACCTACTTTCGCTTTATACTCGAGTA TAAAGCGAAAGTAGGTGA**GTTTTTG-3'
shWwp2_EcoRI_rv	5'-AATTCAAAAA**CTCACCTACTTTCGCTTTATACTC GAGTATAAAGCGAAAGTAGGTGAG** -3'

Table 7: List of DNA oligos for shRNA synthesis

Primers for sequencing

Check_link_Usp24GFP	5'-GCCCTGAGCAAAGACCCCAACG-3'
Check_Usp24_0	5'-GCCTCATGGATGGGTTGTGG-3'
Check_Usp24_1	5'-GGTACAGCCCATGCTAGACC-3'
Check_Usp24_2	5'-GCATTATTCAAGACTTGAAG-3'
Check_Usp24_3	5'-GTTCCACGAACTATTCTACC-3'
Check_Usp24_4	5'-GACTATGAAACAAGGCAGGG-3'
Check_Usp24_5	5'-GTATTTTGATTTGAGATGCC-3'
Check_Usp24_6	5'-CAAGAAAATGGGGAGAGACC-3'
Check_Usp24_7	5'-CCTCAGTCATCCCCTCGGCC-3'
Check_Usp24_8	5'-CTGCTGTTTCTGTAATGAGC-3'
Check_Usp24_9	5'-GGAGCTACATCATCAAGTGC-3'
Check_Usp24_10	5'-TGTCATGCAGAGAGACTCC-3'
Check_Usp24_11	5'-GCATATGAATTCTTTACTAG-3'
Check_USP24_GFP	5'-CGCCACCATGGTGAGCAAGG-3'
CMV_fw	5'-CGCAAATGGGCGGTAGGCGTG-3'
plko.1 seq fw	5'-GATACAAGGCTGTTAGAGAGATAATT-3'
Seq_Wwp2_1	5'-GGAATGAGATCATCATTTTG-3'
Seq_Wwp2_2	5'-CCATGATCCCCTGGGCCCCC-3'
Seq_Wwp2_3	5'-CCTGGATGGCTTCAACGAGG-3'
T7	5'-TAATACGACTCACTATAGGG-3'

Table 8: List of sequencing primers

Primers for qPCR

qPCR_ARPPA_Fw	5'-GCACTGGAAGTCCAACTACTTC-3'
qPCR_ARPPA_Rv	5'-TGAGGTCCTCCTTGGTGAACAC-3'
qPCR_GAPDH_Fw	5'-GAAGGTGAAGGTCGGAGTC-3'
qPCR_GAPDH_Rv	5'-GAAGATGGTGATGGGATTTC-3'
qPCR_Usp24_fw	5'-TGTCTCATCCCAAAGGAATGTTGC-3'
qPCR_Usp24_rv	5'-GCAATGCTAATGGGTGGTCGT TG-3'
qPCR_Wwp2_fl_fw	5'-GCAGTCGCAGCGGAATCA-3'
qPCR_Wwp2_fl_rv	5'-AGGGGATCATGGTCAGTCGA-3'

Table 9: List of qPCR primer sequences

siRNA oligonucleotides

The listed siRNA duplexes were bought from Dharmacon, Lafayette, USA.

silacZ	5'-AAUCGAAGUAUUCCGCGUACGUU-3'

siUsp24#3	5'-GGACGAGAAUUGAUAAAGA-3'
siUsp24#5	Exon 60; NM_015306
siUsp24#6	5'-CCACAGCUUUGUUGAAUGA-3'
siUsp24#7	5'-GUAGAAGCCUUGUUGUUCA-3'

Table 10: List of siRNA target strand sequences

2.1.13 Bacteria

NEB 5-alpha F´I^q Competent E. coli — New England Biolabs, Frankfurt

2.1.14 Cell lines

Cell line	Description	Media	Source
HEK293T	human embryonic kidney cells with SV40 large T-Antigen	DMEM	DSMZ, Braunschweig
HeLa	human cervix carcinoma cells	DMEM	DMSZ, Braunschweig
JJN3	human multiple myeloma cells	RPMI 1460	DMSZ, Braunschweig
LP1	human multiple myeloma cells	IMDM	DMSZ, Braunschweig
OPM2	human multiple myeloma cells	RPMI 1460	DMSZ, Braunschweig
RPMI 2668	human multiple myeloma cells	RPMI 1640	DMSZ, Braunschweig
U2OS	human osteosarcoma cells	McCoy's 5A	ATCC, Virginia, USA
U266	human multiple myeloma cells	RPMI 1640	Kind gift of Dr. Tobia Dechow

Table 11: List of cell lines

L-Glutamine, Penicillin/Streptomycin and 10% FBS were added to all media (3rd panel) – except for HEK293T, whose medium was supplemented with 10% BS instead of FBS.

2.1.15 Cell culture dishes

Dishes and flasks — **Sigma-Aldrich, Taufkirchen**
Greiner CELLSTAR® dish

24-well, 12-well,6-well,T25,T75, 6cm^2, 10cm^2, 15cm2

2.1.16 Membranes

PVDF membrane (Immobilon P) — Millipore, Schwalbach/Ts

2.1.17 Machinery and equipment

Agarose electrophoresis chamber Mini-Sub Cell GT	Bio-Rad, München
Analytical balance ABJ	Kern & Sohn, Balingen-Frommern
Bacterial Shaker	Eppendorf, Hamburg
Calligrapher MiniArrayer	Bio-Rad, München
Centrifuge Multifuge 3SR+	Thermo Scientific, Waltham, USA
CO_2 Incubator Hera cell 150i	Thermo Scientific, Waltham, USA
Cool-centrifuge5417R	Eppendorf, Hamburg
Cool-centrifuge5430R	Eppendorf, Hamburg
A-BOX VX2 Fluorescence Gel Documentation System	Montreal Biotech Inc., Dorval, Canada
FACS Calibur	BD, Franklin Lakes, USA
Fluorescence microscope	Olympus, Hamburg
FluoView FV10i	Olympus, Hamburg
Invitrogen Chamber for Ready Gels	Invitrogen, Karlsruhe
Lightcycler 480 Real-Time PCR System	Roche, Penzberg
Magnetic Thermo Stirrer RCT basic	IKA, Staufen
Microscope Axiovert 40 CFL	Carl, Zeiss AG, Oberkochen
Nano-Photometer	Implen, München
Neubauer hemocytometer	Marienfeld, Lauda-Königshofen
PCR-Thermocycler Primus	Peqlab, Erlangen
pH-meter pH720 InoLab	WTW, Weilheim
Power Supply PAC Basic	Bio-Rad, München
Power Supply PAC HC	Bio-Rad, München
Precision balance 572	Kern & Sohn, Balingen-Frommern
Rotating Wheel 3000	Fröbel Labortechnik, Lindau
Safety cabinet Herasafe KS	Thermo Scientific, Waltham, USA
Scanner V750 Pro	Epson, Meerbusch
SDS-gel Electrophoresis chamber Mini-Protean	Bio-Rad, München
SpeedVac	Eppendorf, Hamburg
Tabletop centrifuge 5425	Eppendorf, Hamburg
Thermomixer	Eppendorf, Hamburg
Waving platform shaker Polymax 1040	Heidolph, Instruments, Kelheim
Western Blotting chamber Trans Blot Cell	Bio-Rad, München

2.1.18 Software and databases

FlowJo cytometry analysis software	Tree Star, Ashland, USA
MacVector Sequence analysis software	Mac Vector, Cary, USA
Oncomine database	Compendia biosciences, Ann Arbor, USA
GraphPad PRISM	GraphPad Software, La Jolla, USA
QuantPrime software	Universität Potsdam, Max-Planck Institut

2.2 Methods

This chapter explains all applied methods. For a better understanding it is subdivided into the sections: molecular biology, protein biochemistry, eukaryotic and bacterial cell culture as well as data mining and processing.

2.2.1 Molecular biology

2.2.1.1 Agarose gel electrophoresis

DNA was visualized by agarose gel electrophoresis. Agarose was dissolved in TBE buffer and heated until the mixture started boiling (s. chapter 2.1.9). After reducing the liquid's temperature under cold water, DNA intercalating chemical – ethidium bromide – was added for visualization of DNA under UV light. The mixture was poured in a chamber and cooled down with subsequent formation of a stable gel. Once potential was applied, DNA could migrate through the gel's pores according to it's charge – proportional to the size of DNA. Resulting bands were visualized under UV light using Gel Documentation System (Montreal Biotech Inc.).

2.2.1.2 DNA restriction

DNA restriction enzymes cleave the sugar-phosphate backbone of DNA. They recognize specific palindromes, i.e. the base sequence reads the same forwards and backwards on complementary strands. By cutting inverted repeat palindromes, they produce sticky ends – overhanging single strand ends – which allows the annealing of two cohesive ends for cloning. For a complete digest enzymatic restriction reactions were incubated for 2 hours at 37°C.

2.2.1.3 DNA ligation

The formation of a phosphodiester between the 3' hydroxyl and 5' phosphate of adjacent DNA residues is catalyzed by DNA ligases. This reaction was used to join dsDNA fragments with sticky or blunt ends to produce recombinant DNA plasmids. For Rapid DNA Ligation Kit (Roche) vector and insert DNA were diluted in 1x concentrated DNA dilution buffer to a final volume of 10µl. After adding ligation buffer and T4 DNA ligase the reaction was incubated at 15°C to 25°C for 5 minutes. For optimal results the molarities of vector and insert DNA were adapted to each other.

2.2.1.4 Molecular cloning and DNA extraction

To clone a selected gene or DNA sequence into a plasmid, functioning as an expression vector, latter was cut with restriction enzymes, recognizing specific palindromes of the multiple cloning site (MCS). Enzymatic reactions were set up according to the manufacturer's instructions and incubated for 2 hours at 37°C for each restriction enzyme. With Agarose Gel Electrophoresis linearized vectors were identified, cut out and purified with a Gel Extraction Kit (Fermentas). For the amplification of the desired gene, primers with short overhanging ends were designed. They encoded for a palindromic restriction site, known to be compatible to the enzymes cutting in the MCS of the vector. Before ligation the amplified gene was purified with a PCR Purification Kit (Fermentas). Ligation was performed with the Rapid DNA Ligation Kit (Roche) at 15 to 25°C for 5 minutes. Ligation reactions were transformed into NEB5α competent E. coli bacteria, followed by incubation at 37°C over night for amplification. An ampicillin resistance of the expression vector allowed the selection of positive clones with ampicillin containing medium. QIAprep Spin Miniprep Kit (Qiagen) was used for smaller amounts and QIAGEN Plasmid Maxi Kit (Qiagen) for larger amounts of DNA to extract the vectors with the inserted DNA fragment. These kits use alkaloid lysis to destroy the bacteria cell wall and anionic columns of silicon dioxide for immobilization of the DNA. After removal of RNA and proteins by increasing salt concentrations and centrifugation, DNA was eluted with high salt buffer. Addition of isopropanol allowed its precipitation. Finally ethanol was used to remove isopropanol and DNA was diluted in elution buffer or distilled H_2O. To verify the insertion of the desired DNA fragment or gene, samples were sent to Eurofins Genomics for sequencing.

2.2.1.5 Gene silencing

For gene silencing short hairpin RNA (shRNA) or small interfering RNA (siRNA) were used. Via interference with mRNA, they inhibit its translation and hence silence the corresponding target gene. siRNAs are double-stranded RNA molecules with 20 to 25 basepairs, which can be directly brought into the cell without need for a vector (s. chapter 2.2.3.1 siRNA silencing). shRNAs are single stranded and have the structure of a hairpin, i.e. two short antiparallel RNA strands with a linker loop in between, allowing the RNA to fold back on itself and to form a double-stranded molecule. Via introduction of a specific DNA sequence, delivered in a vector format,

the target cell can be directed to produce a certain shRNA (s. chapter 2.2.1.4). In this study lentiviral vectors were used, allowing the DNA construct to remain in the nucleus with continuous shRNA production. Thus, a long lasting gene knockdown results. In the end the cell processes shRNA to siRNA (Paddison, Caudy, Bernstein, Hannon, & Conklin, 2002; Rao, Vorhies, Senzer, & Nemunaitis, 2009). siRNA oligos were purchased from Dharmacon. DNA oligos for shRNA synthesis were ordered at Eurofins Genomic with overlapping ends, encoding for the restriction sites of specific enzymes and necessary for the cloning into a certain plasmid (s. chapter 2.1.12).

2.2.1.6 PCR

To amplify a gene or a certain segment of DNA, polymerase chain reaction (PCR) was performed. Forward (fw) and reverse (rv) primers, compatible to the beginning or the end sequence of the gene or DNA fragment, set limits to the area of interest. A mixture of 100ng of target DNA, 1pM fw and 1pM rv primers, 10mM dNTPs for synthesis of the new DNA strands together with the DNA polymerase Pfu Ultra HF (Stratagene) in suitable buffer was prepared. The used PCR protocol started with melting of the DNA double helix at 95°C, followed by annealing of the primers at 55 to 65°C and elongation at 72°C. Since the DNA polymerase Pfu Ultra HF works with a speed of approximately 1kb/min, elongation time was adjusted to the length of the DNA fragment divided by 1000 in minutes. 25 to 30 cycles were performed for exponential amplification of the DNA product.

2.2.1.7 Reverse transcription

To measure the amount of a particular mRNA, it was reversely transcribed to complementary DNA (cDNA). First of all, RNA was extracted with the RNeasy Mini Kit (Qiagen): This kit uses guanidium thiocyanate for cell lysis, a substance, which denaturates RNase enzymes, thus preventing mRNA damage. After addition of Ethanol, to adjust binding conditions, the lysate was loaded on the RNeasy silica membrane and cleaned from DNA and proteins. The remaining RNA was diluted in RNase free water. The concentration of RNA was measured by photometer before starting the reverse transcription to cDNA. For reverse transcription (RT) 1µg RNA was mixed with Random Hexamer primers, binding to multiple mRNA sites, and heated at 70°C for 5 minutes. After cooling down to room temperature dithiothreitol (DTT), a reducing agent, which loosens the secondary structure of RNA, dNTPs,

RNaseOUT (Invitrogen) and Superscript II RT (Invitrogen) were added. The enzymatic reaction was performed at 42°C for 1 hour. For subsequent qPCR, the cDNA was diluted 1:10 in dH_2O.

2.2.1.8 qPCR

Quantitative real time PCR (qPCR) measures the amplification of a target DNA, while PCR is running and not after the reaction is complete, as it is the case for conventional PCR. qPCR experiments were perfomed with SYBR Green qPCR Mix (Roche), that uses SYBR Green as a fluorescent, DNA-intercalating dye. This allows the indirect quantification of the PCR product via the detected fluorescence intensity, set in correlation with the amplification cycles. The corresponding values were displayed as $2^{-\Delta\Delta CT}$. At the same time, expression of ubiquitous genes like GAPDH and ARPPA was measured as a control and used for normalization of the values. qPCR primers were designed with Quant Prime software. Before the qPCR reaction itself could begin, a master mix was prepared, taking into account the number of needed wells: Per well 1,5µl dH_2O, 0,5µl fw primer, 0,5µl rv primer and 5µl SYBR Green qPCR mix were added to a 1,5ml reaction tube. 7,5µl of this master mix were pipetted into each well with subsequent addition of 2,5µl of the diluted cDNA or of dH_2O, serving as a control, respectively. Finally the multiwell plate was covered with aluminium foil and loaded into the Lightcycler 480 Real-Time PCR System (Roche).

2.2.2 Protein biochemistry

2.2.2.1 SDS-gel electrophoresis

SDS-gel electrophoresis was used to separate and visualize proteins according to their electrophoretic mobility. Before loading the protein samples into the pockets of the SDS-gel, they were denatured by heating up in Laemmli buffer (s. chapter 2.1.9). Sodium dodecyl sulfate (SDS) is an anionic detergent. It linearizes proteins and imparts them with a negative charge, correlating with the protein's molecular size. Thus, application of electric potential separates the samples depending on their electrostatic properties. The SDS-gel consists of two parts, a stacking gel and a separating gel. Due to an acidic pH of 6.8 in the stacking gel, glycine as an ingredient of the Laemmli buffer is neutrally charged. Hence, all proteins assemble at the same horizontal barrier in the stacking gel. The pH of the separating gel is alcalic (pH=8.8),

consequently glycine is provided with a negative charge, allowing the proteins to ressolve, according to their electrostatic properties. SDS-PAGE was run at 70 to 120Volt for 1 to 3 hours.

2.2.2.2 Western blot analysis

To make protein samples accessible to antibody detection, they were transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich) after SDS-gel electrophoresis. First of all the PVDF membrane was activated in 100% methanol, to reduce its hydrophobicity. Subsequent western blotting was performed at 70 to 100Volt for 1 to 2 hours or at 30Volt over night: Through application of an electric current, the negatively charged proteins were pulled from the SDS-gel, placed on the cathodic side, onto the membrane, on the anodic side. After their transfer, the proteins were stained with Ponceau S, to check the loading, scanned and destained with washing buffer (WB) (s. chapter 2.1.9). To reduce unspecific antibody binding to the nitrocellulose membrane, latter was blocked with 5% non-fat dry milk in WB for 1 hour. After blocking them, the membranes were incubated with the corresponding primary antibodies (for dilutions s. chapter 2.1.10) either in 5% milk or in 5% BSA for 1 to 2 hours at room temperature or over night at 4°C. Before addition of the secondary antibody, the membranes were washed 3 times for 15 minutes with WB. Incubation with the corresponding secondary antibody, directed against the Fc (fragment crystallizable) domain of the primary antibody, was performed in 5% milk for 1 hour. After rinsing the membranes another 3 times with WB, they were prepared for the development using chemiluminescence substrate by Pierce Protein Biology: The secondary antibody is linked to an enzyme, called horseradish peroxidase, which cleaves hydrogen peroxid in the chemiluminescence substrate, thereby producing luminescence in proportion to the amount of protein. The luminescence signal was detected on photosensitive films, which were scanned afterwards. Density differences were evaluated using publicly available ImageJ[32] program. Size was estimated by comparison of the stained bands to the protein marker of known molecular weight (PAGE Ruler Thermo Scientific) on the membrane. Proteins like beta-Actin and CUL1 were used as loading control.

2.2.2.3 Cell lysis and fractionation

After collection of the cells they were resuspended in NaCl containing lysis buffer (s. chapter 2.1.9) for whole cell lysates. Salt concentration in the lysis buffer was adjusted depending on the requirements of the experiment: To preserve interactions, salt concentration was lowered in the lysis buffer, for very pure fractionation of proteins, it was raised. Other ingredients of the lysis buffer were detergent, to break the cell membrane as well as kinase, protease and phosphatase inhibitors, to prevent enzymatic destruction or modification of the target proteins. For Co-immunoprecipitation and mass spectrometry cells were lysed in 150mM NaCl containing lysis buffer with ocadaic acid, an inhibitor of serin/threonine protein phosphatases, to preserve protein-protein interactions (s. chapter 2.1.9). While 250mM NaCl lysis buffer was used for most other experiments (s. chapter 2.1.9).

In case of fractionating lysis into the nuclear and the cytoplasmic fractions, the cell pellet was first lysed in hypomolar lysis buffer with 10mM KCl for 15 minutes on ice. After centrifugation at top speed for another 15 minutes and transfer of the supernatant - representing the cytoplasmic fraction - into prechilled tubes, nuclei were lysed. Lysis of nuclei was performed using 150mM NaCl lysis buffer (MS/IP lysis buffer), additionally containing benzonase, which hydrolyzes nucleic acids (s. chapter 2.1.9). After lysing the nuclei for 40 minutes on ice, the mixture was centrifugated at top speed for 15 minutes and the supernatant put into cool tubes, representing the nuclear fraction.

Finally protein concentrations were calculated using Lowry assay (Lowry, Rosebrough, Farr, & Randall, 1951) based Bio-Rad DC protein assay (Bio Rad). Lysates were stored at -80°C. To perform SDS page they were denatured with Laemmli buffer at 95°C for 5 minutes.

2.2.2.4 Immunoprecipitation

Immunoprecipitation (IP) is a common method for antigen detection and purification. A specific mono or polyvalent antibody binds to its target antigen. The immune complex of antibody and antigen is then immobilized on agarose or sepharose beads, to which the antibody binds covalently or temporarily. Subsequent centrifugation allows a clear separation of the antigen, bound to heavy beads, from

the other lighter proteins. Finally the antigen can be eluted by denaturation, raising salt concentrations or via the addition of a competitive protein.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) works in a similar way, but it is used to detect interactions of proteins. The interaction partners are not directly bound by the antibody-bead complex but indirectly via the target protein. In this study FLAG M2 agarose beads, monoclonal anti-HA agarose beads (Sigma-Aldrich) and protein A beads (GE Healthcare Life Science) were used. They were washed 4 times with PBS and IP lysis buffer (s. chapter 2.1.9) and afterwards incubated with cell lysates for 1 to 2 hours at 4°C under constant rotating. Protein A beads are not covalently bound to an antibody. Hence, before their addition to the cell lysates, they were incubated with the corresponding antibody at 4°C for 2 hours, thereby allowing temporary binding of the antibody to the beads. To determine the protein concentration for SDS-gel electrophoresis, aliquots were taken out of the samples before adding the beads. After washing off all non-bound proteins with lysis buffer the antigen and its interaction partners were eluted by denaturation in Laemmli buffer (s. chapter 2.1.9) at 95°C for 10 minutes. Finally, SDS-PAGE and western blot were performed as described in chapter 2.2.2.1 and 2.2.2.2.

2.2.2.5 Mass spectrometry

Mass spectrometry (MS) is an important method in the area of proteomics, meaning in the study of proteins. It measures the mass-to-charge ratio (m/z) of ions, thereby identifying and quantifying biomolecules in diverse mixtures. Thanks to methods like Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), the structure of proteins can be studied (Barber, Bordoli, Sedgwick, & Tyler, 1981; Chowdhury, Katta, & Chait, 1990; Fenn, Mann, Meng, Wong, & Whitehouse, 1989) Every mass spectrometer consists of 3 elements: (I) the ion source, (II) a mass analyzer and (III) an ion detector. After loading the samples into the mass spectrometer, they are evaporated and transformed into ions by the ion source. Ionisation is performed with different methods; in this study MALDI technique was used. Thanks to the newly obtained charge, ionized molecules can be accelerated towards the analyzer by application of an electric current. When they encounter the electromagnetic field in the mass analyzer, the path of individual ions

is deflected - depending on their mass to charge ratio (m/z). An orbitrap mass analyzer was utilized in the present study. If the ions were succesfully deviated by the analyzer, they encounter the ion detector, which consequently emits a cascade of electrons (Finehout & Lee, 2004). Finally, a computer software, connected with the detector, extracts peaklists from MS data filing and thus produces graphs, which organize the different ions by their mass-to-charge-ratio. These graphs are processed through databases for identification of the molecules. In this study Mascot Distiller (Matrix Scientific) and the Human IPI database were used for the extraction of peaklists and hence the identification of the proteins.

MS experiments were performed with a LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific) at the laboratory of Prof. Dr. Bernhard Küster. The preparation for mass spectrometry included SILAC (s. chapter 2.2.2.6), anti-FLAG IP (s. chapter 2.2.2.4) and in-gel trypsin digest. Furthermore 1% of the final eluate was visualised by silver staining prior to MS.

2.2.2.6 SILAC

To find interactors of USP24 with a higher sensitivity, stable isotope labeling by amino acids in cell culture (SILAC) was performed by Dr. Bianca-Sabrina Tragosz prior to MS. This method uses different culture media for the trial and the control population. One culture medium contains normal amino acids, the other one amino acids, which are labeled with stable heavy isotopes. In consequence all proteins from the control group with isotope labeled amino acids are heavier than their counterparts from the trial population. This labeling allows not only the mutual analysis of control and trial sample in a mass spectrometer, but also their preparation including anti-FLAG IP, elution and in gel trypsin digest, in one common tube. Thus an identical experimental procedure for both samples is assured. In this study the control population was fed with ^{13}C-Lysine6 and ^{15}N-Arginin10. The trial group, which overexpressed Flag-Strep-Strep-USP24 received culture medium with normal amino acids. After harvesting the cells they were collected in one common tube, lysed and precleared with empty Protein G beads for 45 minutes at 4°C. Anti-FLAG IP was performed with 600µl FLAG-M2-beads for 2 hours at 4°C, followed by 4 washing steps with BT buffer (s. chapter 2.1.9). Proteins were eluted 2 times with 2ml 3xFLAG-Peptide, 4mg/ml, in BT buffer and precipitated with 10% trichloroacetic acid

(TCA precipitation) at 4°C over night. Finally, the pelleted proteins were washed with 100% acetone, dried in a speed vac and sent to the laboratory of Prof. Dr. Bernhard Küster for MS.

2.2.2.7 Ubiquitination assay

For *in vivo* ubiquitination Ha-tagged ubiquitin and the protein of interest were over expressed in HEK293T cells. Usp24 was either over expressed, together with a GFP (green fluorescent protein) tag, or cells were transfected with shUsp24. 24 hours after transfection, cells were treated with MG132 (10μM) for 3 hours. MG132 is a proteasome inhibitor, which prevents degradation of ubiquitin bound substrates and thus preserves ubiquitination. After harvesting the cells, they were lysed for 45 minutes in approximatley 100μl of 250mM lysis buffer (s. 2.1.9), containing ocadaic acid, a phosphatase inhibitor. Samples were centrifugated and supernatants collected. Latter were denatured with 5mM EDTA and 1% SDS, followed by cooking at 95°C for 5 to 10 minutes. To avoid precipitation, samples were cooled down at room temperature, before placing them on ice. For quenching and dilution of the SDS, 900μl of a 1% Triton-X solution (100 μl Triton-X in 10ml 250mM lysis buffer) were added to each sample. After cooling down an immunoprecipitation for the substrate was performed, using anti-FLAG antibodies (s. chapter 2.2.2.4), followed by SDS-gel electrophoresis (s. chapter 2.2.2.1) and western blotting (s. chapter 2.2.2.2)

2.2.3 Eukaryotic cell culture

A humified incubator supplied with 5% CO_2 was used for cell culture. Temperature was set to physiological conditions at 37°C. RPMI (for JJN3, OPM2, RPMI 2668, U266), IMDM (for LP1), DMEM (for HEK293T, HeLa) or McCoy's 5A (for U2OS) medium was supplemented with L-Glutamine, Penicillin/Steptomycin and 10% FBS - except for HEK293T, for which 10% BS was used instead of FBS. Viability and density of the cells were evaluated by counting them after trypan blue staining on a Neubauer hemocytometer under the microscope. To mantain the culture, cells were split at a density of 90%. Adherent cells were scraped from culture dishes and flasks after incubation with trypsin at 37°C for 5 minutes. Latter was washed off and cells were seeded out in a lower density. For freezing cells were centrifugated down to pellets and resuspended in FBS with 10% DMSO. Via

intercalation this substance stabilizes the cell membrane. After addition of isopropanol the suspension was slowly frozen at -80°C and later transferred to liquid nitrogen. Direct freezing in liquid nitrogen would lead to breaking of the cell membranes and should thus be avoided. To unfreeze cells again, they were dissolved in preheated media and DMSO was instantly removed by centrifugation.

2.2.3.1 Plasmid and siRNA transfections

Different methods were used for transfection depending on the cell line and the transfected target.

Calcium phophate transfection

If not differently stated calcium phosphate method was used for transfection of HEK293T cells. This method was developed by Graham and Van der Eb (Graham & van der Eb, 1973) and later modified by Wigler et al. (Wigler et al., 1977). Calcium phosphate is used to form a calcium-phosphate-DNA precipitate, which can attach to the cell surface and be internalized by endocytosis. 20 µg of the plasmid with the target-DNA was diluted in dH_2O; subsequently $CaCl_2$ was added to a final concentration of 250mM. The mixture was left at room temperature for 5 minutes, to allow the formation of ionic bonds between calcium ions and phosphate ions of the DNA's backbone. In a next step, 500µl BES (N,N-Bis-(2hydoxyethyl)-2-aminoethansulfonic acid buffered in NaCl and Na_2HPO_4 to pH 7,1) was added dropwise while vortexing. This phosphate buffer promotes the formation of a fine precipitate. Vortexing helps aeration, to avoid clumping of the DNA, which would not adhere to the cell with the same efficiency. After incubation at room temperature for 20 minutes, the DNA complexes were slowly dropped onto 10cm plates of confluent HEK293T cells with a density of 70 to 90%. The medium was exchanged after 3 to 4 hours. During this time the plasmid was incorporated by the cells. For peGFP expressing plasmids transfection efficiency was assessed by the percentage of green fluorescent cells during the following days.

Lipofectamine transfection/ Lipofection

For other cell lines, e.g. U2OS or HeLa cells, Lipofectamine™ 2000 (Invitrogen) was used for transfection. This method is based on the formation of an aggregate of cationic lipids and the anionic DNA phosphate backbone – together building a

liposome. This liposome can easily fuse with the cell membrane. Lipofection was performed following the Lipofectamine™ 2000 Reagent Protocol 2013 from Invitrogen. As previously described, transfection efficiency was controlled by the percentage of green fluorenscent cells.

siRNA silencing

For siRNA transfection of U2OS HiPerFect reagent (Qiagen) was used according to the manufacturer's instructions. siRNA was diluted in Opti-MEM, a reduced serum medium, with subsequent addition of HiPerFect to a final concentration of 2µM. After incubation for 5 minutes at room temperature, the solution was pipetted onto a confluent cell layer (cell density of 70 to 90%).

2.2.3.2 Lentivirus production

Second generation lentiviral packaging-system, self-inactivating, from Addgene was used for lentivirus production. It includes the packaging plasmid psPAX2, encoding for gag (group specific antigen), pol (lentiviral polymerase), rev (regulator of expression of virion proteins), and tat (transactivator of transcription), as well as the envelope-plasmid pMD2.G, which provides the envelope protein (env). HEK239T cells were plated on gelatinised 10cm plates in an amount, that would allow them, to reach a density of 70 to 90% after 24 hours. For Calcium Phosphate transfection 15µg of the psPAX2 plasmid, 5µg of the pMD2.G plasmid and 20µg of the target DNA vector were diluted in 450µl dH_2O. Previously the target DNA had been cloned between long terminal repeat (LTR) sites of the vector, to later allow its integration into the host's genome. Subsequently 50µl of 2,5M $CaCl_2$ were added. The following steps were performed as described in chapter 2.2.3.1. Medium was exchanged after 3 to 4 hours. For viral infection virus containing medium was taken off at 24 and 48 hours and stored at 4°C.

2.2.3.3 Viral infection

For infection of all cell lines growth medium of transfected HEK293T, collected 48h after transfection, was used. Cells were seeded on 6 well plates, in an amount, that would allow them to reach a density of 40% to 50% for viral infection – permitting further cell division. Cell proliferation is necessary, as viral genetic information can only be integrated into the genome of dividing cells. Polybrene was added to the

virus containing medium for a final concentration of 8µg/ml on the cells. This substance increases viral infection efficiency by reducing the electrostatic repulsion between the viral envelope and the cell membrane (Davis, Rosinski, Morgan, & Yarmush, 2004). 1,5ml of this virus containing medium with polybrene was dropped on each well, followed by centrifugation of the cells at 1200G for 1 hour. This technique is called spin infection. Medium was replaced with new virus-free medium after 3 to 4 hours of incubation. For recovery cells were kept in virus-free medium for 12 to 24 hours and afterwards seeded out in larger plates. The presence of green fluorescence helped to verify viral infection efficiency.

2.2.3.4 Proteasome inhibition

MG132 was used for inhibition of proteasomal degradation, to analyze the ubiquitination of proteins. After dilution in DMSO MG132 was added to the cell's medium to a final concentration of 10µM and left on the cells for 3 to 4 hours. Subsequently they were harvested, pelleted and lysed for *in vivo* ubiquitination (s. chapter 2.2.2.7).

2.2.3.5 Ribosome inhibition

To test protein stability and to determine the half-life of proteins, ribosomal function and hence protein biosynthesis was inhibited with cycloheximide. Latter was dissolved in 100% ethanol to a concentration of 100mg/ml. A final concentration of 100µg/ml was used in culture medium. Cells were plated out in 6-well plates and treated with cycloheximide for up to 8 hours. Levels of proteins, subjected to polyubiquitination and consecutive proteasomal degradation, were preserved upon addition of MG132 for 4 hours. Samples were evaluated by western blotting (s. chapter 2.2.2.2).

2.2.3.6 TGF-beta signaling

For stimulation of the TGF-beta pathway active TGF-beta1 (BPS Biosciences) was used. It was diluted in 0.2M acetic acid with 0.1% BSA to a concentration of 5µg/ml and stored at -20°C. For treatment a 1:1000 dilution with culture medium was prepared and left on the cells for 1 hour.

2.2.3.7 Flow cytometry

Flow cytometry is a laser-based technology, which is used, to analyze the characteristics of single cells. Thanks to fluorescent-labeled antibodies it allows the quantitative evaluation of surface markers or intracellular particles. Furthermore, sorting of cells by size and granularity is possible with a flow cytometer (Adan, Alizada, Kiraz, Baran, & Nalbant, 2016; M. Brown & Wittwer, 2000). To perform flow cytometry, cells were stained, in form of a singe-cell suspension, with fluorochrome-labeled or unlabeled antibodies. After washing the samples with PBS containing buffer, they were analyzed by a flow cytometer (FACS Calibur, Becton Dickinson): Thanks to a narrow nozzle a sheath fluid is created, to send one cell at the time through the laser beam. Detectors measure the light scattered from the cells, as they go through this laser, providing values for the forward scatter (FSC) and the side scatter (SSC). FSC positively correlates with the size of a cell, SSC with its granularity. This allows differentiation of cell populations based on size differences and granularity, as it is done with white blood cells (Adan et al., 2016; M. Brown & Wittwer, 2000). Fluorescence detectors can distinguish cells depending on the expression of a target protein, bound by a fluorochrome antibody. Upon excitation of the antibody by a laser with the corresponding wavelength, it emitts photons of a certain energy. This light is filtered, detected by the photomultiplying tube and converted to a voltage pulse. Latter is measured by the flow cytometer and correlates with the intensity of fluorescence and thus with the quantity of the target. Finally the flow cytometry data was analyzed with FlowJo 10.0.5 software.

Proliferation analysis

To assess cell division, DNA synthesis was evaluated using bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU), a synthetic thymidine analogue. It can be integrated into newly synthesized DNA of dividing cells during the S-Phase. Specific fluorochrome-labeled antibodies can subsequently be used to detect the incorporated BrdU by flow cytometry, thereby allowing an estimation of the proliferation index. For BrdU FACS 10µM BrdU was added to exponentially growing cells. After 1 hour they were harvested, washed with BrdU wash buffer I (s. chapter 2.1.9) and fixed in 70% Ethanol. Latter was taken off followed by denaturation of the DNA for 30 minutes in DNA denaturating buffer (s. chapter 2.1.9). This step is necessary for binding of the antibody to BrdU as part of the DNA. Subsequently, cells were pelleted and resuspended in 1M borate to neutralize the acid as part of the

DNA denaturating buffer. They were washed with BrdU wash buffer II before incubation with the FITC labeled BrdU antibody in a concentration of 20µl/1x10^6 cells at 24°C for 30 minutes. To measure the total DNA amount, cells were treated with 0,5µg/ml of propidium iodine (PI). This substance intercalates into DNA. Hence, evaluation of the distribution over the different cell cycle phases is possible in homogenous populations. Finally, the amount of incorporated BrdU and PI was measured with the flow cytometer FACS Calibur (BD, Franklin Lakes, USA), allowig an assessment of cell proliferation. These experiments were essentially performed by Dr. Bianca-Sabrina Tragosz.

2.2.4 Bacterial cell culture and transformation

LB medium was added to bacteria, which were cultivated at a temperature of 37°C. For an optimal oxygen distribution, they were constantly shaken. To select for transformed clones, 100µg/ml ampicillin was mixed into the medium. Long-term storage was performed at -80°C after addition of 30% Glycerol to an overnight culture.

Transformation

For the amplification of a plasmid, latter was transformed in chemically competent NEB5α E. coli bacteria. Transformation was performed with 2µl of the ligation (s. chapter 2.2.1.3) and 25µl of E.coli. After incubation on ice for 30 minutes, the bacteria were heat shocked for 45 seconds at 42°C, followed by cooling down for two minutes on ice. For recovery the mixture was suplemented with 250µl of SOC (super optimal broth) medium and left on a cell shaker at 37°C for 1 hour. Cells were pelleted by centrifugation at 4000rpm for 2 minutes and, after discarding 200µl of SOC, distributed on LB-Agar plates. Latter contained different antibiotics dependent on the resistance encoded by the respective plasmid - in this study mainly ampicillin was used. For growth of colonies bacteria were incubated for 12 to 24 hours at 37°C.

2.2.5 Data mining and processing

To show a potential role of Usp24 as a tumor suppressor gene in MM, a data base search was performed on Oncomine (Oncomine, 2016). Box blots displaying Usp24 DNA levels as well as student's t-test, giving a p-value for the comparison of Usp24 DNA copy numbers in the control and the cancer group, were obtained

directly through the Oncomine 4.5 software. For evaluation of USP24 protein and mRNA levels, values from 3 independent experiments were processed to graphs using GraphPad PRISM version 6.0e. Corresponding student's t-tests and thus p-values were calculated with the same software. The results from western blot analysis, were scanned and band intensities assessed using publicly available ImageJ[32]. Flow cytometric data was analyzed with the FlowJo 10.0.5 software. MS data peaklists were extracted from MS data filing with Mascot Distiller (Matrix Science) and compared against the Human IPI database 8.8.6.

3 Results

The following data was generated as part of a large collaborative project, directed by the submitting author (Laura Maria Häusler), Dr. Bianca-Sabina Tragosz and Dr. Katharina Engel at the research group of Prof. Dr. Florian Bassermann (Klinikum Rechts der Isar; Technical University of Munich) together with other collaborators. For a better understanding of this study, some of the presented results were provided by other researchers than the submitting author, which will be specified in the text and in the figure legends.

As previously mentioned, MM progression and treatment largely depends on the UPS. To further evaluate its role, this project started off by analysing the available data on DUBs in MM and thereby revealed loss of the Usp24 gene locus in 25% of MM patient samples (Carrasco et al., 2006).

3.1 Loss of the Usp24 gene locus in MM

To verify the data from Carrasco et al. in different methodological approaches, FISH (fluorescence in situ hybridization) analysis, qPCR and western blotting were performed. The results of these experiments are presented in the following 2 sections.

3.1.1 FISH analysis of MM patient samples

FISH analysis of 55 MM patient samples was conducted by Prof. Dr. Sven Perner at the UKSH (Universitätsklinikum Schleswig-Holstein). Green fluorescent probes for the Usp24 gene, mapping to chromosome 1p32.3, were applied on tissue microarrays of the patient samples. Tissue microarray (TMA) is a technique used for the analysis of 1000 or more different tissue samples on one paraffin block. This allows simultaneous examination of molecular targets under the same standardized conditions on a single glass slide (Jawhar, 2009). As a control, centromers were stained red. Figure 9 displays the corresponding FISH results: In 19 of 55 MM patients, representing 33%, Usp24 gene locus deletions were detected. Hence, this data supports the finding from Carrasco et al.: Usp24 is frequently lost in MM.

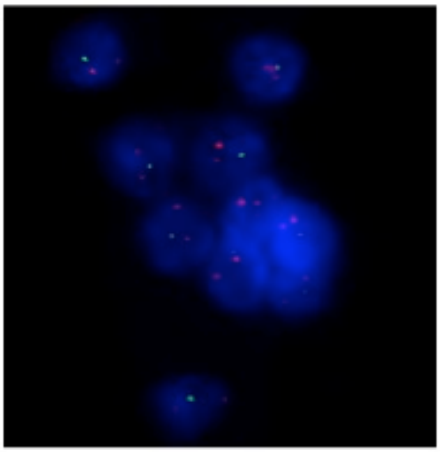


Figure 9: Usp24 gene locus deletions in MM patient samples detected with fluorescence in situ hybridization

For FISH analysis TMAs of 55 MM patient samples were probed for Usp24 (Chr 1p32.3) (green). As a control, centromers were stained (red). In 19 of 55 MM patient samples the Usp24 gene locus was lost. [Data provided by Prof. Dr. S. Perner, UKSH]

3.1.2 Usp24 mRNA and protein levels in different MM cell lines

To measure Usp24 gene expression, unsynchronized MM cells were lysed and RNA was extracted with RNeasy Mini Kit (Qiagen). The extracted mRNA was reversely transcribed to cDNA and latter diluted 1:10 for qPCR. ARPPA served as reference gene for normalization of the results. Figure 10 (a) shows the mean values +/- SEM (standard error of the mean) from 3 independent qPCR experiments. RPMI and U266 are highlighted as MM cell lines with the lowest Usp24 mRNA levels, while LP-1 had the highest expression. The histogram on the right side (b) presents the results from 3 independent western blot analyses of unsynchronized MM cell lines. After lysis they were subjected to anti-USP24 immunoblotting. Signal intensities were assessed with ImageJ[32] and normalized to the loading control beta-Actin. Just as for the mRNA in part (a) RPMI and U266 show the lowest USP24 protein levels (b). Mean values and SEM were calculated using GraphPad PRISM version 6.0e. To summarize, MM cell lines have different expression of Usp24 with some of them downregulating its levels.

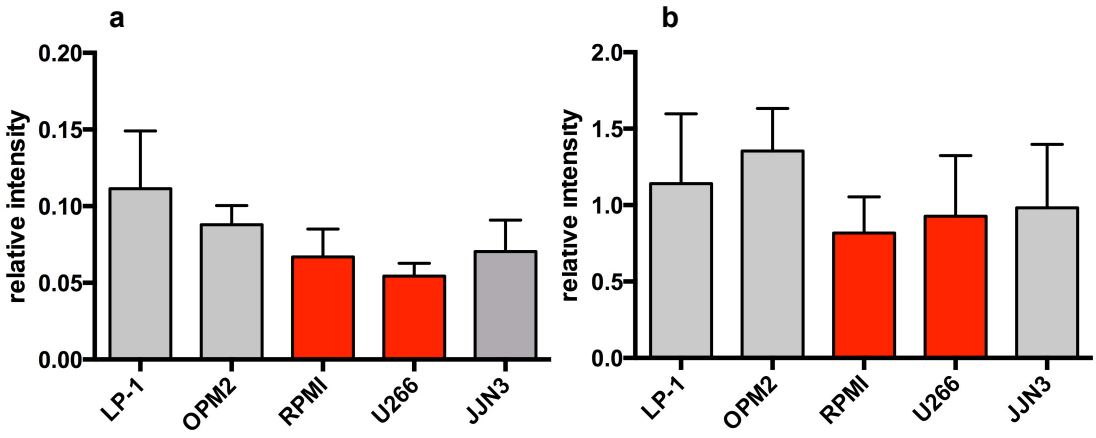
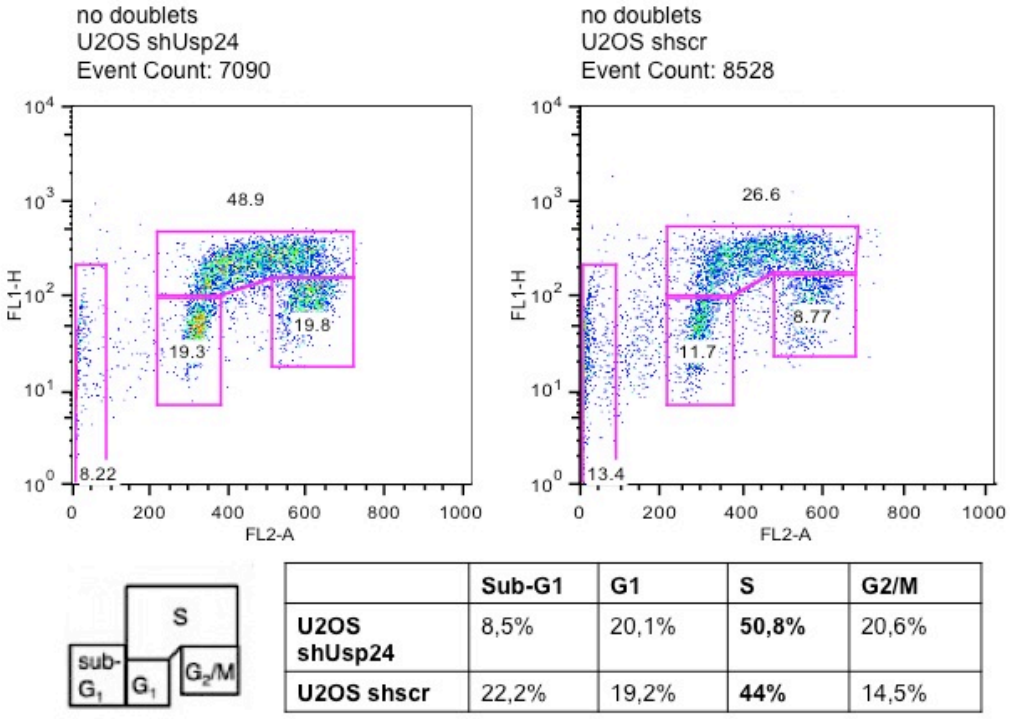


Figure 10: Usp24 expression in different MM cell lines

(a) The left bar chart with the results of 3 independent qPCR experiments shows Usp24 mRNA levels in different MM cell lines. MM cells were lysed and frozen at -80°C for 12 hours. RNA was extracted using RNeasy Mini Kit (Qiagen), measured and reversely transcribed to cDNA. For qPCR cDNA was diluted 1:10 and mixed with primers recognizing the Usp24 gene and the reference gene ARPPA. SYBR Green qPCR Mix (Roche) was added and fluorescence intensity assessed with the Roche Lightcycler 480 Real-Time PCR System. Signal internsities were normalized on the control ARPPA. (b) The right bar chart with the results from western blot analyses shows USP24 protein levels in different MM cell lines. Unsynchronized cells were subjected to anti-USP24 immunoblotting. Intensities were measured with ImageJ[32] and normalized to the loading control beta-Actin. (a)(b) Both histograms present the mean values from 3 independent experiments with the corresponding standard error of the mean (SEM) (n [number of independent experiments]=3; +/- SEM) The diagrams were set with GraphPad PRISM version 6.0e.

3.2 USP24 inhibits proliferation in U2OS cells

As demonstrated in chapter 3.1 (s. figure 9) the gene locus of the orphan DUB USP24 is lost in 33% of MM patient samples. This finding raised the question, whether USP24 plays a role as a tumor suppressor, preventing uncontrolled proliferation of MM cells. In return USP24 depleted cells might show higher proliferation rates. To verify this hypothesis under physiologic conditions, a BrdU-FACS was performed in U2OS with a stable Usp24 gene knockdown. BrdU, a synthetic thymidine analogue, is integrated in the DNA of proliferating cells and afterwards bound by fluorochrome labeled anti-BrdU antibodies for visualization with a fluorescence cytometer. After pulsing the cells with BrdU for 1 hour, their distribution over the four different cell cycle phases Sub-G1, G1, S and G2/M was evaluated. BrdU uptake, indicative of proliferation, increased by nearly 7% in USP24 depleted cells (s. figure 11). 50,8% of the Usp24 knockdown cell population could be localised in the S-phase, compared to only 44% of the control group, transfected with the scrambled shRNA. Hence the number of cells going through mitosis was higher in Usp24sh samples. Taking together USP24 depleted U2OS proliferate more than their normal counterparts, suggesting that USP24 inhibits proliferation in U2OS cells.



	Sub-G1	G1	S	G2/M
U2OS shUsp24	8,5%	20,1%	**50,8%**	20,6%
U2OS shscr	22,2%	19,2%	**44%**	14,5%

Figure 11: BrdU-FACS of USP24 depleted U2OS

For BrdU-FACS U2OS with a stable Usp24 knockdown (shUsp24) were used and compared to U2OS transfected with the scrambled shRNA (shscr). Exponentially proliferating cells were pulsed with 10µM bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) for 1 hour. 0.5µg/ml PI (propidium iodine) was applied, to measure the total amount of DNA and to assess cell cycle distribution. Samples were incubated with fluorochrome tagged anti-BrdU antibodies and analyzed with the flow cytometer (FACS Calibur, Becton Dickinson). Distribution of the cell population over the four different cell cycle phases is shown in the pattern and table below. [Data provided by Dr. B.-S. Tragosz]

3.3 WWP2 as important binding partner of USP24

To find potential binding partners of USP24 an unbiased mass spectrometry based screen for co-immunoprecipitating proteins was performed by Dr. Bianca-Sabrina Tragosz in collaboration with the working group of Prof. Dr. Bernhard Küster (chair of proteomics and bioanalytics, TUM). One population of human embryonic kidney cells (HEK293T) was transfected with a plasmid encoding for N-terminally tagged USP24. The tag consisted of tandem-Strep-single-FLAG peptides and allowed for anti-FLAG IP. Another population of HEK293T cells was transfected with an empty vector and served as a control. Prior to harvesting them, the two populations were fed with different media: The medium for the control population contained stable heavy isotope labeled amino acids (^{13}C-Lysine6 and ^{15}N-Arginin10), making the proteins of this group heavier than their counterparts from the trial group, being fed with normal amino acids. This method, called stable isotope labeling by amino acids in cell culture (SILAC), allows mutual analysis of both groups in the same work flow and thus promotes sensitivity of MS. After harvesting the cells they were collected in one common tube and lysed in MS Lysis Buffer with ocadaic acid, a phosphatase inhibitor, and the detergent NP40 (nonyl phenoxypolyethoxylethanol) (s. chapter 2.1.9). For a detailed description of sample preparation, please refer to chapter 2.2.2.6. To ensure a successful preparation of the sample for mass spectrometry 1% of the final eluate was separated on a SDS-gel and visualized by silver staining technique (s. figure 12). The band detected at 270 kDa corresponds to the USP24 protein. Furthermore, there are several other bands in the USP24 eluate, indicating potential binding partners.

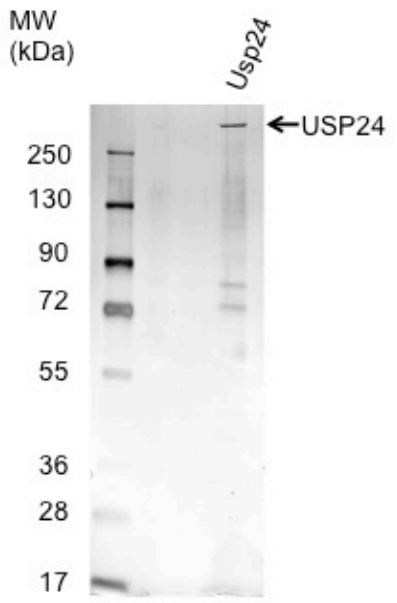


Figure 12: Silver staining of anti-FLAG immunoprecipitated USP24

To find potential interactors of USP24 Tandem-Strep-single-Flag tagged Usp24 was transiently over expressed in HEK293T cells. After harvesting the cells they were subjected to anti-FLAG IP with 600µl FLAG-M2-beads for 2 hours at 4°C, followed by 4 washing steps with BT buffer (s. chapter 2.1.9). USP24 and the attached proteins were eluted 2 times with 2ml 3xFLAG-Peptide (4mg/ml) in BT buffer. 1% of the FLAG eluate was afterwards separated by SDS-PAGE and visualised with silver staining method. [Data provided by Dr. B.-S. Tragosz]

Finally, the eluates were analyzed in the laboratory of Prof. Dr. Bernhard Küster's working group with a LTQ Orbitrap XL mass spectrometer to identify co-immunoprecipitated proteins (s. chapter 2.2.2.5). Table 12 displays the first section of a list of potential interactors with Atrophin-1-interacting protein 2 (AIP2), also known as WWP2, as one of the most abundant. WWP2 is a HECT-domain containing E3-ligase, which belongs to the NEDD-4 family (Chantry, 2011). It harbours four WW-domains (Pirozzi et al., 1997) besides its catalytically active HECT-domain (s. chapter 1.2.2.2). WWP2 has multiple substrates thereunder an epithelial Sodium channel (McDonald et al., 2002) and the transcription factor Oct4, which is essential for the embryonic stem cell fate (Liao & Jin, 2010). Futhermore, the E3-ubiquitin-ligase plays an essential role in tumorigenesis: It controls the degradation of PTEN, a lipid phosphatase often mutated in human cancer (Maddika et al., 2011), and influences the function of SMAD2, 3 and 7, important mediators for the TGF-beta signaling pathway (s. chapter 1.3.2) (Soond & Chantry, 2011). In summary WWP2

presented an interesting and promising interaction partner of USP24, so it was chosen for further investigations.

Ratio H/L Norm	Ratio H/L Count	Intensity	Significance B	Protein IDs	Protein Names	Gene Names	Uniprot
-5,80777	4	18,91061	0,0007756 79	IPI00130 10	Atrophin-1-interacting protein 2	WWP2	O00308
-5,624581	3	18,28464	0,0011719 81	IPI00143 11	Culliin-2	CUL2	Q13617
-5,065014	409	29,27369	0,0038262 56	IPI00902614	Deubiquitinating enzyme 24	USP24	Q9UPU5
-3,291098	10	19,51216	0,0764638 69	IPI00410162	Transcription elongation factor B (SIII), polypeptide 2 (18kDa, elongin B), isoform CRA_a	TCEB2	B7WPD3
-3,108301	5	19,00413	0,0976990 98	IPI00300341	Elongin 15 kDa subunit	TCEB1	Q15369

Table 12: USP24 interaction partners

After transfection of HEK293T with FLAG-Strep-Strep-tagged Usp24 or scrambled shRNA cells of the trial group were fed with normal amino acids, whereas the control population received ^{13}C-Lysine6 and ^{15}N-Arginin10. All cells were pelleted in one common tube and subjected to anti-FLAG immunoprecipitation. Further preparation for mass spectrometry included elution, silver staining and in-gel trypsin digest. Samples were analysed with a LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific) in the laboratory of Prof. Dr. B. Küster. Peaklists were extracted from MS data filing and compared against the Human IPI database for identification. [Data provided by Dr. B.-S. Tragosz and Prof. Dr. B. Küster]

3.3.1 Specific binding of USP24 to WWP2

The interaction between USP24 and WWP2 was further analyzed in embryonic kidney cells (HEK293T cell line). For this purpose the Usp24 gene was cloned into a plasmid encoding for a N-terminal FLAG-tag and Wwp2 into another vector, encoding for a MYC-tag (s. chapter 2.2.1.4). Empty vectors (eV) served as a control in both cases. For co-IP HEK293T cells were transfected either with the Flag-Usp24 plasmid, the Myc-Wwp2 plasmid or in case of the control cells with an empty vector. After cell lysis they were incubated with anti-FLAG or protein A beads, coupled to an

anti-MYC antibody, to pull down either overexpressed USP24 or WWP2 together with their endogenous binding partners (s. chapter 2.2.2.4). The resullts were visualized by western blotting. Figure 13 evidences the strong binding of WWP2 to USP24, regardless of whether FLAG-USP24 or MYC-WWP2 is attached to the beads.

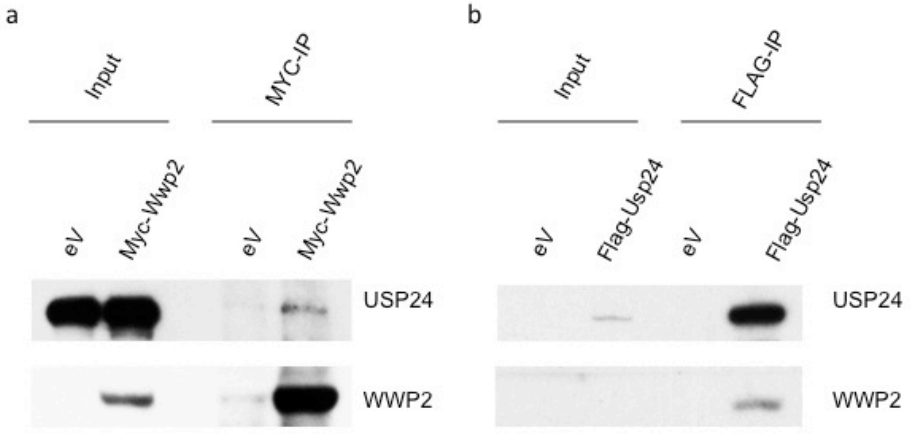


Figure 13: Co-immunoprecipitation of WWP2 and USP24

To verify the mass spectrometric results, a co-IP of USP24 and WWP2 was performed in HEK293T cells. They were transfected with either a Myc-Wwp2 plasmid (a), a Flag-Usp24 plasmid (b) or an empty vector, serving as a control. After 24 hours of gene expression, the cells were subjected to anti-MYC (a) or anti-FLAG IP (b) followed by immunoblotting. [Part a was provided by Dr. B.-S. Tragosz]

To verify, if USP24 is the only DUB binding to WWP2, two other co-IPs were performed with FLAG-tagged USP32 and USP9X in HEK293T. Figure 14 shows the corresponding immunoblots. Only in cells transfected with Flag-Usp24 a band at the molecular size of WWP2 (~ 99 kDa) could be detected with the corresponding antibody. This data suggests, that interaction with WWP2 is a specific property of USP24.

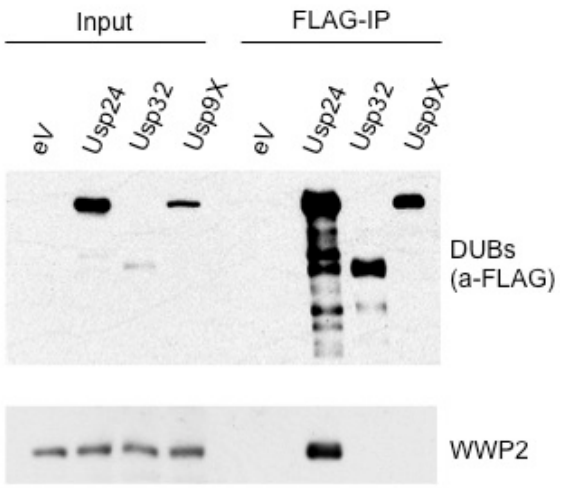


Figure 14: Specific binding of USP24 to WWP2

Co-IPs were performed in HEK293T cells previously transfected with FLAG-tagged Usp24, Usp32 or Usp9X. Calcium phosphate method was used for transfection of the cells. To allow gene expression, they were incubated at 37°C for at least 24 hours prior to harvesting them. Anti-FLAG beads were used to adress the FLAG-tagged substrates and thus, to potentially identify endogenous WWP2 as a binding partner. In the end the protein samples were immunoblotted [Data provided by Dr. B.-S.Tragosz]

3.3.2 WWP2 is not a substrate of USP24

Once WWP2 was identified as a binding partner of USP24, the question came up, whether it could be its substrate or the antagonist E3 ubiquitin ligase. To verify the first hypothesis, an *in vivo* ubiquitination (s. figure 15) and a stability assay with cycloheximide (s. figure 16) were conducted. Both experiments clearly showed, that WWP2 is not a substrate of USP24.

For *in vivo* ubiquitination (s. chapter 2.2.2.7) of WWP2, HEK293T cells were transfected with Ha-Ubiquitin, Flag-Wwp2, Usp24 shRNA or the corresponding empty vectors, respectively. After 24 hours of expression an IP for FLAG- tagged WWP2 was performed. Subsequent western blotting against the HA-tag visualized the level of WWP2 ubiquitination. In the last lane of the corresponding immunoblot (s. figure 15), WWP2 ubiquitination does not increase upon Usp24 knockdown, as it would be expected for the substrate of USP24.

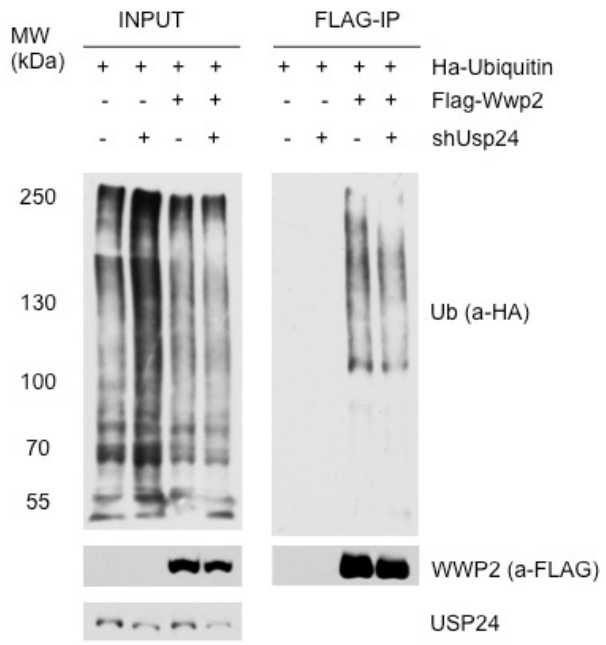


Figure 15: *In vivo* ubiquitination in HEK293T transiently transfected with Flag-Wwp2 and Usp24 shRNA

HEK293T cells were transfected with Ha-Ubiquitin, Flag-Wwp2 or Flag-eV and shUsp24 or scrambled shRNA using calcium-phosphate method. After at least 24 hours of expression, cells were treated with the proteasome inhibitor MG132 and lysed in 250mM NaCl lysis buffer, containing ocadaic acid. Protein samples were denatured and afterwards quenched with Triton-X. After cooling down, an IP for FLAG-tagged-WWP2 was performed. Finally the protein samples were immunoblotted.

Furthermore, a cycloheximide-assay was conducted, to support the result displayed in figure 15. For this experiment U2OS cells were transfected with Usp24 siRNA or siLacZ, serving as a control, using HiPerFect reagent (Qiagen) (s. chapter 2.2.3.1). After a sufficient time of expression cells were treated with cycloheximide, a potent inhibitor of the ribosome and thus of protein biosynthesis, for up to 6 hours. Subsequently they were harvested and subjected to immunoblotting. As shown in figure 16 (b) degradation of WWP2 is not accelerated in USP24 depleted U2OS compared to the control cells transfected with siLacZ. Thus, USP24 has no impact on the stability of WWP2. Part (a) shows the testing of 4 different siRNAs against Usp24 in U2OS cells, to find the most efficient one. siUsp24 number #6 was chosen for the cycloheximide-assay shown in (b). In conclusion, these experiments suggest that WWP2 is not a substrate of USP24.

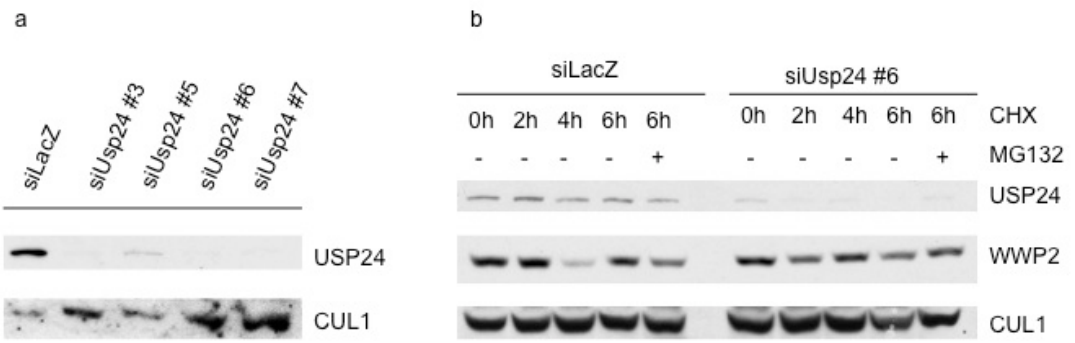


Figure 16.: Cycloheximide-assay in U2OS previously transfected with siUsp24

(a) For testing of siRNAs U2OS cells were transfected with 4 different types of siUsp24 or siLacZ using HiPerFect reagent (Qiagen). siUsp24 #6 was chosen for the stability assay. (b) Just as in part (a) U2OS were transfected with siUsp24 #6 or the control siLacZ. After 12 hours of expression they were plated in 6-well plates at a density of 100 000 cells/well. Another 12 hours later cells were treated with cycloheximide for up to 6 hours. MG132 was added for 4 hours, to inhibit the proteasome for an eventual rescue of the proteins from proteasomal degradation. After 2, 4, or 6 hours of incubation cells were harvested and subjected to cell lysis and western blotting.

3.4 USP24 and proteins of the SMAD family

As described in chapter 1.3.3 WWP2 plays an important role in the regulation of TGF-beta signaling by ubiquitinating SMAD2, 3 and 7. Hypothesizing that one of them is a potential common substrate of USP24 and WWP2, the interaction of USP24 with SMAD proteins was investigated.

3.4.1 Binding of USP24 to activating SMAD2 and 3

To verify, whether proteins of the SMAD family bind to USP24, several co-IPs were performed. Coding sequences of R-Smad2 and 3 as well as I-Smad7 were cloned into the MCS of plasmids, encoding for a FLAG or a HA-tag. After transfection of HEK293T cells with one of the Smad plasmids, Flag-Usp24 or the corresponding empty vectors, 2 samples were treated with TGF-beta1 (5ng/ml) for 1 hour. Subsequently all HEK293T cells were lysed and subjected to anti-FLAG or anti-HA IP. Figure 17 shows the corresponding western blots, indicating that USP24 binds to the activating SMAD2 and 3 (a) but not to the inhibitory SMAD7 (b). Interestingly in the two upper panels (a), the binding of USP24 and SMAD3 decreases upon stimulation with TGF-beta1. Last but not least, the immunoblots illustrate the co-IPs of SMAD2, 3 and 7 with WWP2, as they have been described by Soond et al. (Soond & Chantry, 2011). This data suggests that USP24 interacts not only with the E3 ligase WWP2 but also with two of its substrates: the activating R-SMAD2 and R-SMAD3.

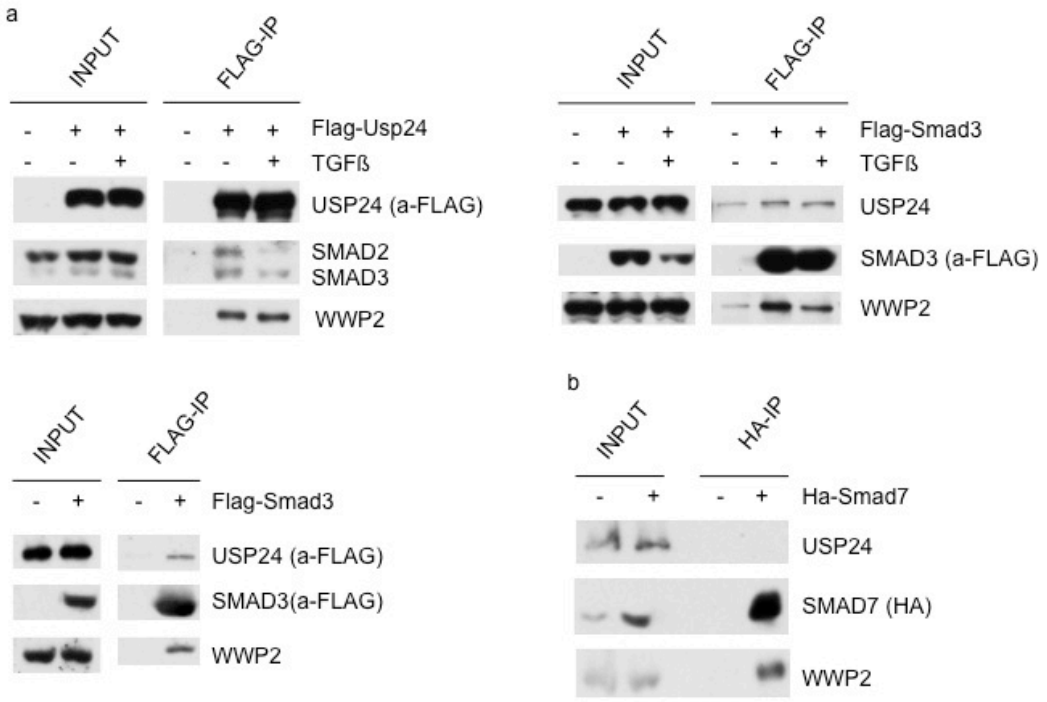


Figure 17: Co-IP of USP24 with proteins of the SMAD family

(a) HEK293T cells were transfected with Flag-Usp24, Flag-Smad3 or Flag-eV, serving as a control, and incubated for 24 hours at 37°C. Prior to harvesting them, the samples of the two upper panels were treated with 5ng/ml TGF-beta1 or acetic acid with 0.1% BSA for 1 hour. All cells were pelleted, lysed and subjected to anti-FLAG IP followed by western blotting. (b) HA-SMAD7 was overexpressed in HEK293T and after 24 hours of gene expression cells were pelleted and lysed. Subsequently an IP for HA-tagged SMAD7 was performed, followed by immunoblotting.

3.4.2 USP24 depletion and phosphorylation of SMAD3

The finding, that USP24 strongly binds to SMAD3, raised the question, whether this DUB plays a role in TGF-beta signaling. As previously described (s. chapter 1.3.2), the activated form of SMAD3 is C-terminally phosphorylated SMAD3 (pSMAD3). pSMAD3 is able to form a heterotrimeric complex with Co-SMAD4, which is subsequently translocated to the nucleus for producing TGF-beta dependent transcriptional responses. To research, if USP24 influences SMAD3 phosphorylation and hence SMAD3-mediated TGF-beta signaling, U2OS cells were plated in 6 well plates and transfected with 2 different Usp24 siRNAs or siLacZ, serving as a control. Afterwards half of the U2OS cells were treated with 10ng/ml TGF-beta1 for 24 hours.

The other half received acetic acid with 0.1% BSA, serving as a control. As expected, the pSMAD3 protein level in the control cells, transfected with LacZ siRNA, augmented upon stimulation with TGF-beta1, which can be seen, when the first and the fourth lane of figure 18 are compared. Besides this, an increasing signal intensity of pSMAD3 was observed in USP24 depleted cells. This effect of siUsp24 becomes most apparent, upon comparison of the first lane, representing U2OS transfected with siLacZ, and lane 2,3,5 and 6, showing U2OS previously transfected with siUsp24. In this experiment there was no extra enhancement of pSMAD3 protein levels, upon addition of TGF-beta1 to USP24 depleted cells. Taken together, this data suggests, that loss of the DUB USP24 promotes SMAD3 phosphorylation in a TGF-beta-indepent manner. Hence Usp24 knockdown might induce SMAD3-mediated TGF-beta signaling responses.

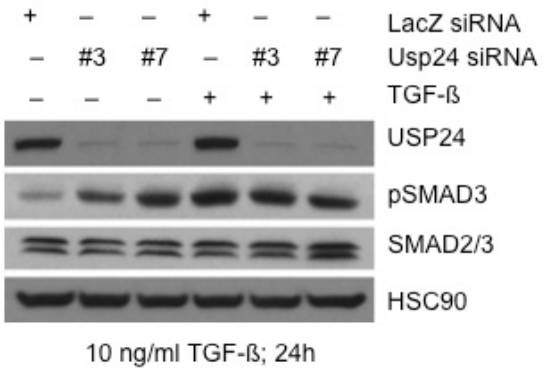


Figure 18: Increased phosphorylation of SMAD3 upon Usp24 knockdown

U2OS cells were plated in 6 well plates at a density of 100 000 cells/well and transfected with 2 different Usp24 siRNAs using HiPerFect reagent (Qiagen). 24 hours later medium was changed and after 36 hours cells were serum starved for 16 hours. In exception of 3 wells, U2OS were treated with 10 ng/ml TGF-beta1 for 24 hours. Subsequently all cells were harvested and subjected to immunoblotting. HSC90 (heat shock cognate 90) served as a loading control. [Data provided by Dr. O. Karpuik]

3.5 Proteins of the SMAD family as possible substrates of USP24

With the objective of finding a common substrate of the DUB USP24 and the E3 ligase WWP2, the interrelation of USP24 with proteins of the SMAD family was further characterized. The following sections display the results of stability assays and *in vivo* ubiquitination experiments of SMAD2 and 3.

3.5.1 USP24 has no influence on SMAD stability

To study the interaction of USP24 and proteins of the SMAD family, it was tested, whether USP24 impacts the stability of SMAD2 and 3, possibly by preventing their proteasomal degradation. For this purpose, a cycloheximide-assay was performed in U2OS cells, transfected with Usp24 siRNA number #6 (s. figure 15) or siLacZ as a control. 48 hours after transfection cells were either harvested or treated with 100µg/ml cycloheximide for 2, 4, or 6 hours. Cycloheximide inhibits the ribosomes and thus protein biosynthesis, thereby allowing to determine the half-life of proteins. The collected pellets were lysed and subjected to immunoblotting. After scanning the results, intensities were measured with ImageJ[32] and normalized to the loading control beta-Actin or CUL1 respectively. The two time courses in figure 19 were calculated using GraphPad PRISM version 6.0e. The X-axis indicates the duration of the cycloheximide treatment, the Y-axis the corresponding relative intensities. The upper diagram (a) shows the graphs for SMAD3 degradation in U2OS transfected with siLaZ (black) or siUsp24 (red). The 2 curves suggest that SMAD3 does not have a longer half-life in USP24 depleted cells, meaning that the DUB USP24 does not prevent degradation of SMAD3. In the lower panel the corresponding graphs for SMAD2 are shown. As for SMAD3, SMAD2 half-life is not signifiantly changed upon Usp24 knockdown. With regard to the presented data USP24 appears to have no impact on the stability of SMAD2 and SMAD3.

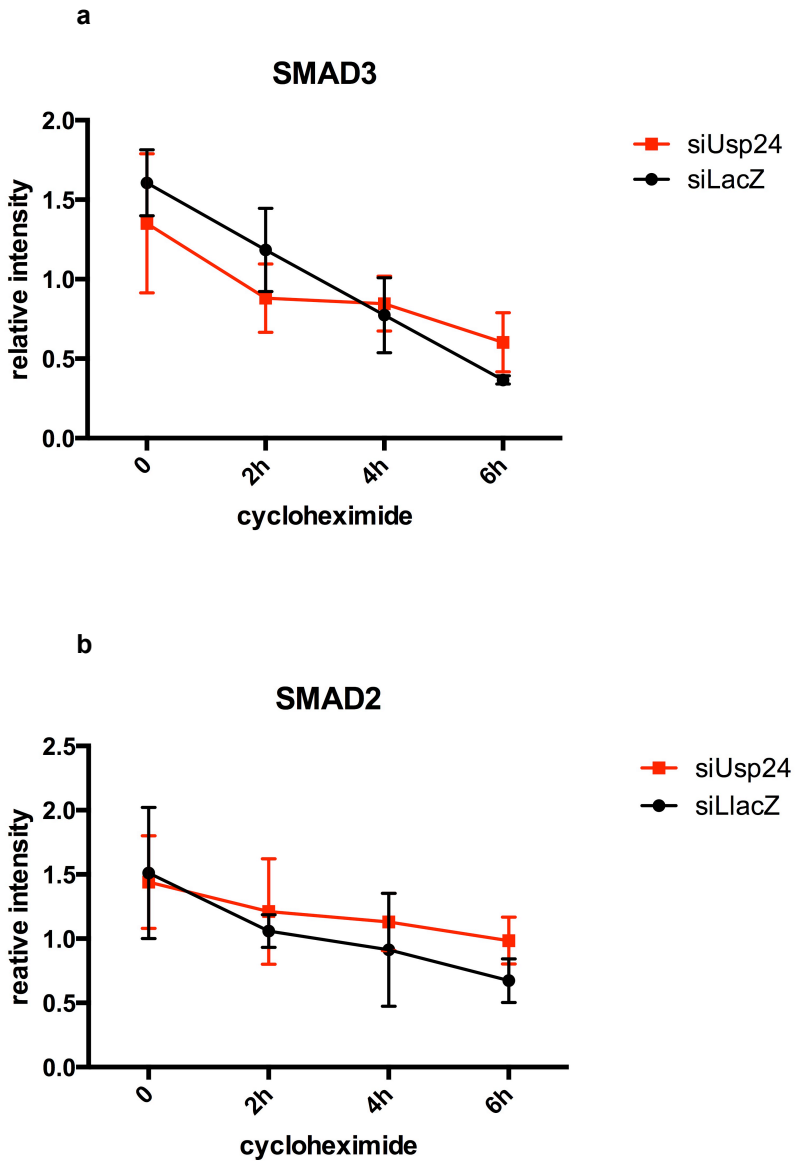


Figure 19: Cycloheximide-assay in U2OS transfected with siUsp24 or siLacZ

U2OS cells were transfected with either siUsp24 number #6 (s. figure 15) or siLacZ using HiPerFect reagent (Qiagen). 12 hours later they were plated in 6 well plates at a density of 100 000 cells/well. 48 hours later U2OS from two wells with siLacZ and siUsp24 were collected. The remaining cells were treated with 100µg/ml cycloheximide (s. chapter 2.2.3.5) and harvested 2, 4 and 6 hours later. Finally the samples were pelleted, lysed and subjected to SDS-PAGE and immunoblotting. After scanning the results, densities were measured with ImageJ[32] and normalized to the loading control. The mean values from 3 independent experiments with the corresponding SEM (n=3; +/-SEM) were processed to graphs using GraphPad PRISM version 6.0e.

3.5.2 SMAD2 is not a substrate of USP24

As shown in figure 17 USP24 binds to SMAD2. To find out, whether SMAD2 is deubiquitinated by USP24, an *in vivo* ubiquitination in HEK293T cells, transiently transfected with Ha-Ubiquitin, Flag-Smad2 and shUsp24 or empty vectors, respectively, was performed. Figure 20, displaying the corresponding immunoblot, shows no increase of SMAD2 ubiqitination in the last lane, upon knockdown of Usp24. Interestingly, the opposite effect, namely the decrease in ubiquitination, was observed, suggesting an indirect regulation of SMAD2 levels by USP24. The bands between 55 and 60kDa correspond to unspecific co-purifying proteins, most likely as a part of the FLAG-M2 beads. The panel on the left side with the inputs, which were not subjected to anti-FLAG immunoprecipitation, shows equal transfection efficiency and loading for all four samples. In conclusion, this experiment suggests that SMAD2 is not a substrate of the DUB USP24.

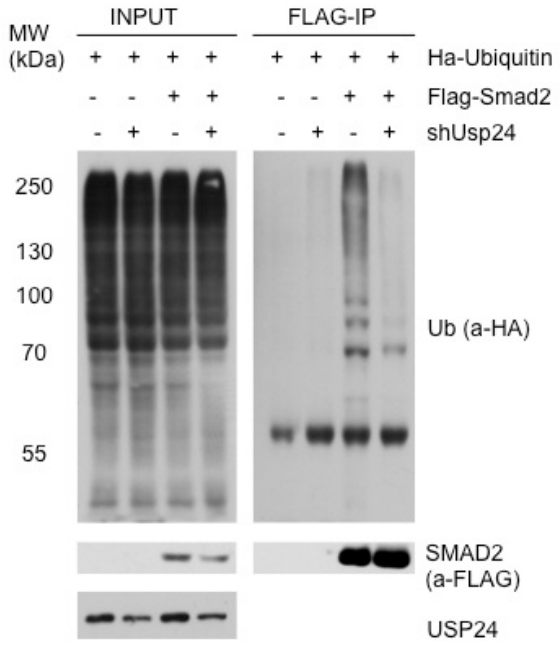


Figure 20: *In vivo* ubiquitination in HEK293T transiently transfected with Flag-Smad2 and shUsp24

HEK293T cells were transfected with Ha-Ubiquitin, Flag-Smad2 or Flag-eV and shUsp24 or scrambled shRNA using calcium phosphate method. 24 hours later they were treated with the proteasome inhibitor MG132, lysed in 250mM lysis buffer, containing ocadaic acid, denatured and finally quenched with Triton-X. Afterwards, an IP for FLAG-SMAD2 was conducted with subsequent immunoblotting.

3.5.3 USP24 antagonizes monoubiquitination of SMAD3

To evaluate SMAD3 as a possible substrate of USP24, HEK293T cells were transiently transfected with Ha-Ubiquitin, Flag-Smad3 and shUsp24 or empty vectors respectively for *in vivo* ubiquitination of SMAD3. Figure 21 shows the corresponding western blot. The protein SMAD3 is generally detected at 55kDa and one ubiquitin molecule weighs 8,5kDa, so the expected molecular weight of monoubiquitinated SMAD3 is 64kDa. Thus, the two distinct bands in the right panel, detected by the HA-antibody at approximately 64 kDa, might correspond to monoubiquitinated SMAD3. In this case, monoubiquitination of SMAD3 increases in the very last lane, representing HEK293T cells with a partial Usp24 knockdown. Hence, this data suggests, that monoubiquitination of SMAD3 is enhanced upon loss of Usp24, meaning that the DUB USP24 antagonizes SMAD3 monoubiquitination. The bands between 55 and 60 kDa correspond to unspecific co-purifying proteins, most likely as a part of the FLAG-M2 beads. On the left side the inputs are shown.

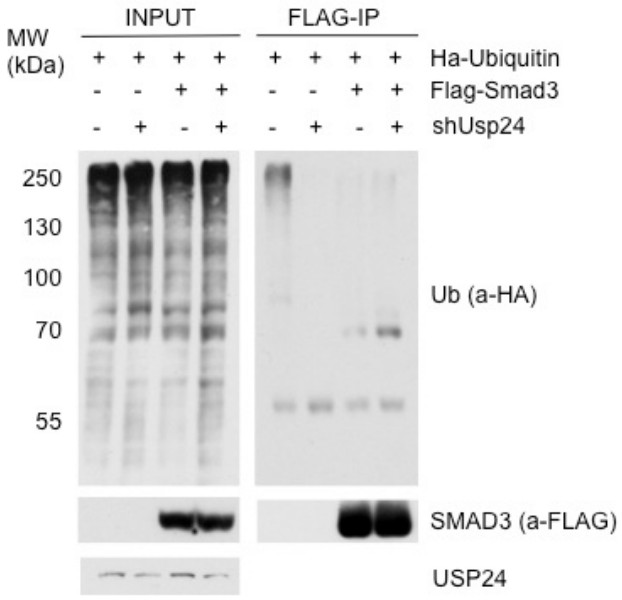


Figure 21: *In vivo* ubiquitination in HEK293T transiently transfected with Flag-Smad3 and shUsp24

Using Calcium Phosphate method, HEK293T cells were transiently transfected with Ha-Ubiquitin, Flag-Smad3 or Flag-eV and shUsp24 or scrambled shRNA, respectively. After 24 hours of gene expression cells were treated with MG132 and lysed with 250mM lysis buffer containing ocadaic acid. Proteins were denatured before quenching with Triton-X and subjected to anti-FLAG IP followed by western blotting.

3.5.4 USP24 interferes with SMAD3/4 complex formation

As described in chapter 1.3.2 SMAD3 binds to SMAD4 once it is activated. This heterotrimeric complex is subsequently translocated to the nucleus for TGF-beta dependent modulation of transcription. Moren et al. and Tang et al. evidenced, that monoubiquitination of SMAD3 or SMAD4 can modulate TGF-beta signaling by attenuating their binding affinity (Moren et al., 2003; Tang et al., 2011). In the present study the orphan DUB USP24 was shown to antagonize monoubiquitination of SMAD3 (s. figure 21). Hence the question came up, whether USP24 interferes with SMAD3/4 complex formation. To verify this hypothesis, HEK293T embryonic kidney cells were transiently transfected with Flag-Smad3 or Flag-eV and shUsp24 or scrambled shRNA. After 24 hours of incubation, the HEK293T cells were pelleted, lysed and subjected to anti-FLAG IP, followed by immunoblotting. Figure 22 (right panel) shows the anti-FLAG IP of HEK293T cells, expressing or lacking the DUB USP24. The last two lanes correspond to cells transfected with Flag-Smad3 and either shscramble (next to last lane) or shUsp24 (last lane). Upon comparison of those lanes it becomes clear, that knockdown of Usp24 (last lane) enhances the co-IP of SMAD3 and SMAD4. Two bands with a higher molecular weight than SMAD4 were detected in the first and second control lanes and correspond to unspecific co-purifying proteins, most likely as part of the FLAG-M2 beads. The inputs are shown on the left panel. In summary, this data suggests, that binding of SMAD3 to SMAD4 increases in USP24 depleted cells. Hence, the DUB USP24 possibly interferes with SMAD3/4 complex formation, an important step in the TGF-beta signaling pathway.

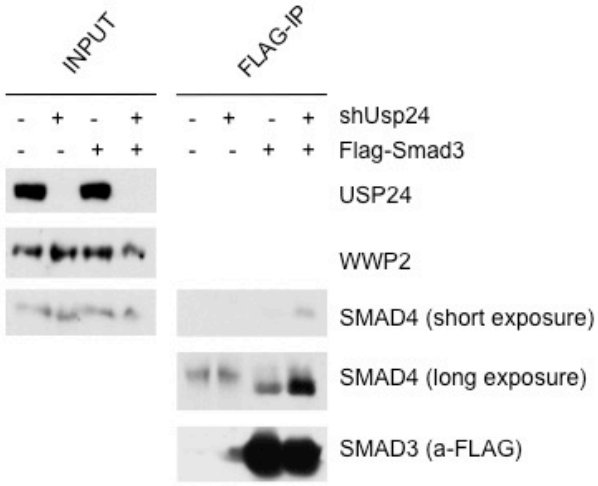


Figure 22: Co-immunoprecipitation of SMAD3 and SMAD4 in HEK293T transiently transfected with Flag-Smad3 and shUsp24.

Embryonic kidney cells were transfected with Flag-Smad3 or Flag-eV and shUsp24 or scrambled shRNA using calcium phosphate method. After 48 hours of expression cells were pelleted, lysed in 150mM lysis buffer and subjected to anti-FLAG IP with subsequent western blotting.

4 Discussion

aCGH arrays and FISH analysis of MM patient samples evidenced deletions of the Usp24 gene locus on chromosome 1p32.3 in up to 33% (Carrasco et al., 2006). This study showed that the corresponding DUB USP24 interacts with proteins of the SMAD family and hence with the TGF-beta signaling pathway under physiologic conditions: By differential modulation of SMAD3 monoubiquitination and phosphorylation, USP24 interferes with SMAD3/4 complex formation. Hence, loss of the Usp24 gene locus, as a common finding in MM, might be associated with deranged TGF-beta signaling in this disease.

In the following sections, the involvement of USP24 in the TGF-beta signaling pathway with potential consequences in normal cells, but also in MM, will be discussed.

4.1 USP24 and its potential role in the TGF-beta signaling pathway

Binding of the largely unknown DUB USP24 to R-SMAD2, R-SMAD3 and I-SMAD7 was characterized, because of its newly identified interaction partner WWP2. Latter had previously been reported to bind and polyubiquitinate SMAD2 and 3 as well as SMAD7, upon stimulation with TGF-beta, and thus designate them for proteasomal degradation. Furthermore Soond et al. evidenced monoubiquitination of R-SMAD2 and 3, mediated by the E3 ligase WWP2 (Soond & Chantry, 2011). Mutual binding of USP24 and WWP2 to SMAD2 and 3 (s. figure 17) raises the question, whether one of these SMAD proteins could be their common substrate. However, SMAD2/USP24 co-IP was only seen in combination with SMAD3. Thus, SMAD2 binding to USP24 could be indirect via SMAD3. Direct interaction between SMAD2 and USP24 was not proven. Moreover, *in vivo* ubiquitination assays, evidencing decreased ubiquitination of SMAD2 upon Usp24 knockdown, exclude SMAD2 as a direct substrate (s. figure 20). This result could indicate, that WWP2 or another E3 ligase, known to ubiquitinate SMAD2, is stabilized by USP24. Although this study demonstrated that WWP2 is not the substrate of USP24 (s. figures 15 and 16), it does not exclude the presence of another WWP2 interactor as a target of USP24. SMAD3/USP24 co-IP instead, was shown independently of SMAD2 and in both directions. Furthermore, SMAD3 monoubiquitination was increased upon Usp24

knockdown (s. figure 21). Hence, SMAD3 can be considered a potential substrate of USP24. As WWP2 binds to USP24 and is known to monoubiquitinate SMAD3, it might be the opposing E3 ligase of USP24 with SMAD3 being their common substrate. Nevertheless, the experimental setting of *in vivo* ubiquitination can not unambiguously prove SMAD3 as a substrate of USP24. Other interaction partners of SMAD3 and USP24 might have produced the observed effect.

Another interesting finding of this study is the enhancement of SMAD3 phosphorylation in USP24 deficient cells, which was observed in unstimulated and stimulated U2OS (s. figure 18). To explain this result, the hypothesis of USP24 as the DUB of a phosphatase, that dephosphorylates SMAD3, can be used. PPM1A acts as a SMAD3 phosphatase with the ability to terminate TGF-beta signaling (Lin et al., 2006). So far, little is known about the regulation and degradation of this phosphatase, which could be deubiquitinated and thus stabilized by USP24. Another possibility is, that USP24 induces stabilization of an E3 ligase, known to polyubiquitinate TbRI, which is the main protein kinase for SMAD2 and 3. In this case Usp24 depletion would lead to stabilization of TbRI and consequent accumulation of p-SMAD3. Among many other E3 ligases, the HECT ligases WWP1 and SMURF2 were shown to induce TbRI degradation (Kavsak et al., 2000; Komuro et al., 2004). However, this second hypothesis seems less plausible, as it does not explain SMAD3 phosphorylation without previous TbRI activation, in the absence of TGF-beta (s. figure 18). Importantly, phosphorylation of SMAD2 and 3 can promote recruitment and stimulation of UPS members and hence favor R-SMAD ubiquitination. For instance, SMAD3 phosphorylation by CDK8 and CDK9 (cyclin dependent kinase 8 and 9) was described, to stimulate its polyubiquitination via the E3 ligase NEDD4L (Alarcon et al., 2009; Gao et al., 2009). SMURF2, already mentioned an E3 ligase, can only monoubiquitinate phosphorylated SMAD3 (Tang et al., 2011). Accordingly, the observed intensification of SMAD3 monoubiquitination, upon Usp24 knockdown, might be caused by its enhanced phosphorylation under these conditions.

The stronger binding of SMAD3 to SMAD4 in USP24 depleted HEK293T cells (s. figure 22) can be explained by two assumptions: (I) First of all, increased phosphorylation of SMAD3, upon Usp24 knockdown, could promote complex formation with SMAD4. TbRI induced phosphorylation of the SXS motif in their C-

terminal MH2 domain activates SMAD2 and 3 and thus enables them to bind SMAD4. Moreover, SMAD2 was shown to release its receptor anchor SARA (SMAD anchor for receptor activation) with subsequent formation of a SMAD2/4 complex and nuclear translocation, upon phosphorylation (Tsukazaki, Chiang, Davison, Attisano, & Wrana, 1998; Wrighton, Lin, & Feng, 2009). (II) On the other hand, enhanced SMAD3 monoubiquitination in USP24 deficient cells could favor SMAD3/4 complex formation. This was previously shown by Moren et al. for SMAD4: Monoubiquitination on its lysine residue K507 increased the binding of SMAD4 to SMAD3 (Moren et al., 2003). Contrariwise multiple monoubiquitinations on several lysine residues in the MH2 domain of SMAD3 by the E3 ligase SMURF2 were associated with the inhibition of heterotrimeric complex formation and thus a negative regulation of TGF-b signaling (Tang et al., 2011). Though, the increased binding of SMAD3 to SMAD4 in Usp24 depleted cells, observed in this study, could be explained by the monoubiquitination of a lysine residue, located outside of the MH2 region.

In conclusion, this study suggests, that USP24 is involved in the regulation of the TGF-beta signaling pathway. USP24 was shown, to antagonize SMAD3 phosphorylation and monoubiquitination as well as complex formation with SMAD4. However, the exact signaling pathway, producing these effects, remains nebulous. Moreover, the presented results were obtained in classical laboratory cell lines (HEK293T, U2OS). Thus, further research is needed for a better understanding of the USP24 function and to depict its relevance for the TGF-beta system in MM.

4.2 USP24 as a novel tumor supressor in MM

Besides the finding, that the Usp24 gene locus is lost in one third of MM patient samples, the present study evidenced higher proliferation rates of U2OS cells, upon Usp24 knockdown. These results raise the question, whether USP24 acts as a novel tumor suppressor in MM, possibly via TGF-beta induced cellular proliferation.

The role of USP24 in TGF-beta signaling was discussed in the last section, though its relevance for MM remains unclear. So far there is no evidence for TGF-beta as a direct stimulator of MM cell proliferation. However, this growth factor promotes MM progression indirectly via increased cytokine secretion and alteration of the bone marrow microenvironment (T. Hayashi et al., 2004; Isufi et al., 2007). In particular, TGF-beta induces IL-6 secretion by MM cells and BMSCs, which plays a fundamental role for MM cell survival, proliferation and drug resistance (Chauhan et al., 2001; Filella et al., 1996; T. Hayashi et al., 2004; Hideshima, Nakamura, Chauhan, & Anderson, 2001). Urashima et al. evidenced increased TGF-beta secretion by MM cells compared to their physiologic counterparts (Urashima et al., 1996). Interestingly, these elevated TGF-beta levels lead to enhanced IL-6 secretion by the malignant plasma cells, thereby making these tumours more aggressive and severe (Filella et al., 1996; Urashima et al., 1996; X. G. Zhang, Klein, & Bataille, 1989). It is known, that TGF-beta stimulates its own secretion in a positive feedback loop in normal and transformed cells. (Van Obberghen-Schilling, Roche, Flanders, Sporn, & Roberts, 1988). However, the mechanism, leading to increased TGF-beta production in MM, has not yet been discovered. TGF-beta gene amplification, mapped to chromosome 19q.13.2, was found in one MM patient collective (n=74) and could be an explanation for some of the cases (NCBI, 2016a; Zhan et al., 2002). Additionally, USP24 might play a role in TGF-beta autoregulation, curbing the positive feedback loop. Hence, loss of the Usp24 gene locus in MM possibly promotes excessive growth factor secretion and thus MM progression. This effect could be caused by enhanced SMAD3 phosphorylation with subsequent activation (s. figure 18) or through monoubiquitination of SMAD3 (s. figure 21) upon Usp24 knockdown, discussed in the previous chapter.

Despite the high TGF-beta levels in MM bone marrow microenvironment, the malignant plasma cells are resistant to TGF-beta mediated inhibiton of cell proliferation and induction of apoptosis. This resistance is attributed to the

deregulation and hence the disruption of the TGF-beta signaling pathway in MM. Baughn et al. described aberrant activation of CDK2 (cyclin-dependent kinase 2) with subsequent phosphorylation of SMAD2 on Threonin8 in MM cells – a PTM linked to impaired SMAD2/SMAD4 complex formation and thus disrupted TGF-beta signaling (Baughn et al., 2009). In addition, the reduction of functional TbRs on the surface of MM cells contributes to the observed TGF-beta resistance (Amoroso, Huang, Roberts, Potter, & Letterio, 1998). However, the TGF-beta signaling pathway stimulates the production of IL-6 by MM cells, described in the last paragraph (Urashima et al., 1996). In this study, Usp24 knockdown in U2OS cells was shown to promote SMAD3 activation and thereby TGF-beta signaling responses, not only upon external stimulation by TGF-beta1 but also independent of such a stimulus (s. figure 18). Hence, USP24 depletion might uncouple several TGF-beta signaling responses like TGF-beta- and IL-6-gene transcription from the external control via TbRs. In this case loss of the DUB USP24, with subsequent deregulation of TGF-beta signaling, could present a critical step in myelomagenesis, MM progression and for drug resistance.

Finally, there is a possibility, that USP24 involvement in MM is TGF-beta-indepedent. Recently Zhang et al. showed, that inhibition of UV-damage-induced apoptosis in USP24 deficient U2OS cells is caused by destabilization of p53 (L. Zhang et al., 2015). Thus, enhanced proliferation, upon knockdown of Usp24, might not be due to changes in the TGF-beta signaling pathway, but rather to the loss of the tumor suppressor p53. Indeed, all the experiments described in this study were performed in classical laboratory cell lines, but not in MM. Thus, it remains unclear if Usp24 gene loss coincidences with increased MM cell growth and progression. Peterson et al. suggested the opposite: In a recent study they found USP24 to promote MM cell survival via stabilization of MCL-1, a key anti-apoptotic protein, in the absence of USP9X (Peterson et al., 2015). Though, the study was mainly performed in three MM cell lines (H929, MM1.S and RPMI-8226), hence the results might not be relevant for all MM patients. Furthermore, several experiments connecting Usp24 knockdown with the decreased survival were conducted in USP9X depleted MM cells, therefore their validity for tumors, not fulfilling this criterion, is arguable.

In conclusion, the question whether Usp24 gene loss in MM promotes or prevents disease progression, cannot yet be answered. USP24 might also play an ambiguous role, depending on the stage of MM and on the pathophysiologic context, just as it is the case for TGF-beta: It switches from tumor suppressor, which inhibits cellular proliferation and promotes apoptosis and differentiation, to pro-oncogenic activities including immunosuppression and angiogenesis but also resistance to apoptosis and cell survival. This functional change is attributed to alterations and disruption of the TGF-beta signaling pathway, discussed in the last paragraph (Massague, 2008; Roberts & Wakefield, 2003). Potentially, Usp24 deletion partly induces the described deregulation of TGF-beta signaling in some MM patients and therefore plays a role in early stages of the disease. Whereas, overexpression of the DUB in more advanced stages or in refractory MM could contribute to drug-resistance via MCL-1 stabilization (Peterson et al., 2015). Hence, USP24 might act as tumor suppressor, whose loss possibly results in enhanced TGF-beta secretion and thus a susceptible bone marrow microenvironment with increased IL-6 and VEGF concentrations, promoting cellular proliferation and local evasion of MM.

5 Summary

Since their development, proteasome inhibitors have revolutionized the therapy of MM. The remarkable improvement of response rates and progression free survival upon introduction of these drugs, revealed the critical role of the UPS for myelomagenesis. Therefore, discovery of ubiquitination pathways, whose deregulation promotes MM cell survival, is essential for understanding MM pathogenesis and to develop novel therapeutic strategies. By identifying Usp24 deletions in one third of MM patients and linking this gene loss with deregulation of the TGF-beta signaling pathway, this study provides additional insights into the function of a UPS member, frequently altered in MM. Further research will hopefully allow verification of these results in MM cells and thus define the exact role of USP24 for this cancer.

6 Literature

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