

From the Department of Cell and Molecular Biology
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FUNCTIONAL STUDIES OF DEUBIQUITINATING ENZYMES

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ABSTRACT

The attachment of ubiquitin to substrate proteins is a key process in regulating cellular events such as cell cycle progression, signal transduction, differentiation, apoptosis, and the clearance of misfolded or aberrant proteins. Like other post-translational modifications, ubiquitination is also reversible. Deconjugation is performed by a family of cysteine- or metallo-proteases collectively known as deubiquitinating enzymes (DUBs). Approximately 100 putative DUBs have been identified in the human genome but only a minority of them has been functionally characterized. The aim of this thesis has been to study the function of selected DUBs in disease-relevant cellular pathways.

Screen of the canonical Wnt-signaling pathway with an RNA interference (RNAi) library targeting the human DUBs identified the ubiquitin-specific protease (USP)-4 as a negative regulator. USP4 interacts with two known components in the pathway: the Nemo like kinase (Nlk) and the T-cell factor 4 (TCF4). NLK promotes nuclear accumulation of USP4 where a subpopulation of TCF4 is a substrate of USP4-dependent deubiquitination. Using a yeast-2 hybrid strategy to search for relevant interactions, we identified the proteasome as a binding partner of USP4. USP4 interacts with the S9 subunit of the 19S regulatory particle (RP) through an N-terminal ubiquitin-like (UBL) domain that resembles, but is functionally distinct from, the UBLs of hHR23a/b and Ubiquilin-1. S9 is as an essential proteasome subunit that may regulate the structural integrity of the 26S complex. Thus, USP4 may play a role in the dynamics of ubiquitination at the proteasome.

A bioinformatics strategy was used to search for membrane-associated DUBs. We found that a putative transmembrane domain targets USP19 to the endoplasmic reticulum (ER). USP19 is a target of the unfolded protein response and rescues ERAD substrate from proteasomal degradation. Moreover, USP19 interacts with the E3 ligases seven in absentia homolog (SIAH) 1 and SIAH2 that mediate USP19 ubiquitination and degradation by the proteasome. Bioinformatics and biochemical analysis revealed the presence in USP19 of a SIAH-interacting motif that is found in a subset of SIAH targets and may function as a degradation signal. A non-enzymatic role of USP19 in the regulation of the reposed to hypoxia was suggested by the finding that wild-type and catalytic mutant USP19 interact with the hypoxia-inducible factor-1 α (HIF-1 α). In the absence of USP19, cells fail to mount a proper response to hypoxia.

LIST OF PUBLICATIONS

- I **Zhao Bin**, Schlesiger C, Masucci MG, Lindsten K.
The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. *J Cell Mol Med.* 2009 Aug;13(8B):1886-9
- II **Zhao Bin**, Velasco K, Sompallae R, Pfirrmann T, Lindsten K.
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Manuscript
- III **Hassink GC, Zhao Bin**, Sompallae R, Altun M, Gastaldello S, Zinin NV, Masucci MG, Lindsten K.
The ER-resident ubiquitin-specific protease 19 participates in the UPR and rescues ERAD substrates. *EMBO Rep.* 2009 Jul; 10(7):755-61.
- IV **Velasco K, Zhao Bin**, Callegari S, Altun M, Liu H, Hassink G, Lindsten K.
A SIAH-interacting motif in the ubiquitin specific protease 19 (USP19) regulates its stability.
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- V **Altun M*, Zhao Bin***, Velasco K, Liu H, Hassink G, Paschke J, Pereira T, Lindsten K.
Ubiquitin-specific protease 19 (USP19) regulates hypoxia-inducible factor 1 α (HIF-1 α) during hypoxia. *J Biol Chem.* 2012 Jan 13;287(3):1962-9.
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LIST OF ABBREVIATIONS

| | |
|----------------|--|
| APC | adenomatous polyposis coli gene product |
| ARF-BP1 | ARF-binding protein 1 |
| ATF6 | activating transcription factor 6 |
| bHLH | basic helix-loop-helix domain |
| BiP | binding immunoglobulin protein |
| CK1 | casein kinase 1 |
| CSN | COP9 signalosome |
| CYLD | cylindromatosis |
| Dvl | dishevelled |
| E1 | ubiquitin activating enzyme |
| E2 | ubiquitin-conjugating enzyme |
| ER | endoplasmic reticulum |
| ERAD | endoplasmic-reticulum-associated degradation |
| fz | frizzled |
| GSK3 | glycogen synthase kinase 3 |
| GST | glutathione S-transferase |
| HDAC | groucho and histone deacetylases |
| HIF-1 α | hypoxia-inducible factor-1 α |
| HRE | hypoxia responsive element |
| HSP90 | heat shock protein 90 |
| ISG15 | interferon-stimulated gene 15 |
| LEF | lymphoid enhancer factor |
| MCM7 | minichromosome maintenance protein |
| Nedd8 | developmentally down-regulated 8 |
| NLK | nemo like kinase |
| IRE1 α | inositol-requiring protein 1 α |
| ODD | O ₂ -dependent degradation domain |
| OTU | ovarian tumor protease |
| PAS | PER-ARNT-SIM domain |
| RACK1 | receptor for Activated PKC kinase 1 |
| RNAi | RNA interference |

| | |
|-------|---|
| RING | really interesting new gene |
| RP | regulatory particle |
| SIAH1 | seven in absentia homolog 1 |
| SIAH2 | seven in absentia homolog 2 |
| SSAT1 | spermidine/spermine N(1)-acetyltransferase 1 |
| SUMO | small ubiquitin-related modifier |
| TβRI | TGF-β receptor type I |
| TAK1 | transforming growth factor-b-activated kinase 1 |
| TCF4 | T-cell factor 4 |
| TMD | transmembrane domain |
| TRAF | TNF receptor-associated factor |
| UAF1 | USP1-associated factor 1 |
| UBL | ubiquitin-like modifiers |
| UCH | ubiquitin C-terminal hydrolases |
| UPR | unfolded protein response |
| USP4 | ubiquitin-specific protease 4 |
| USP19 | ubiquitin-specific protease 19 |
| VHL | von Hippel-Lindau tumor suppressor protein |

1 INTRODUCTION

1.1 *General introduction*

Already in the late 1930s, Rudolph Schoenheimer suggested that the cellular pool of proteins is in a “dynamic state” involving constant synthesis and degradation [1]. We now know that the ubiquitin-proteasome system (UPS) plays a key role in protein degradation and in the regulation of cellular homeostasis.

Protein ubiquitination was discovered in the early 1980s as a post-translational modification involving covalent attachment of ubiquitin, a 76 amino acid long polypeptide highly conserved from yeast to mammals, to a lysine residue of the substrate [2].

The components of the UPS include, in addition to ubiquitin, a family of enzymes that activate (E1), conjugate (E2), and ligate ubiquitin to the substrate (E3), and the proteasome that degrades the ubiquitinated proteins. Ubiquitin is conjugated to the substrates via its C-terminal glycine residue with the help of the E1, E2 and E3 enzymes in a sequential manner. Once the first ubiquitin is added to the substrate, the procedure is repeated to attach more ubiquitin to the previous ubiquitin finally forming a polyubiquitin chain [3]. The poly-ubiquitinated substrates bound to the proteasome are subject to deubiquitination by DUBs, then unfolded and translocated into the inner proteolytic chamber of the proteasome where they can be degraded into small peptides (Figure 1).

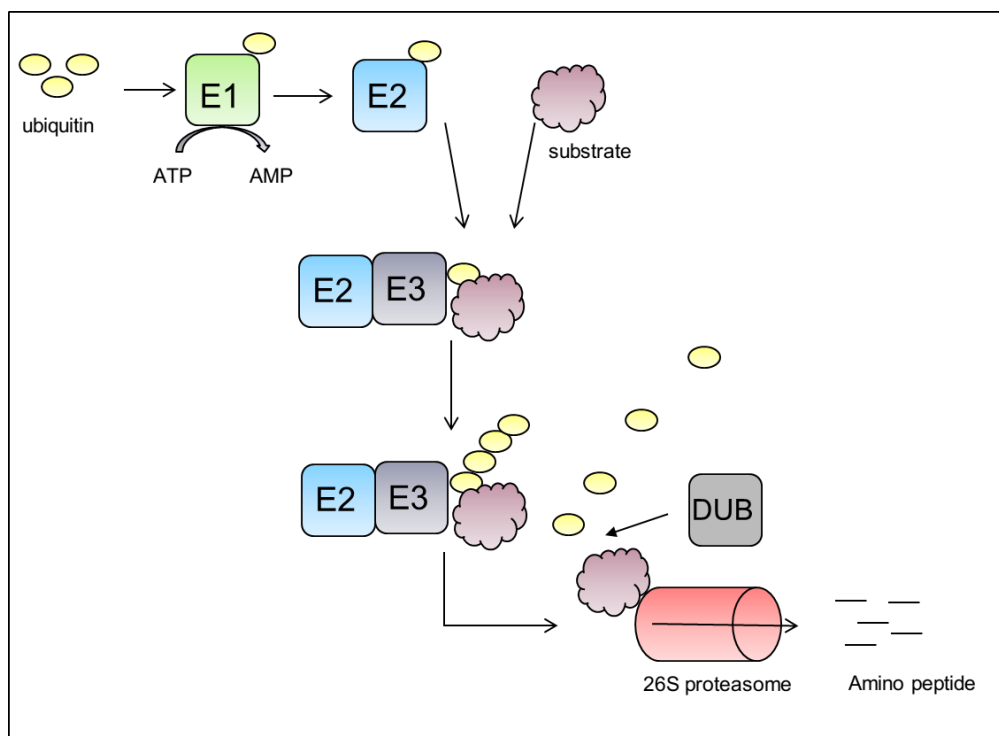


Figure 1. Overview of the ubiquitin-proteasome system

Ubiquitin conjugates to the substrates via its C-terminal glycine residue by the help of the E1, E2 and E3 enzymes. Once the first ubiquitin is added to the substrate, the procedure is repeated, forming a polyubiquitin chain. The poly-ubiquitinated substrate binds to the proteasome where it is deubiquitinated by DUBs, unfolded and translocated into the inner proteolytic chamber for degradation into small peptides.

The UPS plays diverse roles in cellular processes. First, the system controls the degradation of many key proteins involved in cellular events such as cell cycle progression, apoptosis, development and transcription. Second, the UPS plays a role in the immune response since the majority of peptides presented by the major histocompatibility complex (MHC) class I is generated by the proteasome. Last, the UPS has a crucial function in the clearance of aberrant and misfolded proteins. In different cellular stress situation, the UPS protects the cell from toxic accumulation of misfolded and damaged proteins. In addition, the UPS has non-proteolytic roles such as DNA repair, chromatin-remodeling, membrane trafficking, transcription and signaling [4].

1.2 *Ubiquitin-mediated signaling*

1.2.1 Ubiquitin and ubiquitin-like proteins

Ubiquitin is encoded by a gene family that contains monomeric and multimeric ubiquitin genes [5]. The monomeric ubiquitin genes are C-terminally linked to ribosomal proteins. The multimeric ubiquitin genes encode head to tail ubiquitin precursors. The ubiquitin C-terminal hydrolases (UCH) cleave both forms of precursors in the C-terminal Gly residue, forming functional ubiquitin monomers. The crystal structure of ubiquitin shows a distinctive fold characterized by a β -sheet with five antiparallel β -strands, a single helix on top and an exposed C-terminal tail, which is important for conjugation [6]. Ubiquitin is highly expressed in cells and is very stable; it is continuously recycled after tagging protein for degradation.

Ubiquitin-like proteins comprise a group of proteins that share structural similarities with ubiquitin. They can be divided into two groups 1) the ubiquitin-like modifiers (UBLs) and 2) proteins containing ubiquitin-like domain [7]. UBLs such as the small ubiquitin-related modifier (SUMO), the neural precursor cell expressed, developmentally down-regulated 8 (Nedd8) and the interferon-stimulated gene 15 (ISG15) are conjugated to substrates in a process similar to ubiquitination. Modification by UBLs has diverse roles in cellular processes such as DNA repair, protein trafficking and signal activation [8-10]. The proteins containing ubiquitin-like domains play roles in different cellular events and some of them interact with the proteasome.

1.2.2 Ubiquitination

Ubiquitination occurs via attachment of the C-terminal Gly of ubiquitin to a Lys residue, or less commonly to the N-terminus, of the substrate. The process is mediated by the sequential action of three enzymes: the ubiquitin activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin ligase (E3). The first, ubiquitin is activated by the formation of a thiol linkage between its C-terminal Gly and a Cys residue in the E1 enzyme. This first step requires the hydrolysis of ATP.

Then, the activated ubiquitin is transferred to a Cys residue of an E2 enzyme. In the third step an E3 ligase catalyzes the transfer of ubiquitin from the E2 to a Lys residue of the target protein [11]. Once the first ubiquitin is added to the substrate, the procedure is repeated to attach more ubiquitin to the previous ubiquitin forming a polyubiquitin chain [11].

The human genome encodes two E1 [12], dozens of E2 enzyme and more than 1000 E3 ligase [13]. The E3 ubiquitin ligases determine substrate specificity through recognition of degradation signals, also called degrons, which are domains or small motifs present in the substrate [14].

The E3 ubiquitin ligases can be divided into four groups based on their structure and ubiquitination mechanism: the HECT (Homolog of E6-AP C-terminus) E3s, RING (Really Interesting New Gene) domain E3, PHD domain E3s and U-box-type E3s. The HECT E3 and RING domain E3 are two big group of E3 ligases, they regulate diverse cellular events such as cell proliferation, cell cycle arrest and apoptosis [15]. The HECT E3 first loads the ubiquitin from the E2 to an internal Cys residue at the C-terminus of the HECT domain, then passes the ubiquitin to the targeted protein [16]. The RING domain E3 acts like scaffolds that bind the E2 conjugating enzyme via the RING domain, promoting the transfer of ubiquitin directly from the E2 to the substrate [17].

1.2.3 Functions of Ubiquitination

The combination of E2 and E3 enzymes determine the type of ubiquitin chain linkage that will be generated [18]. Proteins can be mono-ubiquitinated in one or multiple Lys residues. Ubiquitin chains can be formed on each of the seven Lys residues of ubiquitin at position 6, 11, 27, 29, 33, 48 and 63 [19]. A very important functions of ubiquitination is to serve as signal for proteasomal degradation [19] but ubiquitination also has diverse non proteolytic functions, including the regulation of protein-protein interactions and subcellular localization [20,21].

Lys48 linked chains are the most abundant ubiquitin chain type in human cells (28%) [19]. Lys48 chain ubiquitinated substrates are degraded by the proteasome while ubiquitin itself is recycled through the activity of deubiquitinating enzymes (DUB). Lys11 ubiquitin chains may also serve as a degradation signal [22,23]. For example, this type of linkage plays an important role in endoplasmic-reticulum-associated degradation (ERAD) [22]. Lys63 ubiquitin chains serve mainly non-proteolytic roles in transcription, intracellular protein trafficking, autophagy, DNA-damage response and cell signaling [24-27].

1.3 *The proteasome*

1.3.1 The 26S proteasome

The proteasome is responsible for selective protein degradation in eukaryotic cells [28], and accounts for approximately to 1% of cellular protein content [29]. The 26S proteasome is a 2.5 MD protein complex composed of more than 30 subunits [28]. It can be divided into two smaller sub-complexes, the 20S core particle (CP) and the 19S regulatory particle (RP). The 19S RP can bind the 20S CP at one or at both ends [29] (Figure 2).

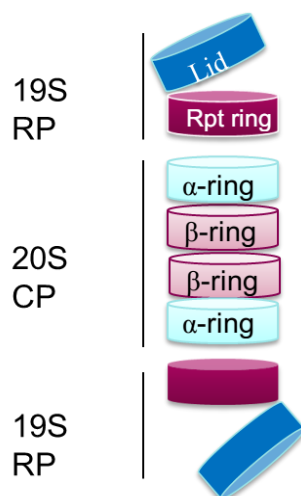


Figure 2. Schematic diagram of the 26S proteasome

The 26S proteasome contains the 20S core particle (CP) and the 19S regulatory particle (RP). The CP is composed of four heteroheptameric rings, two outer α rings and two inner β rings that harbor the catalytic activities. The RP can be divided into the lid and base sub-complexes.

The CP is a barrel-shaped structure that consists of four stacked rings, two outer α -rings and two inner β -rings ($\alpha 1-7-\beta 1-7-\alpha 1-7-\beta 1-7$). The proteolytic active sites are in the two identical β -rings. The $\beta 1$ subunit is a protease with caspase-like activity, which cleaves after acidic amino acids. The $\beta 2$ subunit has trypsin-like activity, cutting after basic residues and the $\beta 5$ subunit possesses chymotrypsin-like activity and prefers to cleave after hydrophobic amino acids [30]. The combination of three different activity sites is essential for degrading a large variety of peptide sequences and also for high efficiency in protein degradation [31].

1.3.2 The 19S regulatory particle

The 19S RP has multiple roles in regulating the activity of the proteasome including ubiquitin chain remodeling and deubiquitination, substrate unfolding and translocation into the 20S catalytic chamber [32]. The 19S RP is composed of at least 19 subunits and can be divided into two sub-complex: the base and the lid.

The base contains six AAA ATPases that form a ring structure, the Rpt1/S7, Rpt2/S4, Rpt3/S6, Rpt4/S10b, Rpt5/S6 and Rpt6/S8 [32]. In addition, the base also contains four non-ATPase subunits: Rpn1/S2, Rpn2/S1, Rpn10/S5a and Rpn13/hRpn13 [33-36]. The ATPases are believed to play an important role in substrate unfolding and thereby assists the translocation of the substrate into the catalytic chamber [32]. One ATPases, Rpt2/S4 is reported to have a role in opening the pore into the 20S CP [37,38]. Another ATPases, Rpt5/S6 is known to play a role in recognizing poly-ubiquitin chains in an ATP dependent manner [39]. Rpn1/S2 and Rpn2/S1 are the largest subunits of the proteasome. They were predicted to form a scaffold or platform unit providing binding capacity to proteasome interacting proteins [40,41]. Rpn1/S2 is known to bind ubiquitin receptor proteins like Rad23, Dsk2 and Ddil. Most of those ubiquitin receptor proteins belong to the UBL-UBA family. They contain a UBL domain that can interact with Rpn1 in the

proteasome, and one or more UBA domains that bind to poly-ubiquitinated chains [42]. These proteins have been proposed to function as shuttling factors that selectively deliver poly-ubiquitinated substrates to the proteasome [43]. Rpn10/S5a and Rpn13/hRpn13 are two well-studied ubiquitin receptors that bind ubiquitin chains [44,45].

The lid is composed of 8 subunits that can be divided into two groups based on their domain structure: Rpn3/S3, Rpn5, Rpn6/S9, Rpn7/S10a, Rpn9/S11 and Rpn12/S14 are predicted to share a PCI domain in their C-terminal, Rpn8/S12 and Rpn11/S13 have a MPN domain in common [46]. Rpn6/S9 is an essential component of the 26S proteasome. Yeast studies show that Rpn6 localizes at the outer rim of lid particle and forms a protrusion reaching down to the ATPases and alpha rings in the 20S RP, functioning thereby as a molecular clamp that holds the 19S RP and the 20S core together [47]. Conditional knock-out of Rpn6 in *S. cerevisiae* impairs the assembly of proteasome thus affecting proteasome activity [46].

One of the major tasks of the 19S RP is to remove the poly-ubiquitin chains from the substrate by cleavage of the isopeptide bond between the substrate and the first ubiquitin in the chain to release the whole chain [45,48]. Rpn11/S13 is the essential DUB responsible for this function [45,48,49]. Two additional DUBs associated with the proteasome participate the deubiquitination process, UCH37/UHL5 and Ubp6/USP14 [50-52]. While Rpn11/S13 promotes substrate degradation, UCH37/UHL5 and Ubp6/USP14 antagonize substrate degradation [53]. These DUBs trim the poly-ubiquitin chain from the distal end. Thus, the longer the chain, the stronger of the interaction of the substrate with the proteasome. By trimming the ubiquitin chain, these DUBs regulate the time of substrate-proteasome interaction thus determining the rate of degradation [50].

1.4 *Deubiquitination*

1.4.1 Deubiquitinating enzyme

Protein modification by ubiquitin is a dynamic and reversible process. The conjugated polyubiquitin chains are removed and disassembled by a group of proteins called DUBs. DUBs are important regulators of diverse cellular processes, such as the cell-cycle, DNA repair and chromatin remodeling. The human genome encodes approximately 100 DUBs that can be divided into six subfamilies: the ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs), Machado-Joseph disease protein domain proteases, JAMM/MPN domain-associated metallopeptidases (JAMMs) and monocyte chemotactic protein-induced protein (MCPIP) families [54]. They are Cysteine proteases, except for the JAMM/MPN+ DUBs, which are Zinc metalloproteases.

DUBs have different functions in the UPS [55,56], including: (1) Ubiquitin precursor processing. DUBs can cleave ubiquitin precursors consisting of multiple ubiquitins formed as tandem repeats or ubiquitin fused to the N-terminus of the L40 and S27 ribosomal proteins [4]; (2) Ubiquitin recycling. DUBs can trim the ubiquitin chain from a substrate prior to degradation. The cleaved off ubiquitin chain will be further disassembled so that recycled ubiquitin re-enter the cellular ubiquitin pool. This trimming of ubiquitin chains on substrates is also essential for (3) the regulation of protein stability. Ubiquitination serves as triggering signal for protein degradation. By trimming ubiquitin chain on a substrate, DUB regulates protein stability. For example, USP10 and USP7 regulate p53 stability via their deubiquitinating activity [57-59]. (4) Non-degradation ubiquitin signal regulation. For example, USP4 removes Lys63 linked polyubiquitin chains from TAK1, which leads to its inactivation and down regulation of NF- κ B activation [60].

DUBs can be regulated at different levels. Including: 1) Transcription level; For example: the protein causing cylindromatosis (turban tumor syndrome), also known as CYLD, is induced by activation of the NF- κ B pathway [61]; 2) Post-translational modification. Phosphorylation or ubiquitin-like modifications can regulate DUB

activity. For example, phosphorylation of CYLD inhibits its ability to deubiquitinate the TNF receptor-associated factor-2 (TRAF2), thus leading to suppression of the NF- κ B pathway [62]; 3) Protein binding partners and allosteric regulation. For example, USP1 shows 35-fold higher activity when bound to UAF1 (USP1-associated Factor 1) [63]. Upon binding to the proteasome, USP14 and UCH37 display hundred folds increased activity [51,64-67]; 4) Degradation. USP20 is ubiquitinated by the E3 ligase pVHL and degraded by proteasome [68].

1.4.2 USP4

USP4 was discovered in the early 1990's in a search for genes located near the Monkeypox virus 20 retroviral insertion site [69]. It was originally called UNP, for ubiquitous nuclear protein, and changed to the new systematic nomenclature for human ubiquitin specific proteases in 1999 [70]. USP4 RNA levels are elevated in several types of human cancers and overexpression of USP4 cDNA leads to oncogenic transformation in NIH 3T3 cells [71,72]. USP4 has regulatory functions in different signaling pathways. By deubiquitinating TRAF2, TARF6 and TAK (transforming growth factor- β -activated kinase)-1, USP4 inhibits the TNF induced activation of NF- κ B [60,73,74]. Recent work implicates USP4, as an inducer of TGF- β signaling via direct interaction and deubiquitination of the TGF- β receptor type I (T β RI). Furthermore, USP4 inhibits p53 through deubiquitination and stabilization of ARF-BP1 (ARF-binding protein-1), an E3 ligase for p53 [72]. Additional interacting partners of USP4 include Ro52, retinoblastoma protein and the A(2A) adenosine receptor [75-80].

USP4 is 936 amino acids long and contains two UBL domains, one in N-terminal next to DUSP domain and one in the middle of the protein, near the catalytic Cys residue. It has been reported that the UBL domain in the middle of USP4 inhibits the deubiquitinating activity [81] (Figure 3).

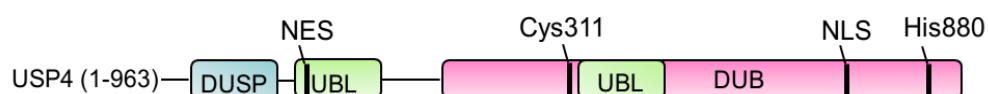


Figure 3. Schematic illustration of USP4

USP4 contains a conserved DUSP domain and two UBL domains. The Cys and His box in the DUB domain form the catalytic active site.

1.4.3 USP19

USP19 was first identified in a screen for DUBs expressed in rat skeletal muscle [82]. USP19 is phylogenetically conserved. Human USP19 has 81% and 95% amino acid identity with rat and mouse USP19 respectively [82]. It has been reported that USP19 plays a positive role in G1/S transition, by binding and stabilizing KPC1, a ubiquitin ligase for p27. Cells depleted of USP19 show reduced growth rate [83,84]. USP19 has also been shown to stabilize c-IAPs (the inhibitor of apoptosis) with consequent inhibition of apoptosis [85].

USP19 is 1357 amino acids long [82]. The USP domain is in the middle of the protein that also contains a Zn-MYND (myeloid translocation protein 8, Nervy and Deaf1) domain that may mediate protein-protein interaction [86,87]. In the N-terminal of the protein, there are two p23-like domains that suggest chaperon functions [88]. The C-terminal transmembrane domain (TMD) is responsible for ER anchoring [89] (Figure 4).

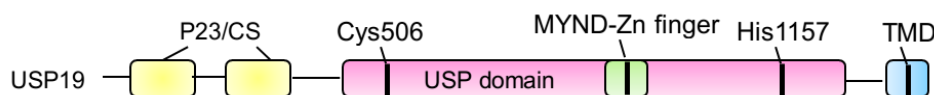


Figure 4. Schematic illustration of USP19

USP19 contains two p23-like domains, a Zn-MYND domain and a C-terminal transmembrane domain (TMD). The Cys and His box in the USP domain form the catalytic active site.

1.5 *Regulation of cellular functions by deubiquitination*

1.5.1 Wnt-signaling

The Wnt signaling pathway regulates a vast array of cellular process such as stem cell maintenance, tissue polarity, cell proliferation and movements [90]. The Wnt pathway is conserved in many species from *C. elegans*, *Drosophila*, *Xenopus* to mammals. Deregulation of Wnt pathway is associated with many hereditary disorders, cancer and other diseases [91]. Mutations in Wnt pathway can be found in various types of cancers such as gastric cancer, colon cancer and breast cancer [91]. Upon ligand binding, different downstream signaling pathways can be activated: the canonical Wnt/ β -catenin pathway, the non-canonical planar cell polarity (PCP) pathway or the Wnt/Ca² pathway [92].

1.5.1.1 The canonical Wnt/ β -catenin pathway

Among the three Wnt pathways, the canonical Wnt/ β -catenin pathway is the best studied. The first step of activation is the binding of the ligand to receptors. Two types of receptor families have important role in Wnt/ β -catenin pathway activation: the frizzled (Fz) seven-pass transmembrane receptors [91], and the LDL receptor-related proteins 5 and 6 [93]. LRP5/6 receptors function with neighboring Fz receptor to form ternary complexes with Wnt ligand [94]. As the key transcriptional activator in the pathway, the cellular level of β -catenin is tightly regulated. It should be noticed that β -catenin, is also a component of adherent junctions, where it plays an important role as binding partner for the cytoplasmic tail of different cadherins [91].

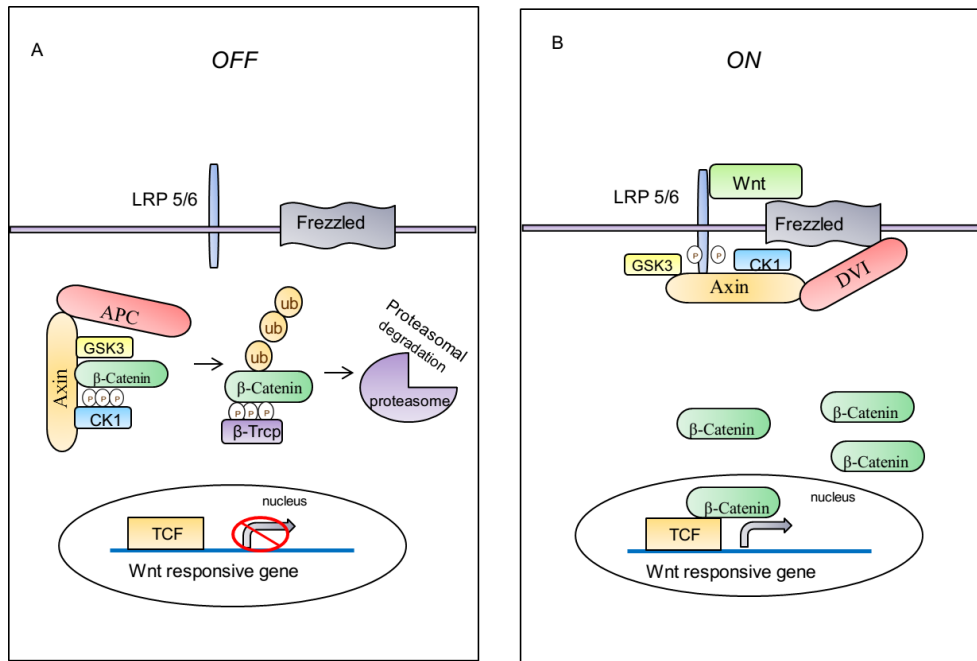


Figure 9. Overview of the canonical Wnt/ β -catenin pathway

A) In the absence of Wnt, β -catenin forms a complex with Axin, APC, GSK3 and CK1 and is phosphorylated by GSK3 and CK1. The phosphorylated β -catenin is recognized and ubiquitinated by the E3 ligase β -TrCP, leading to its proteasomal degradation. B) In the presence of Wnt, Wnt forms a receptor complex with Fz and LRP5/6. The formation of the complex recruits the scaffold protein Dishevelled (Dvl) and leads to LRP phosphorylation. Then Axin is recruited to the LRP5/6, resulting in dissociation of the destruction complex. β -catenin accumulates and translocates to the nucleus where it serves as a transcriptional co-activator for TCF to activate Wnt responsive gene. (Figure adopted from *Dev Cell*. 2009 Jul;17(1):9-26)

When Wnt receptor complexes are not bound by a ligand, the cytoplasmic β -catenin is captured by a destruction complex that consists of Axin, the tumor suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) [93]. The scaffolding protein Axin provides binding sites for β -catenin CK1, GSK3 and APC. CK1 and GSK3 sequentially phosphorylate β -catenin promoting its interaction with the ubiquitin E3 ligase β - TrCP, leading to ubiquitination and degradation by the proteasome [93]. The continuous degradation prevents β -catenin from going into the nucleus with

consequent repression of Wnt target genes by DNA-bound T cell factor (TCF) [95] (Figure 9).

The canonical Wnt/ β -catenin pathway is activated when a ligand binds to its Fz receptor and its co-receptor LRP5/6. The formation of the complex recruits the scaffold protein Dishevelled (Dvl) and leads to LRP phosphorylation. Then Axin is recruited to the LRP5/6, resulting in dissociation of the destruction complex. Unmodified form of β -catenin cannot be recognized by β -TrCP thus leading β -catenin accumulation and subsequent translocation to the nucleus [91]. In the nucleus, β -catenin interacts with transcription factor TCF either by replacing the repressors binding to TCF or by directly binding to TCF. TCF and β -catenin together activate Wnt gene expression [96] (Figure 9). More than 100 direct target genes have been identified, many of them are important regulators of cell cycle progression, determination of cell fate and differentiation [91]

1.5.1.2 Regulation of Wnt signaling by deubiquitinating enzymes

Deubiquitination is an important step in regulating the Wnt pathway. Several DUBs have been reported to have regulatory functions: 1) Trabid belongs to the OTU family of DUBs and was found to be a positive regulator of the Wnt pathway [97]. Trabid interacts with APC and regulates the stability of Lys63-linked ubiquitin chains. Knock-down of Trabid leads to hyper-ubiquitination of APC, suggesting a negative role of Lys63-linked ubiquitination of APC in TCF/LEF mediated gene transcription [97]; 2) USP15, a component of the COP9 signalosome (CSN), mediates deubiquitination and stabilization of APC thus stimulating the degradation of β -catenin [98]; 3) CYLD was identified as a negative regulator of the Wnt signaling pathway as CYLD interacts with and regulates Lys63-linked ubiquitination of Dvl [99,100]. Loss of CYLD enhances the Wnt/ β -catenin pathway [99,100]; 4) UBPY belongs to the USP family of DUBs. By suppressing the Fz receptor trafficking/degradation, UBPY facilitates the Wnt/ β -catenin pathway. Gain and loss of UBPY function led to up and down regulation of the Wnt/ β -catenin pathway [101].

1.5.2 ERAD

The ER is the place where newly synthesized secretory or membrane proteins are folded with the help of molecular chaperones [102]. In the ER, proteins undergo co- and post-translational modifications such as disulfide bond formation and N-linked glycosylation, which are important for proper protein folding. The ER has a sophisticated quality control system. Only properly folded proteins can pass the quality check and be transported out of the ER.

Several DUBs were shown to play a role in ERAD. The otubain YOD1 associates with p97 and serve as a p97-associated ubiquitin processing factor in substrate dislocation from the ER [103]. Expression of a catalytically inactive form of YOD1 hampers the dislocation and promotes the stabilization of ERAD substrates [103]. Another DUB, Ataxin-3 also binds to p97 and regulates retrotranslocation of ERAD substrates. Binding of Ataxin-3 to p97 decreases its interaction with UFD1 and poly-ubiquitin chains, thus hampering the retrotranslocation of substrates from the ER [104].

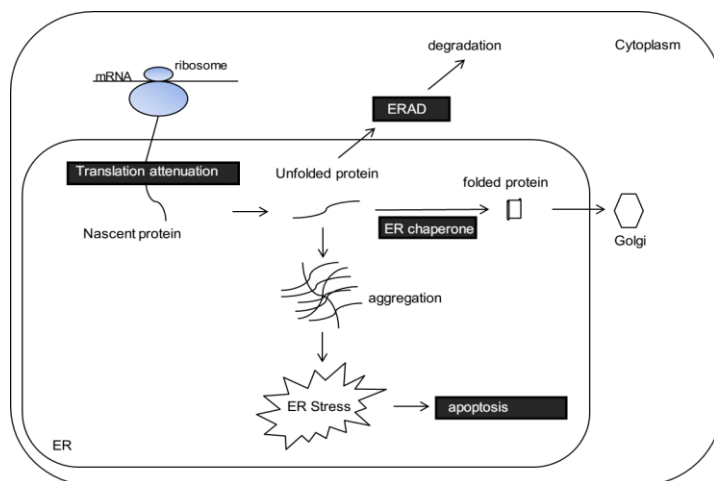


Figure 5. The ER stress response

Accumulation of unfolded or misfolded protein in the ER causes ER stress. Cells coping with ER stress by: 1) translational attenuation; 2) expression of ER chaperons; 3) enhanced ERAD; 4) apoptosis

Physiological and pathologic conditions, such as ER calcium depletion, hypoxia or energy perturbation can cause the accumulation of unfolded or misfolded proteins. This is referred to as “ER-stress”. To deal with this stress situation, the cells activate a complex signaling cascade known as the unfolded protein response (UPR), which aims to resolve the situation by attenuating further protein synthesis, and by up-regulating the expression of ER chaperones, to help protein folding, and ERAD components, to get rid of terminally misfolded proteins (Figure 5). Under severe stress conditions, when the situation cannot be resolved, prolonged UPR signaling will eventually trigger apoptosis [105-108] (Figure 6). Three UPR signal pathways are initiated by three ER-localized stress sensors: inositol-requiring protein 1 α (IRE1 α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor-6 (ATF6). In normal conditions, the sensor proteins are inactivated by binding to the ER chaperone binding immunoglobulin protein (BiP) [109]. In ER stress situations, BiP binds to the unfolded or misfolded proteins thus releasing the sensor proteins, which activates the UPR signaling cascade [109].

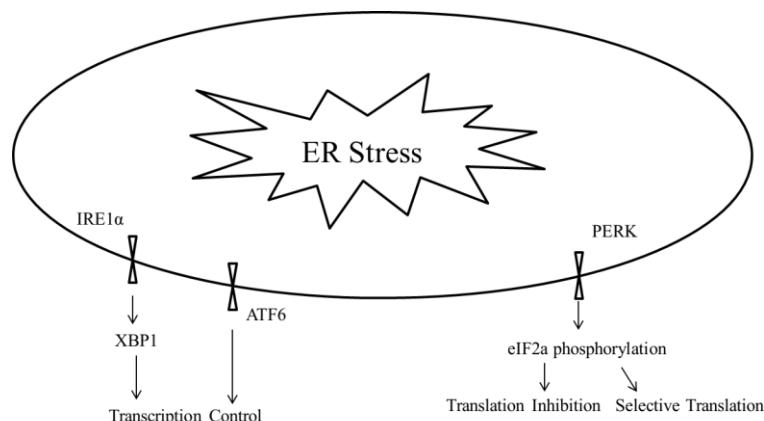


Figure 6. The UPR signaling pathway.

Under ER stress, three ER stress sensors IRE1 α , PERK and ATF6 are activated to regulate the transcriptional and translational program to protect the cell from stress.

1.5.3 The response to hypoxia

Oxygen is required for most organisms including bacteria, yeast, invertebrates and vertebrates to produce energy. Cells use O₂ for mitochondrial ATP generation and

also as electron acceptor in metabolism. Since it is very important to keep a good balance between oxygen supply and consumption, cells have developed a variety of ways to keep oxygen homeostasis.

1.5.3.1 Hypoxia

Hypoxia is a situation when O₂ levels drop below a critical threshold, which hinders the normal function of organs and cellular signaling pathways [110]. Under hypoxic conditions, organs undergo a series of changes to regain O₂ homeostasis to limit the damage caused by low O₂ levels. At the cellular level, various stress proteins are expressed to adapt to the low O₂ level and switch from aerobic metabolism to anaerobic glycolysis.

The hypoxia-inducible factor-1 (HIF-1) transcription factor is a master regulator of the hypoxic response via transcriptional activation of more than 100 genes important for the cellular adaptation to hypoxia [111]. HIF target genes are involved in the regulation of diverse events, such as oxygen transport, iron metabolism, glycolysis, glucose transport, cell survival and proliferation, angiogenesis, invasion and metastasis [112]. HIF-1 activity is deregulated in many cancers where HIF-1 α is often overexpressed. Overexpression of HIF-1 α is associated with increased vascular density, tumor progression and resistance treatment [113]. HIF-1 is a heterodimer consisting of a strictly regulated α subunit and a constitutively expressed β subunit [114]. Three isoforms of the α subunit have been identified: HIF-1 α , HIF-2 α and HIF-3 α , of which HIF-1 α and HIF-2 α are the best characterized. The role of HIF-3 α in hypoxic regulation of gene express is not well understood. All of the HIF subunits belong to the family of the basic helix-loop-helix (bHLH) and PER-ARNT-SIM (PAS) domain containing transcription factors [114]. HIF-1 α contains four functional domains: the bHLH domain, PAS domain, O₂-dependent degradation domain (ODD) and transactivation domain (TAD). The bHLH and PAS domains are responsible for DNA binding and subunit dimerization. It has also been reported that the PAS domain might be responsible for protein-protein interaction [115]. The ODD domain controls the proteasomal degradation of

HIF. The two TAD domains (N-TAD and C-TAD) of HIF-1 α mediate the transcriptional activation of HIF targeted genes.

1.5.3.2 Regulation of HIF-1 α stability

In order to respond to changes in cellular O₂ levels, HIF-1 α undergoes tight post-translational regulations. HIF-1 α is constitutively expressed but the protein levels are kept low by proteasomal degradation [116]. In hypoxic conditions, HIF-1 α starts to accumulate.

Under aerobic conditions, HIF-1 α is hydroxylated by specific prolyl hydroxylases (PHD1, PHD2 and PHD3) at two conserved proline residues (Pro402 and Pro564) in the ODD domain [117,118]. Oxygen is required for this hydroxylation step. The hydroxylated protein is recognized by the substrate adaptor subunit of an E3 ligase complex, the von Hippel-Lindau tumor suppressor protein (pVHL). The VHL ubiquitin ligase, which is a member of the SCF family of ubiquitin ligase complexes, includes the subunits pVHL, elongin C, elongin B, cullin-2 and Rbx1. Together with the E2 enzyme UbcH5, the VHL ligase stimulates HIF-1 α poly-ubiquitination and subsequent degradation by the 26S proteasome [119-121] (Figure 7).

During hypoxia, the low O₂ levels inhibit the activity of PHDs, resulting in HIF-1 α stabilization. The stabilized HIF-1 α is then translocated into the nucleus and dimerizes with the HIF-1 β subunit. With co-activators such as p300, the HIF heterodimer binds to a hypoxia responsive element (HRE) in the promoter region of the target genes to activate transcription (Figure 7).

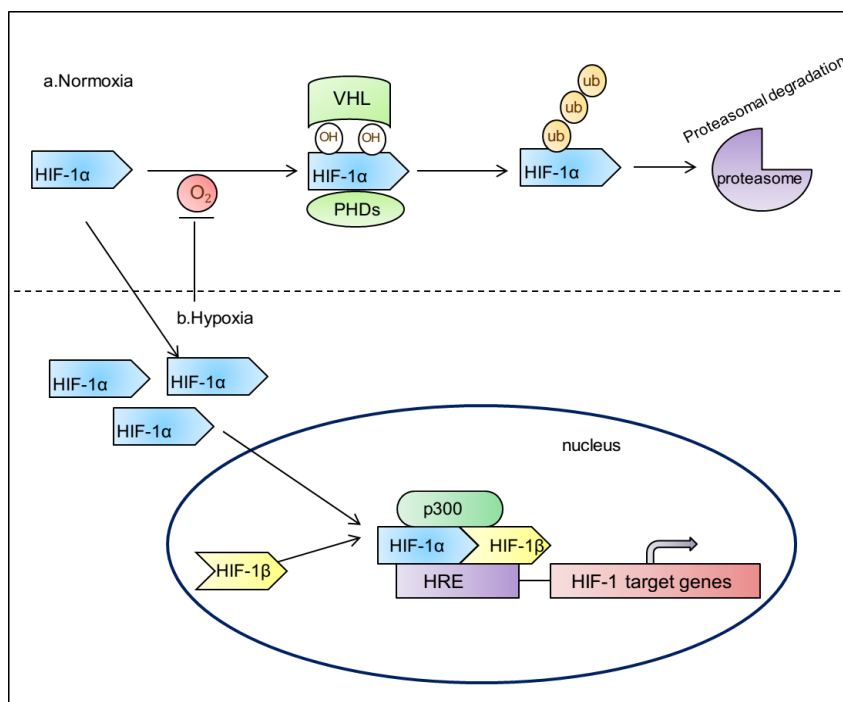


Figure7. Oxygen dependent regulation of HIF-1 α stability

Under normal conditions, HIF-1 is hydroxylated by PHDs, which leads to binding of the pVHL E3 ubiquitin ligase complex. The pVHL E3 complex ubiquitinates HIF-1 α , thus resulting its degradation by the proteasome. Under hypoxic conditions, the activity of PHDs is inhibited, resulting in the stabilization of HIF-1 α . HIF-1 α translocates to the nucleus and dimerizes with HIF-1 β . With co-activators such as p300, HIF binds to the hypoxia responsive element (HRE) and activates the transcription of target genes.

HIF-1 α stability is also regulated by different O₂-independent molecular pathways involving for example Rack1 and Hsp90 (Figure 8).

Receptor for Activated PKC kinase 1 (RACK1) is a multifunctional scaffold protein that plays important roles in different signaling pathways [115]. Overexpression of RACK1 promotes HIF-1 α degradation independent of cellular O₂ level or VHL binding whereas knockdown of Rack1 leads to HIF-1 α stabilization and activation of HIF-1 α target genes [115]. With the help of Spermidine/spermine N(1)-acetyltransferase 1 (SSAT1) Rack1 binds to the PAS-A domain of HIF-1 α , which enables the recruitment of elongin-C and elongin-B thereby promoting HIF-1 α degradation [115,122].

The molecular chaperon heat shock protein 90 (HSP90) stabilizes HIF-1 α by competing with Rack1 for binding to bHLH-PAS domain of HIF-1 α [122].

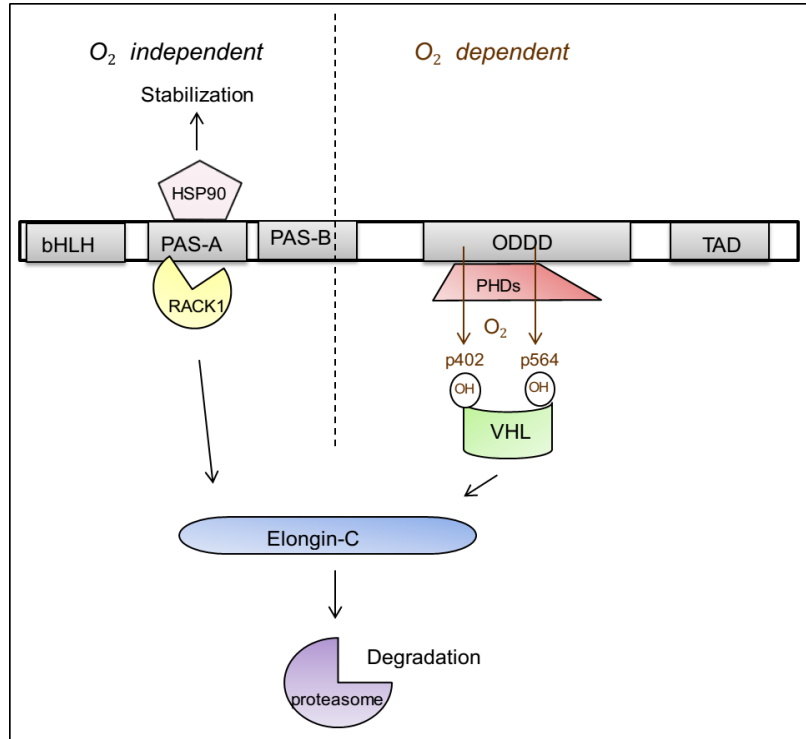


Figure 8. O_2 dependent and O_2 independent regulation of HIF-1 α stability

In the O_2 independent pathway, HSP90 and RACK1 compete for binding to the PAS domain of HIF-1 α . HSP90 binding stabilizes HIF-1 α whereas binding of RACK1 recruits other subunits of the E3 ubiquitin ligase complex and induces degradation by the 26S proteasome. In the O_2 dependent pathway, HIF-1 α is hydroxylated by PHDs, which promotes binding of the pVHL E3 ubiquitin ligase complex. The pVHL E3 complex ubiquitinates HIF-1 α and induces degradation by the proteasome. (figure adopted from Cell Cycle. 2007 Mar 15;6(6):656-9.)

1.5.3.3 Regulation of HIF-1 α by deubiquitination

The ubiquitination of HIF-1 α is regulated by DUBs. USP20 (also known as VDU2) is the first DUB implicated in the regulation of HIF-1 α stability [68]. USP20 binds and deubiquitinates HIF-1 α , which results in stabilization and increase the expression of HIF-1 targeted genes [68]. The pVHL complex can ubiquitinate USP20 and cause its proteasomal degradation. Thus, the cellular levels of HIF-1

appear to be dependent on the balance of its ubiquitination and deubiquitination [68,123]. The relevance of this mode of HIF regulation is not yet understood.

Aside from ubiquitination/ deubiquitination, phosphorylation, SUMOylation as well as subcellular localization, are also involved in the regulation of HIF-1 α [124-128]. However, considering the fact of the various regulation pathways through complex biological mechanisms, this thesis only focus on the role of ubiquitination/ deubiquitination and its following effects on the regulation of HIF-1 α .

2 Aims of this investigation

The overall aim of this study was to investigate the function of selected DUBs in the regulation of disease-relevant cellular functions. To this end my colleagues and I have pursued the following specific aims:

1. Identify new DUBs regulating Wnt pathway and characterize their interaction partners and role in signaling.
2. Identify membrane associated DUBs and characterize their interaction partners and involvement in the cellular response to stress.

3 Methodology

This section describes the major methodology used in this thesis. A detailed description of material and methods is presented in the appended papers.

3.1 *RNAi screening*

RNA interference (RNAi) is a technique that allows to inhibit the activity of targeted genes in living cells. Double-stranded RNAs with sequence complementary to a gene of interest are introduced into the cells, leading to inhibition or reduction of targeted gene expression via sequence-specific degradation or translational interference of mRNA transcripts [129,130]. In mammalian cells, either synthetic siRNAs or vector-based short hairpin RNAs (shRNA) can be used for gene knockdown. RNAi is also widely used in high-throughput screens. High-throughput RNAi screening is a powerful tool for identification of genes associated with a given pathway or specific biological loss-of function phenotypes [129,130]. In paper I, we have used the RNAi library targeting human DUBs to search for new regulators in the canonical Wnt signaling pathway.

3.2 *Bioinformatics*

Bioinformatics is a technique that combine computer technology with biology science to gather, store and integrate biological data such as nucleic acid, protein sequences, structures, functions, pathways and genetic interactions [131]. One of the important applications of bioinformatics is the sequence analysis. The advancement of sequencing technologies in the past decade has made it possible to rapidly sequence hundred of genomes from bacteria to humans. However, much of the sequence data is uncharacterized, and many genes encode proteins of unknown function. Sequence analysis is an effective tool to predict the function of unidentified sequence by means of finding homologous domains or proteins with conserved structural features [131-133]. In paper III and IV, we have used sequence

analysis method to identify DUBs with transmembrane domain and the SIAH interacting motif presented in USP19.

3.3 *Yeast two-hybrid screens*

Yeast two-hybrid screen is a technique that detects physical interactions between two proteins. The technique is based on the activation of a reporter gene by binding of a transcription factor to the activating sequence. The transcription factor consists of two domains, the DNA binding domain and the activating domain. The DNA binding domain is responsible for the binding to a specific DNA sequence. The activating domain is responsible for the assembly of proteins required for transcription. In this screen, a GAL4 transcription factor is split into two separate parts. A protein of interest is fused with a GAL4 DNA-binding domain and transformed in yeast host cells generating the “bait”. A cDNA library of clones that are fused to the GAL4 activation domain carried in yeast cells are called “prey”. Only when the prey and the bait form protein-protein interaction, the down-stream reporter gene will be activated [134,135]. In paper II and V, we have used yeast two-hybrid technique to identify interacting partners of USP4 and USP19.

3.4 *Co-Immunoprecipitation*

Co-immunoprecipitation is a technique to identify protein-protein interactions. An antibody to the protein of interest is added to a cell lysate. Then protein-G coupled sepharose beads are used to capture the antibody-protein complex. These protein complexes can be analyzed by techniques like western blot to identify the binding partners [136,137].

3.5 *GST pull-down assay*

The GST pull-down assay is an *in vitro* technique to determine physical interaction between proteins. It can be used to identify direct protein interactions which can not be achieved by Co-immunoprecipitation. A glutathione S-transferase (GST) tag is commonly fused to the protein of interest. GST-fusion proteins can be produced in *Escherichia coli* as bait. Glutathione-Agarose beads are used to pull down the GST-protein complex. These protein complexes can be analyzed by technique like western blot to identify protein binding partners. [138-140].

3.6 *Western blot*

Western blot is a widely used analytical technique to detect specific proteins in a given sample. SDS-PAGE is used to separate proteins according to the length and charge of the polypeptide. The proteins are then transferred to membranes and specific antibodies are used to detect the protein of interest [141,142].

3.7 *Fluorescence microscopy*

Fluorescence microscopy is a powerful tool for study of protein localization and interactions. Target-specific fluorescent probes or labeled antibodies were used in fixed cells. The fluorescence microscopy permits the visualization of fluorescence in focused area, creating image with single or multi-colors [143,144].

3.8 *Real-Time PCR*

Real-Time PCR (qPCR) is a technique based on PCR. It can amplify and quantify targeted DNA sequence simultaneously. For more specific DNA sequences in a sample, Real-Time PCR enables both detection and quantification. Real time PCR is based on the detection of the increase of fluorescence produced by reporter molecules that bind to double-stranded DNA or sequence specific probes, as proportional to the increase of DNA [145,146].

4 Results and Discussion

4.1 *Paper I*

The canonical Wnt-signaling is highly conserved and regulates a vast array of cellular process such as stem cell maintenance, tissue polarity, cell proliferation and movements [90]. Ubiquitination plays important roles in regulating this pathway. A lot of attention has been paid to the regulatory role of E3 ubiquitin ligases but very little is known on the enzymes that reverse the ubiquitination process. To this end, we have used an RNAi library targeting the family of DUBs to screen the canonical Wnt-signaling pathway for potential new regulators.

In the screening, we identified USP4 as regulator of the canonical Wnt pathway. We have shown that USP4 knock-down increased Wnt signaling in cells where the pathway is activated either by mutations in APC (SW480 cells), by transfection of constitutively active β -catenin or by co-expression of the physiological Wnt1 ligand. To search for potential USP4 interacting partners and the mechanism behind, we performed a set of co-immunoprecipitation experiments and found that USP4 interacts with two components of the Wnt pathway, the transcription factor TCF4 and the Nemo like kinase (Nlk). We have demonstrated that overexpression of a catalytically active Nlk promotes nuclear accumulation of USP4. Moreover, USP4 interacts with a post-translationally modified form of TCF4 that appears to be a substrate of USP4 DUB activity. Taken together, our data identified USP4 as a negative regulator of Wnt pathway. It is likely that USP4 regulates this pathway by going into the nucleus, interfering with the TCF4 transcription regulatory complexes by deubiquitinating TCF4.

The result from this paper suggest that modulation of USP4 expression may provide a new means to interfere with canonical Wnt signaling in a variety of physiological and pathological conditions.

4.2 Paper II

To further explore the functions of USP4 and to identify its interacting partners, we performed a yeast-two hybrid screen using as bait the N-terminal domain of USP4. The C-terminal DUB domain was excluded since it is relatively conserved and also presented in many other DUBs. The most striking interaction identified was with the proteasome subunit S9 that resides in the 19S RP. To confirm the interaction, we performed GST-pull down and *in vitro* binding experiments. Co-immunoprecipitation assays from cell lysates of transfected cells were performed to confirm the interaction *in vivo*. The binding to the proteasome subunit S9 suggests that USP4 might associate with 26S proteasome. We confirmed the USP4-26S proteasome association by co-immunoprecipitation assays.

Many proteins associate with the proteasome via a UBL domain, one of the best-characterized proteasome-interaction motif. More and more evidence supports a role of UBL domains in regulating the activity, specificity or interactions of several DUBs [147,148]. Two DUBs USP14 and UCH37, are known to interact with the proteasome via internal UBL domains. Here we identified USP4 as a third proteasome-interacting DUB. There are two UBL domains in USP4, one embedded in the catalytic site that has autoregulatory functions [81,148], and an N-terminal UBL domain of unknown functions. In order to test whether the UBL domain in USP4 is responsible for the binding to the proteasome we made several truncations/deletions of USP4 constructs and tested their binding to S9 in GST-pull down assays. We have found the N-terminal domain of USP4 is responsible for the binding to S9. This interaction is specific for USP4 and does not involve a conventional UBL-binding domain on S9. The functional significance of the interaction between USP4 and S9 remains to be investigated. USP14 and UCH37 associate with S1 and ADRM respectively and they dock close to the ubiquitinated substrate [28,65]. Their enzymatic activity is dramatically increased upon interaction with the proteasome [51,64], which is likely to restrict their activities to the site where it is needed. Unlike these DUBs, the activity of USP4 was not affected in the presence of S9, suggesting that USP4 is active also in the absence of the proteasome and therefore unlikely to be indiscriminately directed to all

ubiquitinated proteasomal substrates. Compared to other DUBs, the site of interaction of USP4 also suggests a different function. The S9 subunit was recently shown to serve as a molecular clamp that holds the proteasome core and the regulatory particle together [47]. USP4 may play a role in maturation or maintenance of the 26S proteasome complex, or in regulation the turn over of specific substrates.

4.3 Paper III

Ubiquitination of membrane proteins controls cellular events such as endocytosis, and membrane trafficking [149]. While a number of ubiquitin conjugating enzymes and ubiquitin ligases are known to reside in membranes, where they act as important regulators, less is known about the regulatory function of ubiquitin deconjugation in the same processes. To this end, we have used a bioinformatics approach to screen human DUB sequences for putative transmembrane domains and identified three candidates: USP48, USP30 and USP19. USP30 was shown to be a mitochondria-associated protein while USP48 is unlikely to be associated with membranes since the putative transmembrane domain overlaps with the DUB active site. We decided therefore to focus on USP19, previously known to be specifically regulated during muscle atrophy [82].

We have functionally characterized USP19 and shown that it is anchored to the ER membrane via its C-terminal transmembrane domain. Based on the ER localization of USP19, we hypothesized it might be part of UPR and therefore regulated during ER stress. To test this possibility, we induced ER stress and measured USP19 mRNA level by qPCR. Indeed USP19 mRNA was elevated more than two fold upon treatment with ER stress inducing agent. The ER localization and the upregulation after ER stress suggest that USP19 might function in ERAD. To test this possibility, we have examined the turn over of two ERAD substrates CFTR Δ 508 and TCR α in the presence of USP19. We have shown that USP19 interacts with CFTR Δ 508 and TCR α and overexpression of USP19 stabilized both substrates. The presence of two CS/p23 domains is likely to be important for

USP19 function since a catalytic mutant USP19 could stabilize TCR α as well. Through the CS/p23 domain, USP19 may promote protein folding by interacting with HSP90 or it may act as an independent chaperone. This activity of USP19 might be important for the rescue of proteins that needed to survive retrotranslocation.

USP19 is the first example of a membrane-anchored DUB involved in the turnover of ERAD substrates.

4.4 Paper IV

In order to learn more about the function of USP19 and to identify its interacting partners, we have performed a yeast-two hybrid screen. As bait, we used the first 495 amino acids of the N-terminus of USP19 that excludes the relatively conserved USP homology region. More than 50% of the hits in the screen were the ubiquitin ligases SIAH1 and SIAH2.

SIAH1 and SIAH2 are highly conserved RING-type-E3 ubiquitin ligases that regulate a variety of signaling pathways and stress-related cellular events. We have first confirmed that USP19 is an interacting partner of both SIAH1 and SIAH2 independently of both DUB and ligase activity. Next we set out to investigate the functional outcome of this interaction. Our results demonstrated that SIAH can ubiquitinate USP19 and promote its proteasomal degradation. USP19 does not seem to have a comparably strong effect on SIAH, which excludes a simple regulatory loop where the two enzymes could reciprocally regulate their stability. It is noteworthy that only some of the isoforms of USP19 expressed in cells interact with SIAH, suggesting the interaction may selectively affect some of the function of USP19.

Several SIAH interacting proteins contain a conserved RPVAxVxPxxR motif that functions as a degradation signal [150]. To investigate whether such region is present in USP19, we performed multiple sequence alignments with some known

SIAH interacting proteins. We have found that in USP19 contains a SIAH binding motif characterized by the presence of a fully conserved core VxP motif flanked by conserved basic residues. We further confirmed the predicted SIAH interacting motif by showing that deletion of the motif, or mutation of key residues, abolish its interaction with SIAH.

In addition to SIAH, we have identified other USP19 binding partners in our yeast-two hybrid screen including Filamin A, an actin binding protein with multiple cellular functions, and the E3 ligase RING2. This adds to the list of USP19 interacting E3 ligases including previous reported KPC-1, XIAP, c-IAP1 and c-IAP2 [84,85]. Taken together, the identification of several E3 ligases as binding partner, and in some cases substrates, of USP19 underlines the potential involvement of this DUB in a broad variety of ubiquitin-related cellular processes.

4.5 Paper V

The interaction with the hypoxia pathway components SIAH1 and SIAH2 suggests that USP19 may participate in the regulation of the response to hypoxia. To test whether USP19 could interact with additional components in the hypoxia pathway, we performed a set of co-immunoprecipitation experiments and found that USP19 specifically interacts with HIF-1 α . We further mapped the interacting region to the PAS and bHLH domains in the N-terminal of HIF-1 α .

USP19 can rescue c-IAP and ERAD substrates from proteasomal degradation [85,89]. For this reason, we tested whether USP19 can rescue HIF-1 α from degradation. The result demonstrated that both USP19 and a catalytic inactive mutant rescue HIF-1 α from degradation. The effect was reproduced in different cell lines, suggesting that it is not cell type specific. Based on these results, we set out to test whether USP19 was involved in the regulation of the cellular response to hypoxia. The knockdown studies demonstrated that, in the absence of USP19, HIF-1 α is continuously degraded by the proteasome. Furthermore, the HIF-1 α

transcriptional response to hypoxia is significantly reduced in USP19 knockdown cells.

Non-catalytic functions of DUBs are not rare and have recently emerged as an important way for these enzymes to perform their function [151,152]. USP19 may non-catalytically regulate HIF-1 α stability by means of protein interactions or through competitive binding with additional partners. The N-terminal PAS and bHLH domains of HIF-1 α are responsible for the interaction with USP19. Although this region is typically involved in DNA binding and dimerization with HIF- β , other interactions taking place here include the molecular chaperone HSP90 and Rack1 that are associated with the O₂-independent regulation of HIF-1 α and the minichromosome maintenance protein (MCM7) that is involved in O₂-dependent regulation [115]. Our data suggest that USP19 may be part of these regulatory protein complexes.

5 Concluding Remarks

The overall aim of the studies presented in this thesis was to investigate the function of selected DUBs in the regulation of disease-relevant cellular functions. To this end, we have used a combination of bioinformatics and cell biology techniques, which allowed us not only to identify protein-protein interaction but also to investigate the role of DUBs in different molecular signaling pathways.

In paper I and II, we have identified USP4 as a negative regulator of the Wnt pathway that modulates the ubiquitination of TCF4. We also found that USP4 associates with the 26S proteasome via its N-terminal UBL domain. However, the functional significance of this interaction remains unclear. For example it is not known whether USP4 may affects the activity of the proteasome, or whether USP4 might have a role in proteasome assembly.

In paper III we have shown that USP19 is a DUB anchored to the ER membrane. USP19 is induced during ER stress and can rescue ERAD substrates from proteasomal degradation. In papers IV and V we further identified the ubiquitin ligases SIAH1 and SIAH2 and the transcription factor HIF-1 α as USP19 interaction partners. USP19 regulates HIF-1 α stability and is necessary for the appropriate cellular response to hypoxia. However, the mechanism of this regulation remains unclear. We have shown that SIAH regulates the stability of USP19. Since they both control the turnover of the key player of the hypoxia response, HIF-1 α , it would be interesting to test how this interaction contributes to the fine-tuning of the hypoxic response.

We have shown that USP19 is involved in ER stress, and other studies have documented the possible involvement of USP19 in the response to stress in rat skeletal muscles and regulation of apoptosis through rescue of c-IAP [82,85]. Taken together these findings suggest that USP19 may play a cytoprotective role and contribute to the capacity of cells to adapt to different source of stress. In order to have a better understanding of this DUB, future work should be done to identify

additional interacting partners and to reveal new functions in ubiquitin-regulated cellular processes. It is also worth noting that twelve splice variants of USP19 have been identified, only three of which are membrane associated. We have shown that membrane association is critical for the function of USP19 in ERAD but is dispensable for their regulatory role in the hypoxic response. Furthermore, only some of the splice variants have the ability to interact with HIF-1 α and SIAH. Thus, work aiming to understand the specific functions, tissue expression and subcellular localization of the different splice variants of USP19 is likely to yield important insights on the activity of this DUB in different signaling pathways

Collectively, the work described in this thesis has generated new knowledge regarding DUB interacting partners and their function in regulating cellular signaling pathways.

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7 References

1. Olson RE (1997) The dynamic state of body constituents (Schoenheimer, 1939). *J Nutr* 127: 1041S-1043S.
2. Ciechanover A, Hod Y, Hershko A (1978) A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes. *Biochem Biophys Res Commun* 81: 1100-1105.
3. Doherty FJ, Dawson S, Mayer RJ (2002) The ubiquitin-proteasome pathway of intracellular proteolysis. *Essays Biochem* 38: 51-63.
4. Amerik AY, Hochstrasser M (2004) Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta* 1695: 189-207.
5. Aktas O, Prozorovski T, Smorodchenko A, Savaskan NE, Lauster R, et al. (2004) Green tea epigallocatechin-3-gallate mediates T cellular NF-kappa B inhibition and exerts neuroprotection in autoimmune encephalomyelitis. *J Immunol* 173: 5794-5800.
6. Hartmann-Petersen R, Gordon C (2004) Integral UBL domain proteins: a family of proteasome interacting proteins. *Semin Cell Dev Biol* 15: 247-259.
7. Jentsch S, Pyrowolakis G (2000) Ubiquitin and its kin: how close are the family ties? *Trends Cell Biol* 10: 335-342.
8. Jana NR, Zemskov EA, Wang G, Nukina N (2001) Altered proteasomal function due to the expression of polyglutamine-expanded truncated N-terminal huntingtin induces apoptosis by caspase activation through mitochondrial cytochrome c release. *Hum Mol Genet* 10: 1049-1059.
9. Su V, Lau AF (2009) Ubiquitin-like and ubiquitin-associated domain proteins: significance in proteasomal degradation. *Cell Mol Life Sci* 66: 2819-2833.
10. Muller S, Hoege C, Pyrowolakis G, Jentsch S (2001) SUMO, ubiquitin's mysterious cousin. *Nat Rev Mol Cell Biol* 2: 202-210.
11. Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 82: 373-428.
12. Jin J, Li X, Gygi SP, Harper JW (2007) Dual E1 activation systems for ubiquitin differentially regulate E2 enzyme charging. *Nature* 447: 1135-1138.
13. van Wijk SJ, Timmers HT (2010) The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *FASEB J* 24: 981-993.
14. Laney JD, Hochstrasser M (1999) Substrate targeting in the ubiquitin system. *Cell* 97: 427-430.
15. Shi D, Grossman SR (2010) Ubiquitin becomes ubiquitous in cancer: emerging roles of ubiquitin ligases and deubiquitinases in tumorigenesis and as therapeutic targets. *Cancer Biol Ther* 10: 737-747.
16. Bernassola F, Karin M, Ciechanover A, Melino G (2008) The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell* 14: 10-21.
17. Lipkowitz S, Weissman AM (2011) RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nat Rev Cancer* 11: 629-643.
18. Clague MJ, Urbe S (2010) Ubiquitin: same molecule, different degradation pathways. *Cell* 143: 682-685.

19. Komander D (2009) The emerging complexity of protein ubiquitination. *Biochem Soc Trans* 37: 937-953.
20. Bhat KP, Greer SF (2011) Proteolytic and non-proteolytic roles of ubiquitin and the ubiquitin proteasome system in transcriptional regulation. *Biochim Biophys Acta* 1809: 150-155.
21. Haglund K, Dikic I (2005) Ubiquitylation and cell signaling. *EMBO J* 24: 3353-3359.
22. Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, et al. (2009) Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 137: 133-145.
23. Jin L, Williamson A, Banerjee S, Philipp I, Rape M (2008) Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell* 133: 653-665.
24. McCullough J, Clague MJ, Urbe S (2004) AMSH is an endosome-associated ubiquitin isopeptidase. *J Cell Biol* 166: 487-492.
25. Sato Y, Yoshikawa A, Yamagata A, Mimura H, Yamashita M, et al. (2008) Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. *Nature* 455: 358-362.
26. Hofmann RM, Pickart CM (1999) Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* 96: 645-653.
27. Duwel M, Hadian K, Krappmann D (2010) Ubiquitin Conjugation and Deconjugation in NF-kappaB Signaling. *Subcell Biochem* 54: 88-99.
28. Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, et al. (2012) Complete subunit architecture of the proteasome regulatory particle. *Nature* 482: 186-191.
29. Xie Y (2010) Structure, assembly and homeostatic regulation of the 26S proteasome. *J Mol Cell Biol* 2: 308-317.
30. Navon A, Ciechanover A (2009) The 26 S proteasome: from basic mechanisms to drug targeting. *J Biol Chem* 284: 33713-33718.
31. Kisselev AF, van der Linden WA, Overkleeft HS (2012) Proteasome inhibitors: an expanding army attacking a unique target. *Chem Biol* 19: 99-115.
32. Bar-Nun S, Glickman MH (2012) Proteasomal AAA-ATPases: structure and function. *Biochim Biophys Acta* 1823: 67-82.
33. Smith DM, Chang SC, Park S, Finley D, Cheng Y, et al. (2007) Docking of the proteasomal ATPases' carboxyl termini in the 20S proteasome's alpha ring opens the gate for substrate entry. *Mol Cell* 27: 731-744.
34. Tomko RJ, Jr., Funakoshi M, Schneider K, Wang J, Hochstrasser M (2010) Heterohexameric ring arrangement of the eukaryotic proteasomal ATPases: implications for proteasome structure and assembly. *Mol Cell* 38: 393-403.
35. Elsasser S, Gali RR, Schwickart M, Larsen CN, Leggett DS, et al. (2002) Proteasome subunit Rpn1 binds ubiquitin-like protein domains. *Nat Cell Biol* 4: 725-730.
36. Rosenzweig R, Bronner V, Zhang D, Fushman D, Glickman MH (2012) Rpn1 and Rpn2 coordinate ubiquitin processing factors at proteasome. *J Biol Chem* 287: 14659-14671.
37. Hanna J, Finley D (2007) A proteasome for all occasions. *FEBS Lett* 581: 2854-2861.

38. Kohler A, Cascio P, Leggett DS, Woo KM, Goldberg AL, et al. (2001) The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. *Mol Cell* 7: 1143-1152.
39. Lam YA, Pickart CM, Alban A, Landon M, Jamieson C, et al. (2000) Inhibition of the ubiquitin-proteasome system in Alzheimer's disease. *Proc Natl Acad Sci U S A* 97: 9902-9906.
40. Song S, Jung YK (2004) Alzheimer's disease meets the ubiquitin-proteasome system. *Trends Mol Med* 10: 565-570.
41. Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 78: 477-513.
42. Wilkinson CR, Seeger M, Hartmann-Petersen R, Stone M, Wallace M, et al. (2001) Proteins containing the UBA domain are able to bind to multi-ubiquitin chains. *Nat Cell Biol* 3: 939-943.
43. Hartmann-Petersen R, Seeger M, Gordon C (2003) Transferring substrates to the 26S proteasome. *Trends Biochem Sci* 28: 26-31.
44. Mayor T (2003) [A cryptic protease couples deubiquitination and degradation by the proteasome]. *Med Sci (Paris)* 19: 401-403.
45. Verma R, Aravind L, Oania R, McDonald WH, Yates JR, 3rd, et al. (2002) Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. *Science* 298: 611-615.
46. Kim T, Hofmann K, von Arnim AG, Chamovitz DA (2001) PCI complexes: pretty complex interactions in diverse signaling pathways. *Trends Plant Sci* 6: 379-386.
47. Pathare GR, Nagy I, Bohn S, Unverdorben P, Hubert A, et al. (2012) The proteasomal subunit Rpn6 is a molecular clamp holding the core and regulatory subcomplexes together. *Proc Natl Acad Sci U S A* 109: 149-154.
48. Yao T, Cohen RE (2002) A cryptic protease couples deubiquitination and degradation by the proteasome. *Nature* 419: 403-407.
49. Guterman A, Glickman MH (2004) Complementary roles for Rpn11 and Ubp6 in deubiquitination and proteolysis by the proteasome. *J Biol Chem* 279: 1729-1738.
50. Lee MJ, Lee BH, Hanna J, King RW, Finley D (2011) Trimming of ubiquitin chains by proteasome-associated deubiquitinating enzymes. *Mol Cell Proteomics* 10: R110 003871.
51. Leggett DS, Hanna J, Borodovsky A, Crosas B, Schmidt M, et al. (2002) Multiple associated proteins regulate proteasome structure and function. *Mol Cell* 10: 495-507.
52. Stone M, Hartmann-Petersen R, Seeger M, Bech-Otschir D, Wallace M, et al. (2004) Uch2/Uch37 is the major deubiquitinating enzyme associated with the 26S proteasome in fission yeast. *J Mol Biol* 344: 697-706.
53. Nag DK, Finley D (2012) A small-molecule inhibitor of deubiquitinating enzyme USP14 inhibits Dengue virus replication. *Virus Res* 165: 103-106.
54. Liang J, Saad Y, Lei T, Wang J, Qi D, et al. (2010) MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF-kappaB signaling. *J Exp Med* 207: 2959-2973.
55. Katz EJ, Isasa M, Crosas B (2010) A new map to understand deubiquitination. *Biochem Soc Trans* 38: 21-28.
56. Hussain S, Zhang Y, Galaray PJ (2009) DUBs and cancer: the role of deubiquitinating enzymes as oncogenes, non-oncogenes and tumor suppressors. *Cell Cycle* 8: 1688-1697.

57. Khoronenkova SV, Dianova, II, Ternette N, Kessler BM, Parsons JL, et al. (2012) ATM-dependent downregulation of USP7/HAUSP by PPM1G activates p53 response to DNA damage. *Mol Cell* 45: 801-813.
58. Yuan J, Luo K, Zhang L, Cheville JC, Lou Z (2010) USP10 regulates p53 localization and stability by deubiquitinating p53. *Cell* 140: 384-396.
59. Jochemsen AG, Shiloh Y (2010) USP10: friend and foe. *Cell* 140: 308-310.
60. Fan YH, Yu Y, Mao RF, Tan XJ, Xu GF, et al. (2011) USP4 targets TAK1 to downregulate TNFalpha-induced NF-kappaB activation. *Cell Death Differ* 18: 1547-1560.
61. Yoshida H, Jono H, Kai H, Li JD (2005) The tumor suppressor cylindromatosis (CYLD) acts as a negative regulator for toll-like receptor 2 signaling via negative cross-talk with TRAF6 AND TRAF7. *J Biol Chem* 280: 41111-41121.
62. Reiley W, Zhang M, Wu X, Granger E, Sun SC (2005) Regulation of the deubiquitinating enzyme CYLD by IkappaB kinase gamma-dependent phosphorylation. *Mol Cell Biol* 25: 3886-3895.
63. Villamil MA, Chen J, Liang Q, Zhuang Z (2012) A noncanonical cysteine protease USP1 is activated through active site modulation by USP1-associated factor 1. *Biochemistry* 51: 2829-2839.
64. Yao T, Song L, Xu W, DeMartino GN, Florens L, et al. (2006) Proteasome recruitment and activation of the Uch37 deubiquitinating enzyme by Adrm1. *Nat Cell Biol* 8: 994-1002.
65. Qiu XB, Ouyang SY, Li CJ, Miao S, Wang L, et al. (2006) hRpn13/ADRM1/GP110 is a novel proteasome subunit that binds the deubiquitinating enzyme, UCH37. *EMBO J* 25: 5742-5753.
66. Li T, Duan W, Yang H, Lee MK, Bte Mustafa F, et al. (2001) Identification of two proteins, S14 and UIP1, that interact with UCH37. *FEBS Lett* 488: 201-205.
67. Hu M, Li P, Song L, Jeffrey PD, Chenova TA, et al. (2005) Structure and mechanisms of the proteasome-associated deubiquitinating enzyme USP14. *EMBO J* 24: 3747-3756.
68. Li Z, Wang D, Messing EM, Wu G (2005) VHL protein-interacting deubiquitinating enzyme 2 deubiquitinates and stabilizes HIF-1alpha. *EMBO Rep* 6: 373-378.
69. Gupta K, Copeland NG, Gilbert DJ, Jenkins NA, Gray DA (1993) Unp, a mouse gene related to the tre oncogene. *Oncogene* 8: 2307-2310.
70. Baker RT, Wang XW, Woollatt E, White JA, Sutherland GR (1999) Identification, functional characterization, and chromosomal localization of USP15, a novel human ubiquitin-specific protease related to the UNP oncoprotein, and a systematic nomenclature for human ubiquitin-specific proteases. *Genomics* 59: 264-274.
71. Gray DA, Inazawa J, Gupta K, Wong A, Ueda R, et al. (1995) Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. *Oncogene* 10: 2179-2183.
72. Zhang X, Berger FG, Yang J, Lu X (2011) USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. *EMBO J* 30: 2177-2189.
73. Zhang L, Zhou F, Drabsch Y, Gao R, Snaar-Jagalska BE, et al. (2012) USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-beta type I receptor. *Nat Cell Biol* 14: 717-726.

74. Xiao N, Li H, Luo J, Wang R, Chen H, et al. (2012) Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNF α -induced cancer cell migration. *Biochem J* 441: 979-986.
75. Zezula J, Freissmuth M (2008) The A(2A)-adenosine receptor: a GPCR with unique features? *Br J Pharmacol* 153 Suppl 1: S184-190.
76. Wada K, Kamitani T (2006) UnpEL/Us4 is ubiquitinated by Ro52 and deubiquitinated by itself. *Biochem Biophys Res Commun* 342: 253-258.
77. Milojevic T, Reiterer V, Stefan E, Korkhov VM, Dorostkar MM, et al. (2006) The ubiquitin-specific protease Us4 regulates the cell surface level of the A2A receptor. *Mol Pharmacol* 69: 1083-1094.
78. Wada K, Tanji K, Kamitani T (2006) Oncogenic protein UnpEL/Us4 deubiquitinates Ro52 by its isopeptidase activity. *Biochem Biophys Res Commun* 339: 731-736.
79. DeSalle LM, Latres E, Lin D, Graner E, Montagnoli A, et al. (2001) The deubiquitinating enzyme Unp interacts with the retinoblastoma protein. *Oncogene* 20: 5538-5542.
80. Blanchette P, Gilchrist CA, Baker RT, Gray DA (2001) Association of UNP, a ubiquitin-specific protease, with the pocket proteins pRb, p107 and p130. *Oncogene* 20: 5533-5537.
81. Luna-Vargas MP, Faesen AC, van Dijk WJ, Rape M, Fish A, et al. (2011) Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain. *EMBO Rep* 12: 365-372.
82. Combaret L, Adegoke OA, Bedard N, Baracos V, Attaix D, et al. (2005) USP19 is a ubiquitin-specific protease regulated in rat skeletal muscle during catabolic states. *Am J Physiol Endocrinol Metab* 288: E693-700.
83. Lu Y, Bedard N, Chevalier S, Wing SS (2011) Identification of distinctive patterns of USP19-mediated growth regulation in normal and malignant cells. *PLoS One* 6: e15936.
84. Lu Y, Adegoke OA, Nepveu A, Nakayama KI, Bedard N, et al. (2009) USP19 deubiquitinating enzyme supports cell proliferation by stabilizing KPC1, a ubiquitin ligase for p27Kip1. *Mol Cell Biol* 29: 547-558.
85. Mei Y, Hahn AA, Hu S, Yang X (2011) The USP19 deubiquitinase regulates the stability of c-IAP1 and c-IAP2. *J Biol Chem* 286: 35380-35387.
86. Lutterbach B, Sun D, Schuetz J, Hiebert SW (1998) The MYND motif is required for repression of basal transcription from the multidrug resistance 1 promoter by the t(8;21) fusion protein. *Mol Cell Biol* 18: 3604-3611.
87. Huibregtse JM, Scheffner M, Beaudenon S, Howley PM (1995) A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* 92: 5249.
88. Felts SJ, Toft DO (2003) p23, a simple protein with complex activities. *Cell Stress Chaperones* 8: 108-113.
89. Hassink GC, Zhao B, Sompallae R, Altun M, Gastaldello S, et al. (2009) The ER-resident ubiquitin-specific protease 19 participates in the UPR and rescues ERAD substrates. *EMBO Rep* 10: 755-761.
90. Tauriello DV, Maurice MM (2010) The various roles of ubiquitin in Wnt pathway regulation. *Cell Cycle* 9: 3700-3709.
91. Klaus A, Birchmeier W (2008) Wnt signalling and its impact on development and cancer. *Nat Rev Cancer* 8: 387-398.
92. Clevers H, Nusse R (2012) Wnt/beta-Catenin Signaling and Disease. *Cell* 149: 1192-1205.

93. He X, Semenov M, Tamai K, Zeng X (2004) LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* 131: 1663-1677.
94. Semenov MV, Tamai K, Brott BK, Kuhl M, Sokol S, et al. (2001) Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr Biol* 11: 951-961.
95. MacDonald BT, Tamai K, He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17: 9-26.
96. Cruciati CM, Ohkawara B, Acebron SP, Karaulanov E, Reinhard C, et al. (2010) Requirement of prorenin receptor and vacuolar H⁺-ATPase-mediated acidification for Wnt signaling. *Science* 327: 459-463.
97. Tran H, Hamada F, Schwarz-Romond T, Bienz M (2008) Trabad, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. *Genes Dev* 22: 528-542.
98. Huang X, Langelotz C, Hetfeld-Pechoc BK, Schwenk W, Dubiel W (2009) The COP9 signalosome mediates beta-catenin degradation by deneddylation and blocks adenomatous polyposis coli destruction via USP15. *J Mol Biol* 391: 691-702.
99. Tauriello DV, Haegbarth A, Kuper I, Edelmann MJ, Henraat M, et al. (2010) Loss of the tumor suppressor CYLD enhances Wnt/beta-catenin signaling through K63-linked ubiquitination of Dvl. *Mol Cell* 37: 607-619.
100. Komander D (2010) CYLD tidies up dishevelled signaling. *Mol Cell* 37: 589-590.
101. Mukai A, Yamamoto-Hino M, Awano W, Watanabe W, Komada M, et al. (2010) Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. *EMBO J* 29: 2114-2125.
102. Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13: 1211-1233.
103. Ernst R, Mueller B, Ploegh HL, Schlieker C (2009) The otubain YOD1 is a deubiquitinating enzyme that associates with p97 to facilitate protein dislocation from the ER. *Mol Cell* 36: 28-38.
104. Zhong X, Pittman RN (2006) Ataxin-3 binds VCP/p97 and regulates retrotranslocation of ERAD substrates. *Hum Mol Genet* 15: 2409-2420.
105. Marciniak SJ, Ron D (2006) Endoplasmic reticulum stress signaling in disease. *Physiol Rev* 86: 1133-1149.
106. Zhao L, Ackerman SL (2006) Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol* 18: 444-452.
107. Welihinda AA, Tirasophon W, Kaufman RJ (1999) The cellular response to protein misfolding in the endoplasmic reticulum. *Gene Expr* 7: 293-300.
108. Wu J, Kaufman RJ (2006) From acute ER stress to physiological roles of the Unfolded Protein Response. *Cell Death Differ* 13: 374-384.
109. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2: 326-332.
110. Hockel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 93: 266-276.
111. Semenza GL, Wang GL (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 12: 5447-5454.

112. Semenza GL (2004) Intratumoral hypoxia, radiation resistance, and HIF-1. *Cancer Cell* 5: 405-406.
113. Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. *Nature* 441: 437-443.
114. Wang GL, Jiang BH, Rue EA, Semenza GL (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A* 92: 5510-5514.
115. Liu YV, Baek JH, Zhang H, Diez R, Cole RN, et al. (2007) RACK1 competes with HSP90 for binding to HIF-1 α and is required for O₂-independent and HSP90 inhibitor-induced degradation of HIF-1 α . *Mol Cell* 25: 207-217.
116. Galban S, Kuwano Y, Pullmann R, Jr., Martindale JL, Kim HH, et al. (2008) RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1 α . *Mol Cell Biol* 28: 93-107.
117. Harten SK, Ashcroft M, Maxwell PH (2010) Prolyl hydroxylase domain inhibitors: a route to HIF activation and neuroprotection. *Antioxid Redox Signal* 12: 459-480.
118. Wenger RH, Camenisch G, Stiehl DP, Katschinski DM (2009) HIF prolyl-4-hydroxylase interacting proteins: consequences for drug targeting. *Curr Pharm Des* 15: 3886-3894.
119. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, et al. (2001) Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292: 468-472.
120. Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, et al. (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the β -domain of the von Hippel-Lindau protein. *Nat Cell Biol* 2: 423-427.
121. Jung CR, Hwang KS, Yoo J, Cho WK, Kim JM, et al. (2006) E2-EPF UCP targets pVHL for degradation and associates with tumor growth and metastasis. *Nat Med* 12: 809-816.
122. Baek JH, Liu YV, McDonald KR, Wesley JB, Zhang H, et al. (2007) Spermidine/spermine N(1)-acetyltransferase-1 binds to hypoxia-inducible factor-1 α (HIF-1 α) and RACK1 and promotes ubiquitination and degradation of HIF-1 α . *J Biol Chem* 282: 33358-33366.
123. Li Z, Wang D, Na X, Schoen SR, Messing EM, et al. (2002) Identification of a deubiquitinating enzyme subfamily as substrates of the von Hippel-Lindau tumor suppressor. *Biochem Biophys Res Commun* 294: 700-709.
124. Flugel D, Gorlach A, Michiels C, Kietzmann T (2007) Glycogen synthase kinase 3 phosphorylates hypoxia-inducible factor 1 α and mediates its destabilization in a VHL-independent manner. *Mol Cell Biol* 27: 3253-3265.
125. Carbia-Nagashima A, Gerez J, Perez-Castro C, Paez-Pereda M, Silberstein S, et al. (2007) RSUME, a small RWD-containing protein, enhances SUMO conjugation and stabilizes HIF-1 α during hypoxia. *Cell* 131: 309-323.
126. Berta MA, Mazure N, Hattab M, Pouyssegur J, Brahimi-Horn MC (2007) SUMOylation of hypoxia-inducible factor-1 α reduces its transcriptional activity. *Biochem Biophys Res Commun* 360: 646-652.
127. Huang C, Han Y, Wang Y, Sun X, Yan S, et al. (2009) SENP3 is responsible for HIF-1 transactivation under mild oxidative stress via p300 de-SUMOylation. *EMBO J* 28: 2748-2762.
128. Kallio PJ, Okamoto K, O'Brien S, Carrero P, Makino Y, et al. (1998) Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment

- of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *EMBO J* 17: 6573-6586.
129. Mohr SE, Perrimon N (2012) RNAi screening: new approaches, understandings, and organisms. *Wiley Interdiscip Rev RNA* 3: 145-158.
 130. Echeverri CJ, Perrimon N (2006) High-throughput RNAi screening in cultured cells: a user's guide. *Nat Rev Genet* 7: 373-384.
 131. Hogeweg P (2011) The roots of bioinformatics in theoretical biology. *PLoS Comput Biol* 7: e1002021.
 132. Krogh A, Brown M, Mian IS, Sjolander K, Haussler D (1994) Hidden Markov models in computational biology. Applications to protein modeling. *J Mol Biol* 235: 1501-1531.
 133. Ye X, Wang G, Altschul SF (2011) An assessment of substitution scores for protein profile-profile comparison. *Bioinformatics* 27: 3356-3363.
 134. Gietz RD, Triggs-Raine B, Robbins A, Graham KC, Woods RA (1997) Identification of proteins that interact with a protein of interest: applications of the yeast two-hybrid system. *Mol Cell Biochem* 172: 67-79.
 135. Young KH (1998) Yeast two-hybrid: so many interactions, (in) so little time. *Biol Reprod* 58: 302-311.
 136. Phizicky EM, Fields S (1995) Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 59: 94-123.
 137. Berggard T, Linse S, James P (2007) Methods for the detection and analysis of protein-protein interactions. *Proteomics* 7: 2833-2842.
 138. Udomsinprasert R, Pongjaroenkit S, Wongsantichon J, Oakley AJ, Prapanthadara LA, et al. (2005) Identification, characterization and structure of a new Delta class glutathione transferase isoenzyme. *Biochem J* 388: 763-771.
 139. Allocati N, Federici L, Masulli M, Di Ilio C (2009) Glutathione transferases in bacteria. *FEBS J* 276: 58-75.
 140. Beckett GJ, Hayes JD (1993) Glutathione S-transferases: biomedical applications. *Adv Clin Chem* 30: 281-380.
 141. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76: 4350-4354.
 142. Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112: 195-203.
 143. Opas M (1999) Fluorescence tracing of intracellular proteins. *Biotech Histochem* 74: 294-310.
 144. Pedley KC (1997) Applications of confocal and fluorescence microscopy. *Digestion* 58 Suppl 2: 62-68.
 145. VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 44: 619-626.
 146. Schefe JH, Lehmann KE, Buschmann IR, Unger T, Funke-Kaiser H (2006) Quantitative real-time RT-PCR data analysis: current concepts and the novel "gene expression's CT difference" formula. *J Mol Med (Berl)* 84: 901-910.
 147. Zhu X, Menard R, Sulea T (2007) High incidence of ubiquitin-like domains in human ubiquitin-specific proteases. *Proteins* 69: 1-7.
 148. Faesen AC, Luna-Vargas MP, Sixma TK (2012) The role of UBL domains in ubiquitin-specific proteases. *Biochem Soc Trans* 40: 539-545.

149. Borgese N, Brambillasca S, Colombo S (2007) How tails guide tail-anchored proteins to their destinations. *Curr Opin Cell Biol* 19: 368-375.
150. House CM, Hancock NC, Moller A, Cromer BA, Fedorov V, et al. (2006) Elucidation of the substrate binding site of Siah ubiquitin ligase. *Structure* 14: 695-701.
151. Hanna J, Hathaway NA, Tone Y, Crosas B, Elsasser S, et al. (2006) Deubiquitinating enzyme Ubp6 functions noncatalytically to delay proteasomal degradation. *Cell* 127: 99-111.
152. Peth A, Besche HC, Goldberg AL (2009) Ubiquitinated proteins activate the proteasome by binding to Usp14/Ubp6, which causes 20S gate opening. *Mol Cell* 36: 794-804.