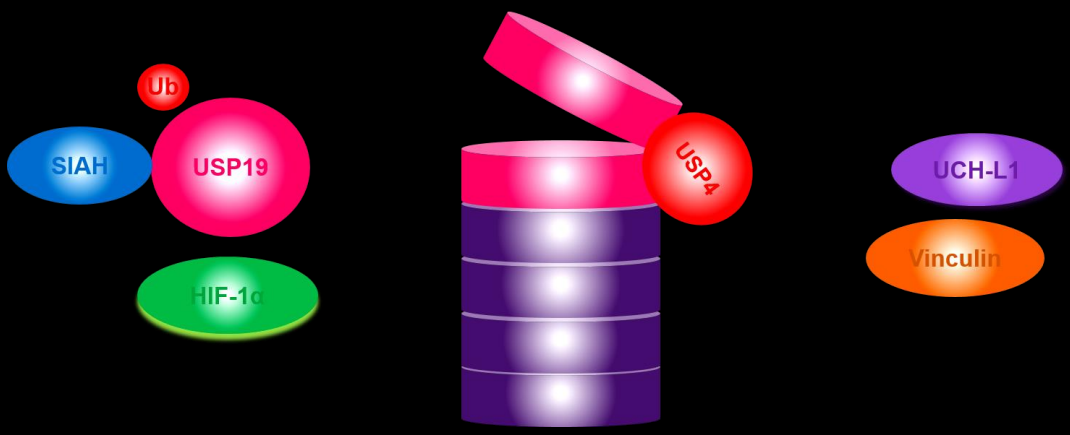
FUNCTIONAL STUDIES OF THE DEUBIQUITINATING ENZYMES USP19, USP4 AND UCH-L1



KELLY MARCELA VELASCO PINTO

UNIVERSIDAD DEL ROSARIO

FACULTAD DE CIENCIAS NATURALES Y MATEMÁTICAS

BOGOTÁ

2013

FUNCTIONAL STUDIES OF THE DEUBIQUITINATING ENZYMES USP19, USP4 AND UCH-L1

KELLY MARCELA VELASCO PINTO

Thesis for doctoral degree (Ph.D.)

Professor
María Marcela Camacho Navarro, M.D., Ph.D.

Kristina Lindsten, Ph.D.

Professor
Maria Grazia Masucci, M.D., Ph.D.

UNIVERSIDAD DEL ROSARIO
FACULTAD DE CIENCIAS NATURALES Y MATEMÁTICAS
BOGOTÁ
2013

Aunque debo todo lo que soy a cada una de las personas con las que he tenido la fortuna de compartir algo a lo largo de mi vida, y de una u otra forma estoy muy agradecida con todas ellas, dedico este trabajo a las personas que siempre me han acompañado y apoyado con su inmensurable y desinteresado amor, porque este trabajo también es fruto de sus esfuerzos. Primero, a mis cuatro abuelos: Gloria, Miguel, Pola y Ricardo. Segundo, a mis padres: Rick y Ede. Tercero, a mi hermano Gerson. Y cuarto, a mis tíos: Ingrid, Gina, Vicky, Gloria, Ana Virginia, Miguel Ángel y Flor. Sin ellos, no sería.

También dedico este trabajo a mis primos y sobrinas, especialmente a los que son más jóvenes que yo, como un testimonio de que pueden llegar a ser y a hacer lo que siempre soñaron y lo que nunca imaginaron. No tengan miedo, luchen por lo que quieren, conserven un corazón bondadoso, reconozcan su propio valor y el de las personas a su alrededor… amen, rían, abracen, besen, hablen, muévanse… ¡vivan! Nunca es tarde para empezar.

I would like to acknowledge to the Centro Internacional de Física (Bogotá, Colombia) its support to start my doctoral studies in the Universidad del Rosario (Bogotá, Colombia) and to carry out my thesis as an exchange student in the Karolinska Institute (KI, Stockholm, Sweden). I would like to offer my special thanks to Prof. Marcela Camacho, Dr. Martha Lucia Posada, Dr. Eduardo Posada, and my colleagues Lina Maria Garcia and Diana Carolina Ochoa, among many others.

I wish to express my sincere gratitude to Dr. Kristina Lindsten and Prof. Maria Masucci, my supervisors at KI, and Dr. Teresa Frisan for their invaluable guidance and support carrying out this project. Deepest gratitude are also due to my co-workers and colleagues at KI for their permanent assistance, especially Bin Zhao, Linda Thörn, Stefano Gastaldello, Giuseppe Coppotelli, Siamak Akbari, Nouman Mughal, Simone Callegari, Xinsong Chen, Javier Avila-Cariño, Riccardo Guidi and Laura Levi.

Heartfelt thanks to my colleagues and friends Daniela Gödderz, Roberta Biasiotto, Philipp Klambauer, Hicham Lamniri, Natalia Ortiz, Sandra Zlodej, Iván Hernández, Daniel León and many others for their help proof-reading numerous texts, their technical, and professional advises and discussions, and for encouraging and motivating me.

Last but not the least, big thanks to Sven Le Moine-Bauer for spicing my life up the last years, for his kindness and support, his help proof-reading this text, and his statistical advice.

This work was possible thanks to the efforts of many persons from Universidad del Rosario, Karolinska Institute, and several others institutions involved in the development of the ERACOL (Erasmus-Columbus 2013) project, which provided the funding for my academic exchange.

Other institutions that sponsored this work are mentioned in the appended articles.

CONTENTS

GLOSSARY

19S REGULATORY PARTICLE (19S RP): A sub-complex of the 26S proteasome composed of two subunits, the base and the lid. It flanks each extremity of the 20S CP controlling the access of the substrates to the internal chamber and removing ubiquitin from the substrate committed to degradation.

20S CORE PARTICLE (20S CP): A sub-complex of the 26S proteasome, composed of 28 subunits forming a hollow cylinder that contains inside a catalytic chamber where internalized substrates are degraded.

26S PROTEASOME: A large and complex enzyme that degrades substrates tagged with certain ubiquitin signals. It is composed of two sub-complexes, a 20S core particle (20S CP) and a 19S regulatory particle (19S RP)

CATALYTIC TRIAD: Three amino acid residues, within the active site of the protease, that work together to break peptide bonds.

COLONY FORMING UNIT (CFU): Estimate of viable cells present, assessed by counting the amount of colonies visible on the plate.

CO-IMMUNOPRECIPITATION: Technique to study protein-protein interactions under physiological conditions by using specific antibodies that recognize the proteins of interest.

DAPI: Fluorescent counterstain for nucleus and chromosomes. It binds strongly to A-T rich regions in DNA emitting blue fluorescence. Used extensively in fluorescence microscopy.

DEGRON: Specific recognition motifs within the structure of proteins, ought to be degraded through the ubiquitin pathway, which are identified by particular E3 ligases.

DEUBIQUITINATING ENZYMES (DUBs): A diverse group of proteases responsible for the deconjugation of ubiquitin.

DOXYCYCLINE: Semisynthetic antibiotic, tetracycline derivative.

FOCAL ADHESION COMPLEX: Integrin-based macromolecular complexes that connect the cell cytoskeleton with extracellular ligands.

FOCAL ADHESION KINASE (FAK): Tyrosine kinase recruited at early stages of focal adhesions formation. It integrates many cellular signals.

HECT E3 LIGASES: E3 ligases that harbor an HECT domain within its structure. In opposition to the RING E3 ligases, they bind the ubiquitin carried by its cognate E2 to its own catalytic cysteine before transferring it to the target protein.

HYPOXIA INDUCIBLE FACTOR (HIF)-1: Key transcription factor of the hypoxia response.

IMMUNOFLUORESCENCE: Technique to detect the location and relative abundance of proteins of interest in the cells, by using specific fluorescent-labeled antibodies.

INTEGRINS: Family of cell surface adhesion receptors. They operate as transmembrane proteins with large extracellular and short cytoplasmic domains that connect the extracellular matrix to the cell cytoskeleton.

LIPOPOLYSACCHARIDE (LPS): The major component of the outer membrane of Gram-negative bacteria.

mCHERRY: Monomeric red fluorophore with peak absorption/emission at 578 nm and 610 nm, respectively.

MULTIPLICITY OF INFECTION (MOI): The ratio of infectious agents (in this case bacteria) to infection targets (cells).

PHALLOIDIN: Toxin that binds F-actin with high-affinity. It is conjugated to fluorescent dyes, such as fluorescein (FITC) to visualize the actin cytoskeleton of the cells.

POST-TRANSLATIONAL MODIFICATIONS (PTMs): Chemical modifications in the proteins structure. They alter the functions of their substrates by regulating their activity, cellular location and interaction with other molecules.

PULL-DOWN ASSAY: Technique to study physical protein-protein interactions in *in vitro* conditions.

PUROMYCIN: Antibiotic that inhibits protein synthesis.

P120-CATENIN: Protein that regulates the abundance and activity of the cadherin cell adhesion receptors.

RING E3 LIGASES: E3 ligases that harbor a RING domain within its structure. In opposition to the HECT E3 ligases, they promote the discharge of ubiquitin directly from its cognate E2 to the substrate, without binding the ubiquitin to its own catalytic site.

SEVEN IN ABSENTIA HOMOLOG (SIAH) 1 and 2: E3 ubiquitin-protein ligases.

TET-ON SYSTEM: Inducible gene expression system, in which the expression of the gene of interest is triggered by tetracycline.

TETRACYCLINE: Broad-spectrum antibiotic. One of its derivatives is doxycycline.

TRIGGER MECHANISM: Bacterial invasion strategy, in which the bacteria inject protein effectors into the host cytoplasm that promote their internalization.

UBIQUITIN: A small protein of 76 amino acids that operates as a post-translational modification.

UBIQUITINATION: The enzymatic cascade that conjugates ubiquitin to its substrates.

UBIQUITIN ACTIVATING ENZYME (E1): The first enzyme in the ubiquitination cascade that activates the C-terminal glycine of ubiquitin, allowing its subsequent binding to the other enzymes and its final substrate.

UBIQUITIN BINDING DOMAINS (UBDs): Domains harbored on effector proteins downstream the ubiquitin pathway, known as ubiquitin receptors, which bind non-covalently to ubiquitin recognizing the signal it conveys and eliciting the appropriate response.

UBIQUITIN CONJUGATING ENZYME (E2): The second enzyme in the ubiquitination cascade that takes the activated ubiquitin from the E1 enzymes and brings it in proximity of a specific E3 enzyme, to aid in the transference of ubiquitin to its final substrate.

UBIQUITIN LIGASE (E3): The third enzyme in the ubiquitination cascade that catalyzes the formation of an isopeptide bond between the activated C-terminal glycine of ubiquitin and usually a lysine within the substrate.

UBL-SPECIFIC PROTEASES (ULPs): Proteases responsible for the deconjugation of ubiquitin-like modifiers (UBLs)

UBIQUITIN-DOMAIN PROTEINS (UDPs): Proteins that bear domains related to ubiquitin, but contrary to ubiquitin they cannot be processed and conjugated to other substrates as modifiers.

UBIQUITIN PROTEASOME SYSTEM (UPS): The coupled action of ubiquitination and proteasomal degradation, where ubiquitination targets the substrate protein for degradation within the 26S proteasome.

UBIQUITIN-LIKE MODIFIERS (UBLs): Ubiquitin-like proteins that can be covalently conjugated to substrates working as PTMs in a manner analogous to ubiquitin.

UBIQUITIN-LIKE PROTEINS: Proteins related to ubiquitin in sequence, structure or function

Ub-VS: Functional probe that labels active DUBs.

VINCULIN: Component of the focal adhesion complexes. It carries out a stabilizing function.

YEAST-TWO-HYBRID SCREEN: Method to detect weak and transient physical protein-protein interactions *in vivo*.

ZIPPER MECHANISM: Bacterial invasion strategy, in which bacteria exploit host cell membrane surface receptors to induce their internalization.

FIGURES

APPENDICES

ABBREVIATIONS

19S RP: 19S regulatory particle

20S CP: 20S core particle

AD: Activating domain

ARF: ADP ribosylation factor

ARF-BP1: ARF-binding protein 1

ATM: Ataxia telangiectasia mutated

ATR: Ataxia telangiectasia and Rad3-related protein

bHLH: Basic-helix-loop-helix domain

CFU: Colony forming units

c-IAP1/2: Cellular inhibitor of apoptosis protein 1/2

DBD: DNA binding domain

DOX: Doxycycline

DUB: Deubiquitinating Enzyme

DUSP: Domain present in ubiquitin specific proteases

ECM: Extracellular matrix

EMT: Epithelial-to-mesenchymal transition

ER: Endoplasmic reticulum

ERAD: Endoplasmic reticulum associated degradation

FAK: Focal adhesion kinase

E1: Ubiquitin activating enzyme

E2: Ubiquitin conjugating enzyme

E3: Ubiquitin ligase

GST: Glutathione S-transferase

HCV: Hepatitis C virus

HECT: Homologous to E6AP C terminus

HGF: Hepatocyte growth factor

HIF-1: Hypoxia inducible factor-1

His: Poly-histidine

HPV: Human papilloma virus

JAMM: Josephins and JAB1/MPN/MOV3 metalloenzyme

KPC1: Kip1 ubiquitination-promoting complex 1

LPS: Lipopolysaccharide

MCPIP: Monocyte chemotactic protein-induced protein

MOI: Multiplicity of infection

MYND domain: Myeloid translocation protein 8, Nervy, and DEAF-1

NEMO: NF-κβ essential modulator

NLS: Nuclear localization sequence

NF-κβ: Nuclear factor kappa-light-chain enhancer of activated B cells

OTU: Ovarian tumor protease

PAS: Per-Arnt-Sim domain

PFA: Paraformaldehyde

PHD: Prolyl hydroxylase

PTM: Post-translational modification

PuroR: Puromycin selection marker

PVDF: Polyvinylidene difluoride

p23/CS domain: p23-like CHORD and Sgt1 domain

RING: Really interesting new gene

rtTA3: Tetracycline-controlled transcription factor

SIAH: Seven in absentia homolog

TAK: Transforming growth factor-β-activated kinase 1

TβRI: TGF-β type I receptor

TF: Transcription factor

TGF-β: Transforming growth factor β

TMD: Transmembrane domain

TNF: Tumor necrosis factor

TRAF2/3/6: TNF-receptor associated factor 2/3/6

TRE: Tetracycline-inducible promoter

Ub: Ubiquitin

UBC: Human ubiquitin C

UBD: Ubiquitin binding domain

UBL: Ubiquitin-like

ULP: UBL-specific protease

UCH: Ubiquitin C-terminal hydrolase

UCH-L1: Ubiquitin C-terminal hydrolase-L1

UPS: Ubiquitin proteasome system

USP: Ubiquitin-specific protease

USP4: Ubiquitin specific protease 4

USP19: Ubiquitin specific protease 19

VHL: Von Hippel-Lindau tumor suppressor protein

WGA: Wheat germ agglutinin

ABSTRACT

The conjugation of ubiquitin to proteins, known as ubiquitination, has different cellular functions; they include targeting proteins for degradation by the proteasome, regulation of DNA damage repair signaling, membrane receptor signaling and endocytosis (1). The ubiquitin moieties can be de-conjugated from their substrates or other ubiquitin moieties by a large group of proteases named deubiquitinating enzymes (DUBs) (2). DUBs are essential for the maintenance of the ubiquitin homeostasis in the cell and regulation of the ubiquitination status of the different substrates. The diversity of these proteases hints on their specificity for certain targets and participation in particular cellular pathways. Although several DUBs have been thoroughly studied, at present the targets and physiological roles of most of them remain unknown. Here, we studied the functional roles of the ubiquitin specific protease 19 (USP19), USP4 and the ubiquitin C-terminal hydrolase (UCH-L1), using several cellular and molecular techniques. We found that, i) USP19 can be regulated by SIAH ubiquitin ligases, ii) USP19 is important for controlling the key regulator of response to hypoxia, HIF-1α, iii) USP4 is a proteasome-interacting DUB, iv) an mCherry-UCH-L1 chimera reproduces only partially previous phenotypes described for UCH-L1, and v) UCH-L1 promotes *Yersinia pseudotuberculosis* internalization.

Keywords: Ubiquitin, deubiquitinating enzymes, USP19, USP4, UCH-L1

RESUMEN

El marcaje de proteínas con ubiquitina, conocido como ubiquitinación, cumple diferentes funciones que incluyen la regulación de varios procesos celulares, tales como: la degradación de proteínas por medio del proteosoma, la reparación del ADN, la señalización mediada por receptores de membrana, y la endocitosis, entre otras (1). Las moléculas de ubiquitina pueden ser removidas de sus sustratos gracias a la acción de un gran grupo de proteasas, llamadas enzimas deubiquitinizantes (DUBs) (2). Las DUBs son esenciales para la manutención de la homeostasis de la ubiquitina y para la regulación del estado de ubiquitinación de diferentes sustratos. El gran número y la diversidad de DUBs descritas refleja tanto su especificidad como su utilización para regular un amplio espectro de sustratos y vías celulares. Aunque muchas DUBs han sido estudiadas a profundidad, actualmente se desconocen los sustratos y las funciones biológicas de la mayoría de ellas. En este trabajo se investigaron las funciones de las DUBs: USP19, USP4 y UCH-L1. Utilizando varias técnicas de biología molecular y celular se encontró que: i) USP19 es regulada por las ubiquitin ligasas SIAH1 y SIAH2 ii) USP19 es importante para regular HIF-1α, un factor de transcripción clave en la respuesta celular a hipoxia, iii) USP4 interactúa con el proteosoma, iv) La quimera mCherry-UCH-L1 reproduce parcialmente los fenotipos que nuestro grupo ha descrito previamente al usar otros constructos de la misma enzima, y v) UCH-L1 promueve la internalización de la bacteria *Yersinia pseudotuberculosis*.

Palabras clave: Ubiquitina, enzimas deubiquitinizantes, USP19, USP4, UCH-L1

INTRODUCTION

Before the 1950s, it was believed that the set of proteins within our body were building our organs as static blocks. Nowadays we know that body proteins are in a dynamic state, being synthesized and degraded extensively in a process known as protein-turnover. This conception started to be slowly accepted after pioneering experiments of Rudolf Schöenheimer. Using the stable isotope ^{15}N to label amino acids, he demonstrated that the ingested proteins are not completely metabolized and excreted, but an important portion of them is retained in body proteins (3). Further support to the concept of protein-turnover came in the mid-1950s, when Christian de Duve discovered the lysosome, an organelle able to digest intracellular proteins; thus providing a mechanism for protein degradation (4). However, protein synthesis was still perceived as the most relevant component of the protein-turnover. Most of the studies were focused on how genes are transcribed to RNA and translated to proteins, thus neglecting the area of protein degradation (5).

Nevertheless, the principles described for lysosomal degradation did not match with some observations regarding protein breakdown. A breakthrough came in the 1980s, when Aaron Ciechanover, Avram Hershko, and Irwin Rose discovered that a small protein named ubiquitin (Ub) is responsible for regulated protein degradation in cells in an energy-dependent process (6-8). The discovery of this protein degradation pathway was awarded with the Nobel Prize in 2004 by virtue of its critical importance in maintaining the internal balance of most eukaryotic cells, as well as in the control of cellular events. On top of that, this findings demonstrated that protein degradation is as important and tightly regulated as protein synthesis. Successive studies have established further non-proteolytic roles of Ub conjugation to cellular proteins. To date, we have learned that Ub is a key modulator of several signaling pathways, participating in virtually every cellular process (1).

Deubiquitinating enzymes (DUBs), a diverse group of proteases responsible for the deconjugation of Ub, are essential in the dynamics and regulation of the Ub pathway. The research work described in this document focused on the functional study of three of them: USP19, USP4 and UCH-L1.

1. THEORETICAL FRAMEWORK

This section gives an account of the fundamental concepts underlying this study, starting with the identity, mode of operation and functions of Ub and DUBs. Subsequently, USP19, USP4 and UCH-L1 are discussed in further detail, followed by the presentation of the aims pursued in this work.

1.1 POST-TRANSLATIONAL MODIFICATIONS BY UBIQUITIN AND UBIQUTIN-LIKE MOLECULES

1.1.1 Ubiquitin and ubiquitin-like molecules

The last decade of research has shown that the human genome contains less information than it was first expected, with an estimation of about 20.000 genes (9). However, we know now that the proteome, which actually represents more accurately the identity of a cell, its state, and behavior, is highly complex. This complexity is achieved through different mechanisms that modify in several different ways a gene product, such as post-transcriptional modifications (e.g. alternative splicing) and post-translational modifications (PTMs). PTMs are chemical modifications made in the structure of proteins. They alter proteins functions by regulating their activity, cellular location and interaction with other molecules. Currently there are more than 300 different types of PTMs identified; some of the most prominent ones are the addition of phosphate, methyl and acetyl groups known as phosphorylation, methylation and acetylation, respectively (10).

Ub is a small protein of 76 amino acids that operates as a PTM. After its discovery, several proteins related to it in sequence, structure or function have been described. These proteins are termed Ub-like proteins, and most of them share the tertiary structure ubiquitin fold, also termed β-grasp fold (11).

The Ub-like proteins fall into two separate classes: First, Ub-like modifiers (UBLs) are proteins that can be covalently conjugated to substrates functioning as PTMs in an analogous manner to ubiquitin. Second, Ub-domain proteins (UDPs) bear domains related to ubiquitin that cannot be processed and consequently conjugated to other substrates as modifiers. Instead, UDPs fulfil an adaptor function binding non-covalently to ubiquitin or UBLs affecting protein-interactions, enzymatic activity, stability or sub-cellular localization of the protein that contains them (12).

PTMs by ubiquitin and UBLs participate in a variety of physiological processes. In general, each one of this modifications display different mechanisms of conjugation, their own set of conjugating enzymes and adaptors, and distinct actions on their substrates. To date more than ten UBLs have been identified. The small-ubiquitin-related modifier (SUMO), and the neural precursor cell-expressed developmentally downregulated-8 (NEDD8) are the best known, but other UBLs are under intensive investigations, such as the F-adjacent transcript-10 (Fat10) and the interferon-stimulated gene-15 (ISG15).

1.1.2 The conjugation cascade

Ub and UBLs have related biochemical mechanisms for conjugation to their substrates. Precursors of ubiquitin and most of the UBLs need to be processed by deubiquitinating-enzymes (DUBs) in the case of ubiquitin, and UBL-specific proteases (ULPs) in the case of UBLs to generate mature forms. These mature forms usually terminate with a signature di-glycine sequence, which is the site of conjugation to their substrates (13,14).

The conjugation of ubiquitin to the substrates is named ubiquitination (Figure 1). It involves the sequential action of three enzymes: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a substrate specific ubiquitin ligase (E3) (15). First, the E1 forms a thio-ester with the C-terminal glycine of ubiquitin in an ATP requiring reaction causing the activation of the C-terminus of ubiquitin. This reaction allows the subsequent binding of the activated ubiquitin to the active-site cysteine of an E2 enzyme. Finally, the E3 bound specifically to its cognate substrate, binds a ubiquitin loaded-E2 and catalyzes the formation of an isopeptide bond between the activated C-terminal glycine of ubiquitin and usually a lysine (K) within the substrate (16). These PTMs are then recognized, leading to specific downstream events, which vary depending on the identity of the PTM and the location and identity of substrates. Most of the functional consequences of these PTMs are unknown (14).

Although the ubiquitin pathway keeps a hierarchical structure, its components operate in an intricate network. In humans there are two known E1s for ubiquitin (UBA1 and UBA6) (17), around 35 active E2s have been described (18) and there is an estimated of more than 600 putative E3s (19). Presumably those E1s are able to activate and transfer all the ubiquitin necessary for every E2 in the cell. One to a few E2s interact with each E3, and most E2s interact with several E3s. Likewise, many E3s interact with different substrates, and in turn some substrates can be targeted by more than one E3 (20). The accurate operation of this pathway is accomplished by means of the tight regulation of its components and high

specificity for its substrates. Many of these components are further regulated in a spatial and temporal way often by the action of PTMs, including ubiquitination itself (21,22).

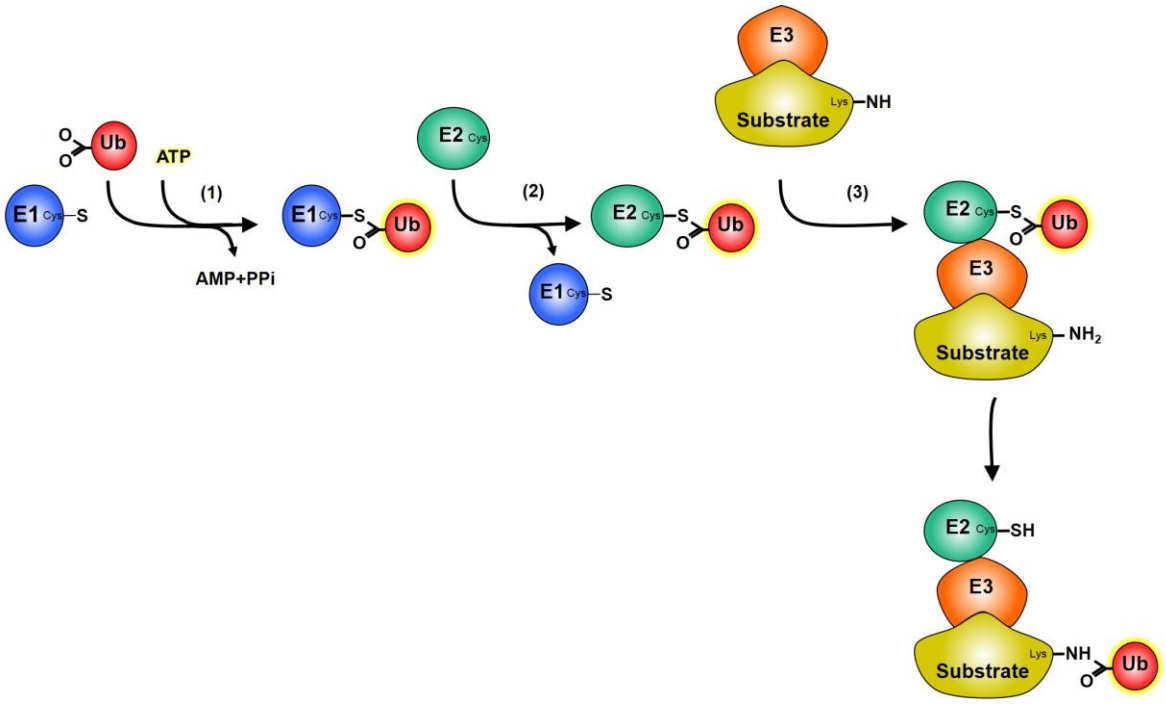


Figure 1. Ubiquitination cascade. (1) E1 forms a thio-ester with Ub in an ATP-requiring reaction, causing the activation of Ub. **(2)** Later, the Ub bound to the E1 is transferred to the active-site Cys of an E2. **(3)** The E2 cooperates with a substrate specific E3 to conjugate the Ub to the substrate through an isopeptide bond between the C-terminal glycine of Ub and a Lys within the substrate. Ub: Ubiquitin, E1: Ubiquitin activating enzyme, E2: Ubiquitin conjugating enzyme, E3: Ubiquitin ligase, Cys: cysteine, Lys: Lysine.

The outstanding diversity of the E3s among the ubiquitin pathway components hints on their large contribution to the specificity of ubiquitination. These ligases can identify within their substrates specific recognition motifs, named degrons when ubiquitination results in substrate degradation. Two major types of E3s have been defined according to the presence of either a HECT (homologous to E6AP C terminus) or a RING (Really interesting new gene)/U-box (Ufd2-box) domain. Furthermore, these groups have divergent mechanisms of ubiquitin transfer: the RING E3s function as a scaffold, binding to E2 enzymes and promoting the discharge of its bound ubiquitin directly on the substrate. In contrast, the HECT E3 enzymes are ubiquitinated by the cognate E2 to its own catalytic Cys before transferring ubiquitin to the target (23,24) (Figure 2). Besides the HECT and RING ligases, additional E3s with different structural and/or mechanistic characteristics have been identified (25,26).

Additionally, ubiquitinated substrates are recognized by any of the numerous small ubiquitin-binding domains (UBDs) harbored on effector proteins known as ubiquitin receptors, which bind non-covalently to ubiquitin signals and elicit the appropriate downstream event. Many of them have been shown to specifically recognize certain ubiquitin linkages (27).

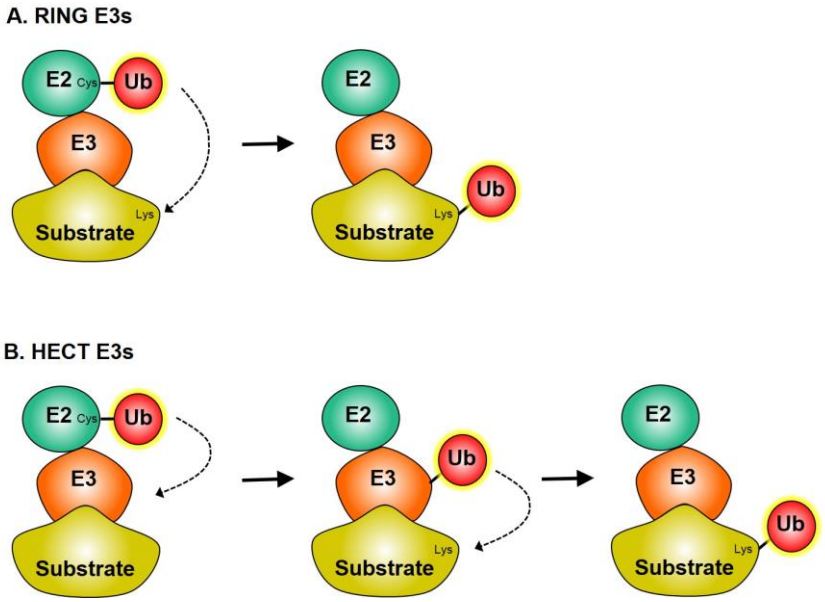


Figure 2. RING and HECT E3 ubiquitin ligases. A. RING E3s ligases promote the transfer of Ub from the E2 directly to the substrate. **B.** HECT E3 enzymes are first ubiquitinated by the cognate E2 and from there Ub is finally transferred to the substrate. Ub: Ubiquitin, E2: Ubiquitin conjugating enzyme, E3: Ubiquitin ligase, Lys: Lysine.

1.2 FUNCTIONS OF UBIQUITINATION

The presence of ubiquitin in most eukaryotic cells and outstanding high evolutionarily conservation may reflect a strong selective pressure on this protein. Ubiquitin modify a plethora of cellular proteins, being critical for cellular functions such as cell cycle progression, inflammatory response and antigen presentation among many (13).

Ubiquitin can be found conjugated to the substrates either as monomers or as chains of ubiquitin molecules with a variety of lengths and topologies. Ubiquitin chains can be linked by any of the possible ubiquitination sites within the ubiquitin itself, which are the lysines

K48, K63, K11, K33, K27, K6, K29 and the methionine M1 (28,29). The polyubiquitin chains can be homogenous if the same residue is modified during elongation, or mixed if different linkages alternate at succeeding positions of the chain (Figure 3).

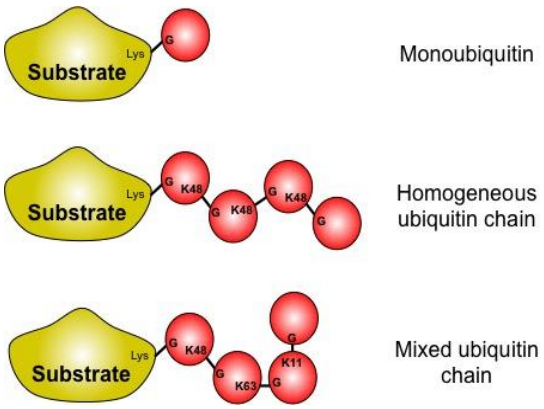


Figure 3. Topologies of ubiquitin chains. Ubiquitin can be found conjugated to the substrates as monomers (monoubiquitin), or as chains of ubiquitin molecules of different lengths. In turn, the chains can be linked by the same lysine in each ubiquitin molecule (homogenous ubiquitin chain), or by different lysines (mixed ubiquitin chain). Red circumferences: Ubiquitin, K/Lys: Lysine, G: Glycine.

Although it is known that such diversity of ubiquitin chains is important for targeting the substrates to different fates, the functional implications of each type of chain and the mechanisms used to polymerize ubiquitin chains are not clearly determined yet (30). For instance, monoubiquitination can regulate DNA repair, gene expression, receptor transport and viral budding (31). Polyubiquitin chains of at least four subunits regulate protein stability by proteasomal degradation, which is explained below in the section 1.2.1. The canonical signal for targeting substrates to proteasomal degradation are K48 linked chains, although they can produce other outcomes (32). Moreover, other non-K63 linked chains are also involved in proteasomal degradation (33). K63 chains lead instead to modification of protein function on different cellular processes such as kinase activation, DNA damage tolerance, signal transduction and endocytosis (32) (Figure 4).

1.2.1 Proteasomal-dependent degradation

The coupled action of ubiquitination and proteasomal degradation is known as ubiquitin proteasome system (UPS) (Figure 4, middle panel). The UPS is one of the major protein

degradation pathways in the cell. It degrades damaged, misfolded and short-lived regulatory proteins related to numerous cellular pathways (5).

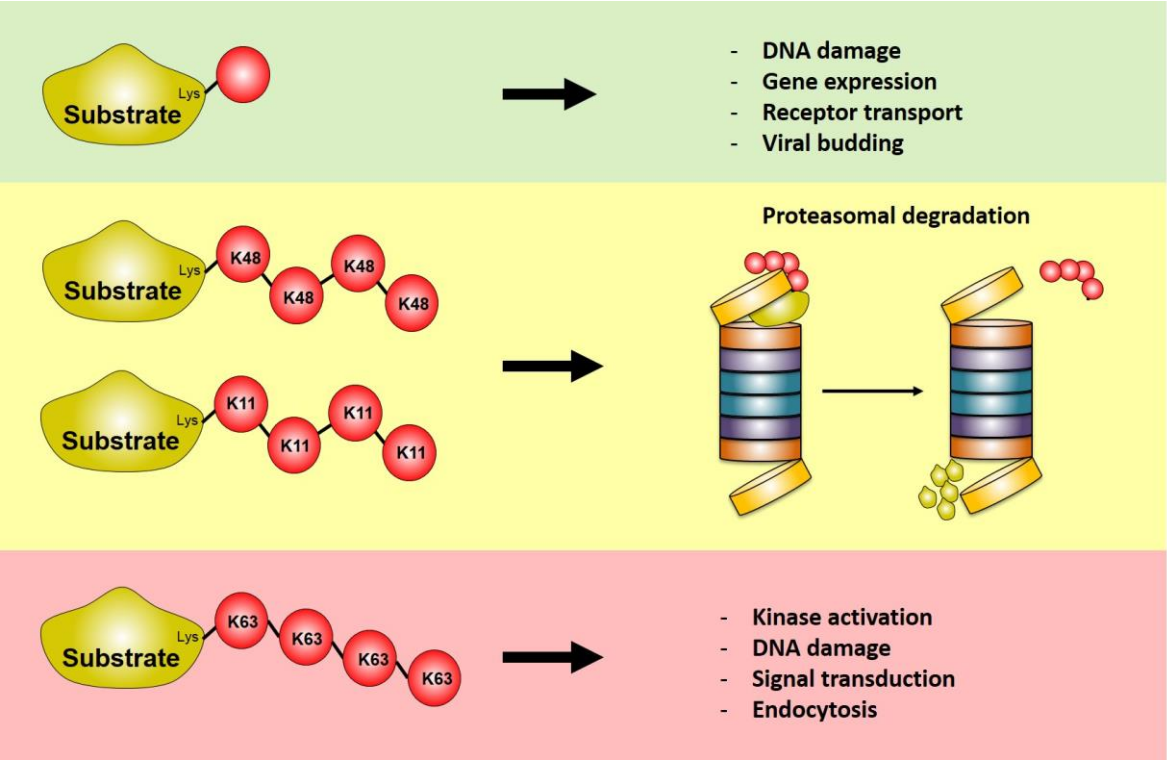


Figure 4. Functions of ubiquitination. Substrates can be destined to different fates according to the length and topology of Ub chains. Red circumferences: Ubiquitin, Lys/K: Lysine.

In the UPS pathway, ubiquitination targets the substrate for degradation within the 26S proteasome, a large and complex enzyme composed of two sub-complexes, a 20S core particle (20S CP) and a 19S regulatory particle (19S RP) (20) (Figure 5). The 20S CP is a 28-subunit hollow cylinder that contains inside a catalytic chamber where the internalized substrates are degraded. The subunits are arranged on four stacked rings. Each ring contains seven subunits; the outer rings are composed of α subunits, while the inner rings possess β subunits, resulting in the two-fold symmetric structure $\alpha_{1-7}\,\beta_{1-7}\,\beta_{1-7}\,\alpha_{1-7}$ (20,34). The β-rings are responsible of the substrate degradation, three subunits of each β-ring harbor active catalytic sites with different cleavage site preferences facing towards the inside of the 20S core particle, ensuring an efficient protein degradation (34). The α-rings aid to control the passage of the substrates and degradation products into and out the catalytic chamber, forming a network with their N-termini that acts as a narrow pore. The

α-subunits also possess cavities or pockets that participate on the binding with the 19S RP (35,36).

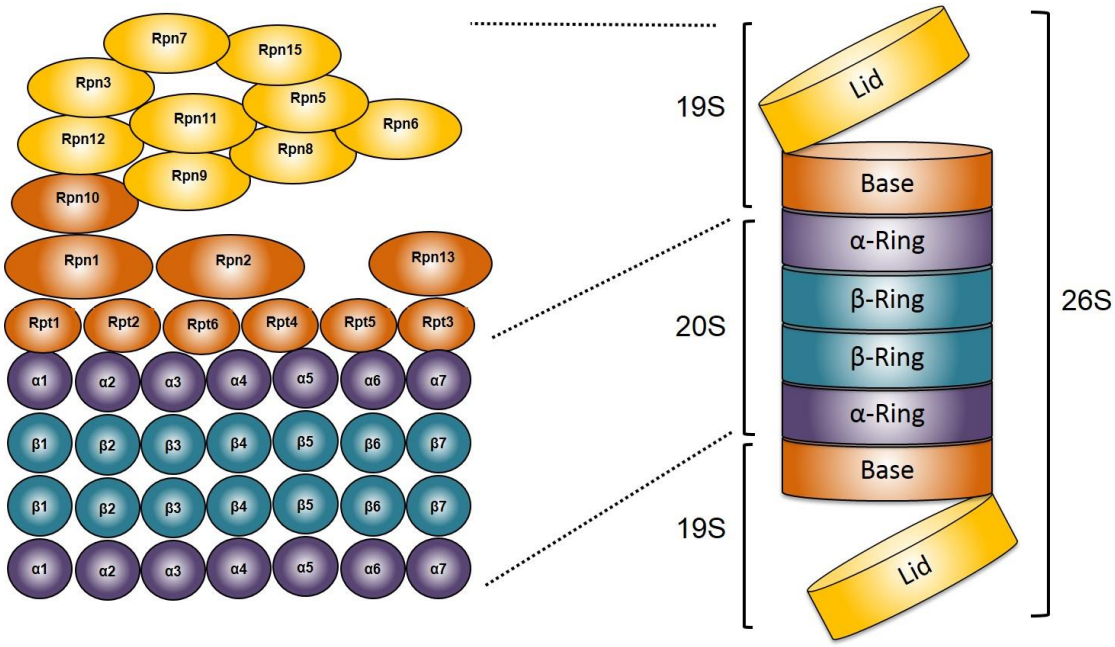


Figure 5. Representation of the proteasome structure. On the left is shown a schematic representation of the components of the holoenzyme 26S proteasome. On the right is presented the subunit composition of each complex (19S and 20S) of the 26S proteasome.

The 19S RP is in turn composed of two parts: the lid and the base (Figure 5). This sub-complex flanks each extremity of the 20S CP controlling the access of the substrates to the internal chamber, and removing the ubiquitin from the substrate committed to degradation (37). The base contains six AAA+ (ATPase associated with a variety of cellular activities)-ATPases, named Rpt1-Rpt6 in yeast. They form a pseudo-symmetrical ring and use the ATP energy to pull, unfold and translocate the substrate into the catalytic chamber (38). Moreover, the ATPases are critical for the RP-CP complex formation and the opening of the CP pore as their C-termini insert into the α-subunit cavities in presence of ATP (39,40). Additionally, the base contains four non-ATPase subunits: the scaffolding proteins Rpn1, Rpn2 and the ubiquitin receptors Rpn10 and Rpn13, from which Rpn10 is essential to connect the lid with the base (35). The additional ubiquitin receptors Rad23, Ddi1 and Dsk2 are recruited to the base through interactions with Rpn1, which also binds the non-essential deubiquitinated protein (DUB) (a class of enzymes that will be discussed in section 1.3) Ubp6 (USP14 in mammals) (41). Ubp6 can act removing the ubiquitin signal from the substrate before it is committed to degradation, antagonizing substrate breakdown (35).

The lid consists of nine non-ATPase proteins (Rpn3, Rpn5-Rpn9, Rpn11, Rpn12 and Rpn15). Rpn11 (POH1 in mammals) is a DUB that deubiquitinates the substrate only in the presence of ATP, which indicates that, in contrast to Ubp6, Rpn11 acts only on the substrates committed to degradation. Furthermore, the DUB action of Rpn11 speeds up the degradation of the substrates besides helping on ubiquitin recycling (42). Rpn5 also stabilizes the interface between the lid and the RP (35).

1.3 DEUBIQUITINATING ENZYMES

Ubiquitination is a dynamic process. Ubiquitin can be deconjugated by the action of a large group of proteases named deubiquitinating enzymes (DUBs), that cleaves specifically the link between ubiquitin and the substrate (2,43). This ability empowers the DUBs to participate in every process where ubiquitin is involved. DUBs can perform proofreading functions by removing ubiquitin signals before the substrate is committed to proteasomal degradation, leading to its rescue. Alternatively, other DUBs release ubiquitin from substrates engaged to degradation, promoting ubiquitin recycling and facilitating the entry of the substrate into the catalytic chamber of the proteasome. DUBs also provide a way to modulate non-proteolytic ubiquitin-dependent processes. Additionally, these enzymes are used in the processing of ubiquitin precursors, which are transcribed as chains of ubiquitin joined to other ubiquitin molecules or ribosomal proteins (2,43,44) (Figure 6).

About 98 functional DUBs are predicted to be encoded in the human genome. According to their mechanisms of catalysis they are either cysteine or metallo-proteases. Furthermore, based on the similarity of their ubiquitin protease domains they have been grouped into six families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor proteases (OTUs) and monocyte chemotactic protein-induced protein (MCPIP) are cysteine-proteases, whereas the Josephins and JAB1/MPN/MOV3 metalloenzymes (JAMMs) are metallo-proteases (45).

To date, most of the putative DUBs that have been tested display ubiquitin protease activity in vitro and many evidences support their specificity: First, they are very diverse; second, they appear to regulate a limited number of proteins and pathways; third, structural studies have demonstrated that some of them experience conformational changes once they are bound to their substrate in order to display catalytic activity; fourth, some of these enzymes have been found to be included in multi-complexes, what may help them localizing their targets and driving the catalysis (43,44,46); fifth, they contain

additional domains that could work as ubiquitin binding domains and protein-protein interaction domains facilitating the recognition and binding to the substrates and multi-complexes (44,47,48).

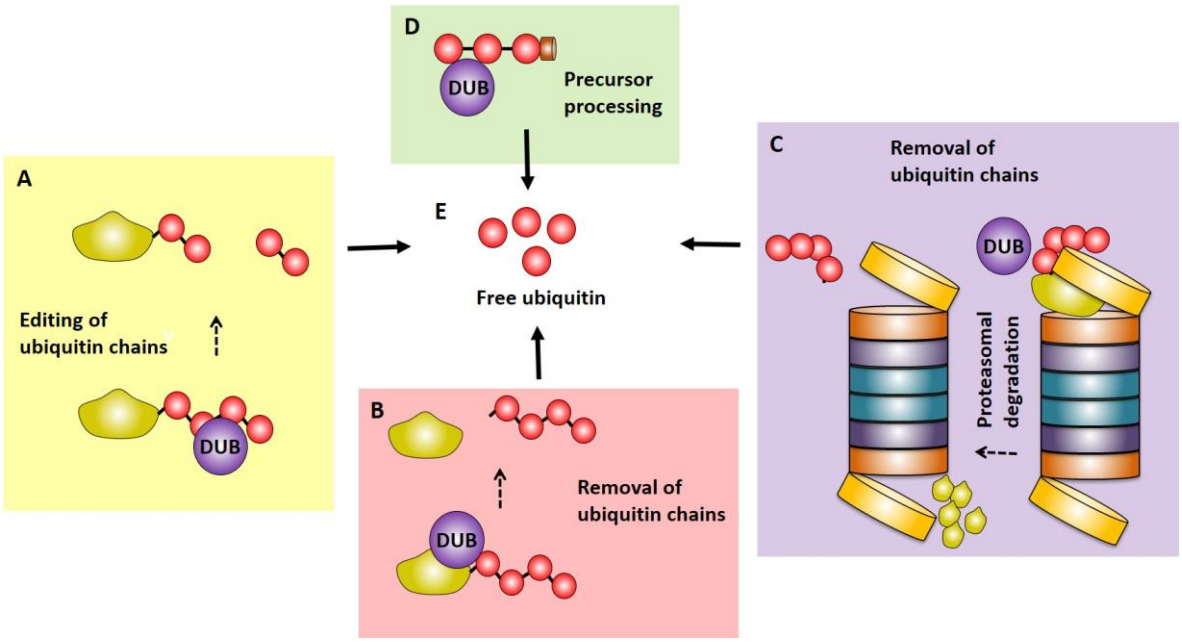


Figure 6. Function of DUBs. A. and B. DUBs can deconjugate ubiquitin moieties with different purposes: They can edit or remove ubiquitin chains rescuing substrates for degradation or modifying their fates in other pathways. **C.** DUBs release Ub from substrates engaged to degradation facilitating their entry into the proteasome. **D.** DUBs also process ubiquitin precursors. **E.** Through all this actions DUBs aid on the recycling of ubiquitin. Red circumferences: Ubiquitin, DUB: Deubiquitinating enzyme, yellow form: substrate.

Although several DUBs have been thoroughly studied, the mode of regulation, targets and physiological role of most of them is far from being completely deciphered. In general, DUBs can be regulated at transcriptional level, by post-transcriptional modifications, allosteric interactions and subcellular localization (2,48). A better comprehension of their operation comes from the identification of binding partners that may act for instance as substrates, regulators or scaffolds (47). The general aim of this work is to contribute to the functional characterization of three DUBs: the ubiquitin specific protease 4 and 19 (USP4 and USP19, respectively), and the ubiquitin carboxy-terminal hydrolase L1 (UCH-L1).

1.3.1 Ubiquitin specific protease 19 (USP19)

USP19 belongs to the largest family of DUBs, the USPs. This enzyme of 150 kDa possesses a canonical USP domain harboring the catalytic triad that comprises the residues C506, H1157 and D1189, and a MYND domain (Myeloid translocation protein 8, Nervy, and DEAF-1) that could mediate protein-protein interactions. Close to the N-terminal region of USP19 lie two p23-like CHORD and Sgt1 (p23/CS) domains that might exert chaperone functions although their exact functions in USP19 are still unknown. Additionally, this is one of the few DUBs with an identified transmembrane domain (TMD). This domain, located close to the C-terminus, anchors the protein to the ER membrane (49) (Figure 7).

USP19 has DUB activity *in vitro* and shows preference towards K63-linked ubiquitin chains over K48-linked chains, which may indicate its involvement in non-proteolytic signaling events (50). Although USP19 has been implicated in diverse pathways, its physiological role and substrates have not been clearly elucidated. This enzyme was first identified from rat skeletal muscle and subsequently found to be strongly induced during muscle atrophy, however the mode of regulation and substrates targeted in this process remain unknown (51).

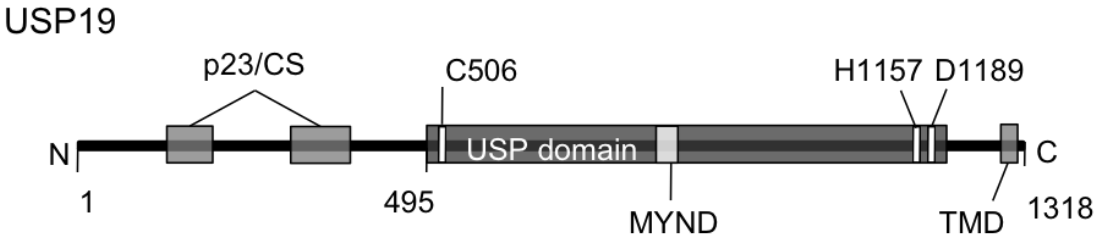


Figure 7. Schematic representation of the protein domains of USP19. The N-terminal region of USP19 harbors two p23-like CHORD and Sgt1 (p23/CS) domains. The MYND domain (myeloid translocation protein 8, Nervy, and DEAF-1) and the catalytic triad (comprising the residues C506, H1157 and D1189) are found within the USP domain as indicated. The transmembrane domain (TDM) is located close to the C-terminus

Its DUB activity has been implicated in the regulation of some cellular functions. The active enzyme stabilizes the ubiquitin ligase Kip1 ubiquitination-promoting complex 1 (KPC1), which promotes the proliferation of rat fibroblasts (52). During hepatitis C virus (HCV) infection, re-localization of USP19 to the virus replication compartments hampers its ability to rescue proteasomal substrates. This is dependent on the interaction of USP19 with

the non-structural proteins NS5A and is likely to mediate the cell-proliferation inhibitory properties of NS5A since infection with a recombinant virus lacking the USP19 interaction site failed to decrease cell growth (53). Interestingly, non-catalytic functions of USP19 have been reported. The inactive enzyme stabilizes the cellular inhibitor of apoptosis protein 1 and 2 (c-IAP1 and c-IAP2, respectively) (54). Furthermore, our group previously described a non-catalytic effect of USP19 in the rescue of endoplasmic reticulum associated degradation (ERAD) substrates (49).

Similar to many DUBs, USP19 can regulate its own ubiquitination (54), but additional mechanisms involved in the transcriptional and post-translational regulation remain largely unknown. Splice variants of the human USP19 gene code for at least four isoforms, some of which lack the TMD (49). In addition, USP19 is phosphorylated by the protein kinase ataxia telangiectasia mutated (ATM) or ataxia telangiectasia and Rad3-related protein (ATR) in response to DNA damage (55).

Hence, albeit some cellular and physiological roles, and substrates of USP19 have been identified, its mode of operation and regulation remain largely unknown. In order to contribute to the knowledge of these aspects we aimed to identify interacting partners of this DUB, and subsequently to study the significance of the interactions that could potentially unveil details on how USP19 operates.

1.3.2 Ubiquitin specific protease 4 (USP4)

USP4 belongs to the USP family. This DUB of 963 amino acids long harbors in the N-terminal a domain present in ubiquitin specific proteases (DUSP) and a ubiquitin-like (UBL) domain, both with unknown function. Within the USP domain, it contains the catalytic residues C311 and H880, another UBL domain close to the catalytic cysteine residue that has auto-regulatory functions (56), and a nuclear localization sequence (NLS) (Figure 8).

USP4 regulates various cellular functions through the stabilization of substrates and the regulation of protein activity within signaling pathways by cleaving K48- and K63-linked ubiquitin chains respectively (57). For instance, USP4 cleaves K63-linked Ub chains from TNF(tumor necrosis factor)-receptor associated factor 2 (TRAF2), TRAF6 and transforming growth factor-β-activated kinase 1 (TAK1) to downregulate the tumor necrosis factor α (TNFα)-mediated activation of the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), which is downstream of those three proteins (58-60). Among the broad range of cellular responses regulated by NF-κB, the action of USP4 has

been linked with migration of lung cancer cells and immune response (59,60). Another example is the stabilization of the p53 inhibitor ARF-binding protein 1 (ARF-BP1), which might regulate apoptosis, senescence, and oncogenic transformation (61). USP4 also participates in the regulation of transforming growth factor β (TGF-β) signaling, important in embryogenesis and tissue homeostasis, by stabilizing the TGF-β type I receptor (TβRI) and thus inducing the signaling of the TGF-β pathway with possible pro-tumorigenic effects (62).

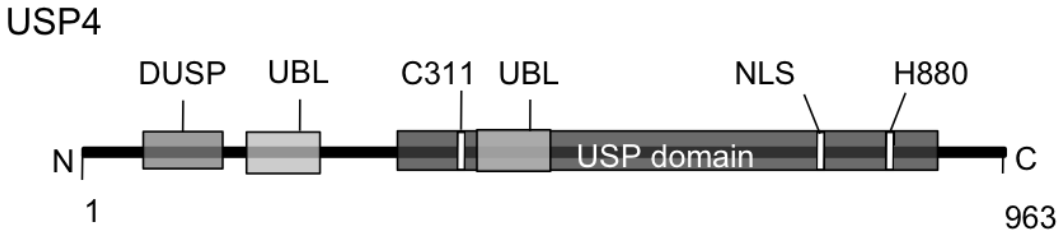


Figure 8. Schematic representation of the protein domains of USP4. The N-terminal region of USP4 harbors a domain present in ubiquitin specific proteases (DUSP) and a UBL domain. Within the USP domain contains the catalytic resides C311 and H880, another UBL domain and a nuclear localization sequence (NLS).

As the information presented above suggest, USP4 has potential roles in cancer. Additional evidences show that its mRNA levels are elevated in various types of cancers (61). Furthermore, its overexpression promotes tumorigenesis in mice (63). Nevertheless, USP4 role in cancer is controversial. USP4 dowregulates the NF-κB pathway and its knockdown activates Wnt signaling, suggesting that USP4 represses signaling pathways that can have tumorigenic activity (59,64). In addition its expression levels were decreased in lung cancer cells (65). Therefore, some authors suggest that its role in cancer might be dependent on the biological context.

In order to understand better the functions of USP4, we tried to identify unknown interacting partners of this DUB in a yeast-two-hybrid screen, and to further study the most relevant findings.

1.3.3 Ubiquitin carboxy-terminal hydrolase L1 (UCH-L1)

UCH-L1 is a member of the UCH family of DUBs. UCH-L1, also known as PGP 9.5, is 223 amino acids long and possesses a UCH homology domain harboring the catalytic residues C90 and H161 (66,67). This DUB has no accessory domains identified (Figure 9).

UCH-L1 participates in the maintenance of the pool of free ubiquitin through the processing of ubiquitin precursor proteins (67), the stabilization of monoubiquitin independently of its catalytic activity (68,69), and the generation of monomeric ubiquitin by cleaving a peptide bond at the ubiquitin C-terminal glycine (67). The analysis of the crystal structure of UCH-L1 shows that the active site is hidden behind a small aperture and a loop that covers it, making it not easily accessible. This suggests that UCH-L1 can either cleave only small moieties or must undergo conformational changes to expose the catalytic site to its substrates (70). Additionally, UCH-L1 might act also as a ubiquitin ligase, recognizing α-synuclein as its substrate (71).

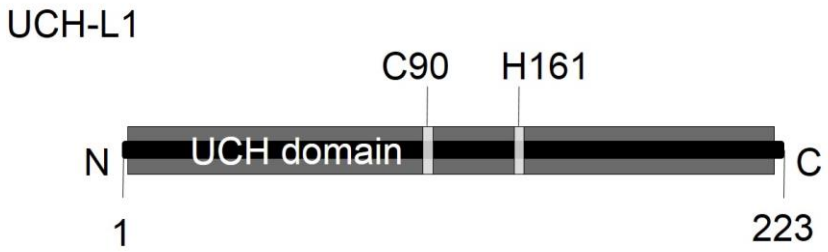


Figure 9. Schematic representation of the protein domains of UCH-L1. UCH-L1 possesses a UCH homology domain harboring the catalytic residues C90 and H161.

Although the functions of UCH-L1 are not clearly defined yet, and its substrates have been hard to identify, this DUB is implicated in important human diseases. UCH-L1 is one of the most abundant proteins in the brain, and is present in other tissues at lower levels, such as testis and ovaries (66,72). Several lines of evidence suggest that it is related to neurodegenerative conditions. Its spontaneous mutation in mice leads to neuronal dysfunction and neurodegeneration (73,74). In humans UCH-L1 has been implicated in Alzheimer´s and Parkinson´s diseases (71,75-77). Recent studies suggests that the human papillomavirus (HPV) recruits UCH-L1 on basal keratinocytes to suppress the adaptative immune response by removing K63 ubiquitin chains from the tumor-necrosis factor receptor-associated factor-3 (TRAF3) and promoting the degradation of the NF-KB essential modulator (NEMO) (78). In addition, UCH-L1 has been found to be over-expressed in several human cancers (79). Multiple evidences show that UCH-L1 is implicated in the regulation of phenotypic properties associated with malignant growth such as epithelial-to-mesenchymal transition (EMT), cell motility and invasion (80,81). Conversely, other studies report that UCH-L1 is downregulated by methylation in nasopharyngeal and breast cancer, what prevents its DUB activity to stabilize the tumor suppressor p53 (82,83).

1.4 AIMS

The information presented above expose the need to discover unknown substrates and improve our knowledge of the biological roles of USP19, USP4 and UCH-L1. In order to contribute to the functional characterization of these DUBs, the present work pursued the following specific aims:

Aim 1. Identification of new interacting partners of USP19, and study of the biological significance of the interactions.

Aim 2. Identification of new interacting partners of USP4, and study of the biological significance of the interactions.

Aim 3. Study of the molecular events by which UCH-L1 regulates membrane proximal events associated with cell adhesion and bacteria internalization.

Due to the implication of these DUBs in important cellular pathways, many of them cancer-related, this study not only contributes to their functional characterization, but can further provide some insights on how they could be manipulated to treat human diseases in which they might be involved. DUBs are an attractive potential therapeutic target and there has been an increasing interest from the pharmaceutical industry to target these enzymes in drug development (45).

2. METHODOLOGY

This section contains a brief description of the main techniques used during the experimental part of this work, and the general principles behind them. Additional information about the protocols followed are found in the Materials and Methods sections of the respective papers (Appendix A, B, C and D).

2.1 YEAST-TWO HYBRID SCREEN (Appendix A, B and C)

Given that the majority of proteins have to interact with other ones to exhibit a proper biological function, the study of interacting partners is fundamental for the understanding of protein functions. There is a wide variety of methods and approaches to study protein-protein interactions. Yeast-two hybrid screen is a versatile and sensitive method, able to detect relatively weak and transient physical protein interactions *in vivo*.

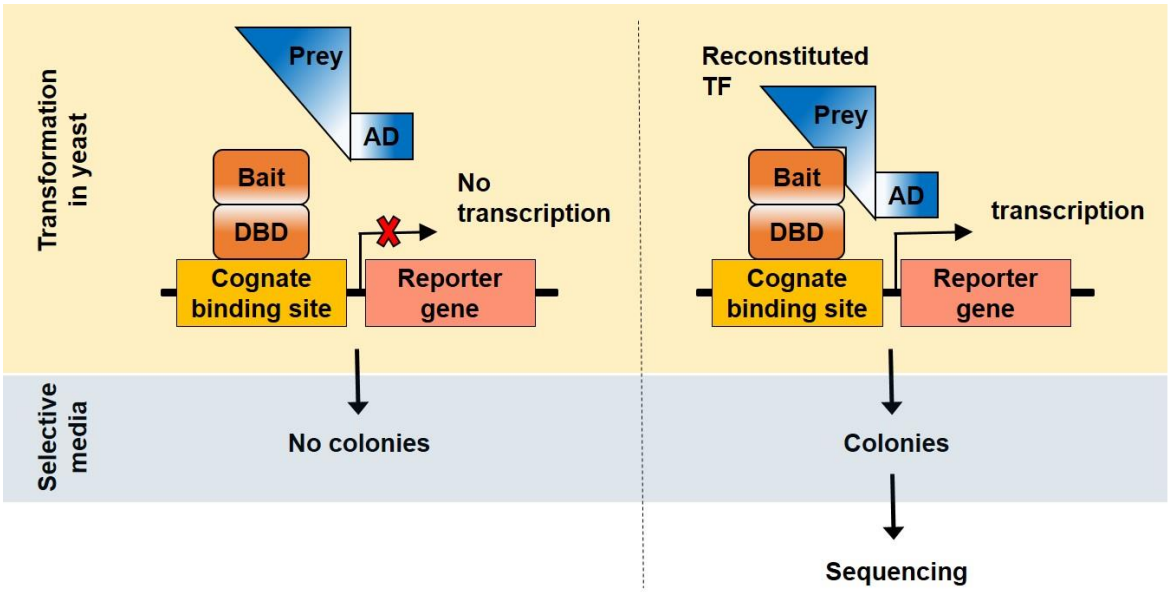


Figure 10. Principle of the yeast-two hybrid screen. Two proteins under evaluation, called bait and prey, are fused respectively with the DNA binding domain (DBD) and the activating domain (AD) of a transcription factor (TF), and transformed into yeast. The interacting proteins come to close proximity reconstituting the TF (right panel), and activating the expression of a reporter gene that allows the yeast to grow on selective media. The interacting partners are identified by sequencing the corresponding expression vectors in the selected yeast colonies.

This assay is performed in yeast strains carrying a reporter gene that is activated upon reconstitution of its functional transcription factor (TF) when the two proteins under evaluation interact physically. This is possible because the TF, in this case Gal4, is divided into two separate parts: the DNA binding domain (DBD) and the activating domain (AD). The protein of interest, or bait, is fused with the DBD. The protein that binds to the bait, known as prey, is fused to the AD. Each construct is cloned in a different yeast expression vector, and is subsequently transformed into yeast. If the prey happen to be a binding partner of the bait, upon interaction, the DBD and the AD are brought in close proximity and the TF is reconstituted. This activates in consequence, the expression of the reporter gene. Plating the transformed yeast co-expressing the bait and the prey on selective media makes possible to screen for individual clones that express the reporter gene, indicating interacting protein pairs (84). The identity of the interacting partners is then obtained by sequencing the corresponding expression vectors in the selected yeast colonies (Figure 10).

Here we used as bait the unique N-terminal regions of USP19 (Appendix A and B) and USP4 (Appendix C) since they exclude the USP consensus domain. These regions were cloned in-frame with the Gal4 DBD and Myc tag in the yeast expression vector pGBKT7 (Clontech), which was transformed in a yeast strain. As prey we used the Matchmaker™ pre-transformed cDNA HeLa library (Clontech) (Appendix A, B and C). In this way the technique enables the sampling of several potential binding partners.

Although the high sensitivity of this technique is an advantage, it produces a high rate of false positives. Additionally, the interactions observed could be a product of conditions created by the yeast model rather than the reflection of events produced in the real context. For these reasons it is indispensable to validate the obtained results using other techniques. It is noteworthy that this technique only allows the identification of pairs of proteins interacting directly (85,86).

2.2 CO-IMMUNOPRECIPITATION (Appendix A, B, C and D)

Co-immunoprecipitation is another technique to study protein-protein interactions. It is one of the most commonly used methods for verification of interactions. To start, an antibody recognizing specifically the protein of interest is added to a cell lysate. After a period of incubation, protein G attached to sepharose beads is used to capture the antibody associated to the protein of interest and its binding partners (Figure 11). The beads are then washed to remove all the proteins not binding specifically to the antibody, and boiled to elute the beads, antibody and proteins associated. The bounded proteins are visualized by

western blot (section 2.2). This technique is sensitive to transient interactions, and has the advantage of being performed *in vivo*, providing the conditions to find the proteins on their native conformation and with the modifications that they might need to interact with their binding partners (85,87). However, it is convenient to verify if the interaction is taking place in the cell, and not as a consequence of cell lysis, using other assays e.g. evaluating its co-localization or the effect of mutants on the interaction. Furthermore, it is important to consider that the interactions identified with this technique can be direct or proceed through another protein that links both of them (86).

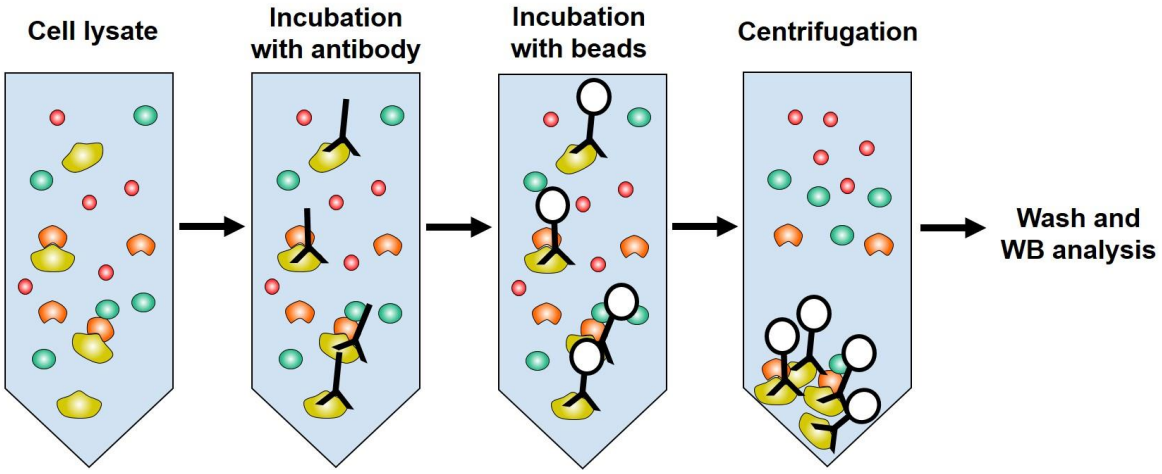


Figure 11. Principle of co-immunoprecipitation. A cell lysate is incubated with an antibody that specifically recognizes the protein of interest. Later, a protein G attached to sepharose is added to capture the antibodies. After incubation the beads are precipitated by centrifugation and the supernatant removed. The beads are washed several times with a suitable buffer and re-suspended in loading buffer to be analyzed by western blot.

2.3 CROSSLINKING (Appendix D)

Although co-immunoprecipitation is a technique suitable to study protein-protein interactions under physiological conditions, the stringent wash conditions applied to avoid false positives can disrupt and make invisible labile and transient interactions (88), which occur very often in *in vivo* conditions to facilitate signaling or metabolic functions. One approach that makes possible the study of those kind of interactions is the employment of cross-linking reagents in intact cells with the purpose of freezing those bindings in place and sizing them in a complex sufficiently stable for isolation and characterization by downstream methods such as Western blot, immunoprecipitation and mass spectrometry.

This results in an increased sensitivity to detect weak protein-protein interactions (87). Cross-linking reagents are molecules that contain two or more reactive end groups that bind specific functional groups of proteins. If two proteins physically interact with each other, those reagents will form covalent bonds between them. After the fixation step, highly stringent conditions can be used during cell lysis and enrichment, minimizing the risk of identifying false positives (89).

Several cross-linkers varying in length, reaction groups and other properties are available. One of the most commonly used is formaldehyde. It reacts primarily with the residues of the basic amino acid lysine (89). Additionally, it is highly permeable towards cell membranes enabling a rapid cross-linking in the intact cell. The small size of formaldehyde ensures that only closely associated proteins will be cross-linked. This is important because this technique could detect any protein in close vicinity which may not be in direct contact (87,88).

2.4 PULL-DOWN ASSAY (Appendix C)

The pull-down assay is a technique, analogous to co-immunoprecipitation, used to discover or confirm physical protein-protein interactions. In contrast to co-immunoprecipitation, the interactions are tested in *in vitro* conditions, and a bait protein is used instead of an antibody to capture the protein complexes. The bait protein usually is fused to a protein-reactive tag, such as glutathione S-transferase (GST) or poly-histidine (His), to facilitate the collection of the protein complexes.

To start, the recombinant fusion tagged protein or bait is generated. This can be done by expressing and purifying it from an appropriate expression system such as bacteria. Subsequently, the bait is incubated with the putative prey proteins. The source of the prey proteins varies according to the objective of the experiment, they can be either a cell lysate or recombinant purified proteins. The addition of an affinity ligand corresponding to the tag used (i.e. glutathione in the case of GST) coupled, for instance, to agarose or sepharose beads allows the pull-down and collection of the complexes by centrifugation. These complexes are then washed, eluted and analyzed by standard protein detection methods such as western blotting (90).

The pull-down assay is useful when other techniques are not sensitive enough to detect an interaction thanks to the possibility of concentrating the tested proteins in the production and purification step. It also allows the study of interacting proteins for which

antibodies have not been produced or where the antibodies available interfere with the interaction under investigation. Additionally the *in vitro* conditions allows to control the conditions of the interaction. This helps for the identification of additional factors needed for an interaction to occur, such as co-factors or accessory proteins. Some disadvantages of the technique are the possible failure to find or confirm an interaction when it needs unknown factors or conditions not present in the incubation step. It is worth to notice that it can also become more laborious as it requires the production and purification of at least the bait.

2.5 WESTERN BLOTTING (Appendix A, B, C and D)

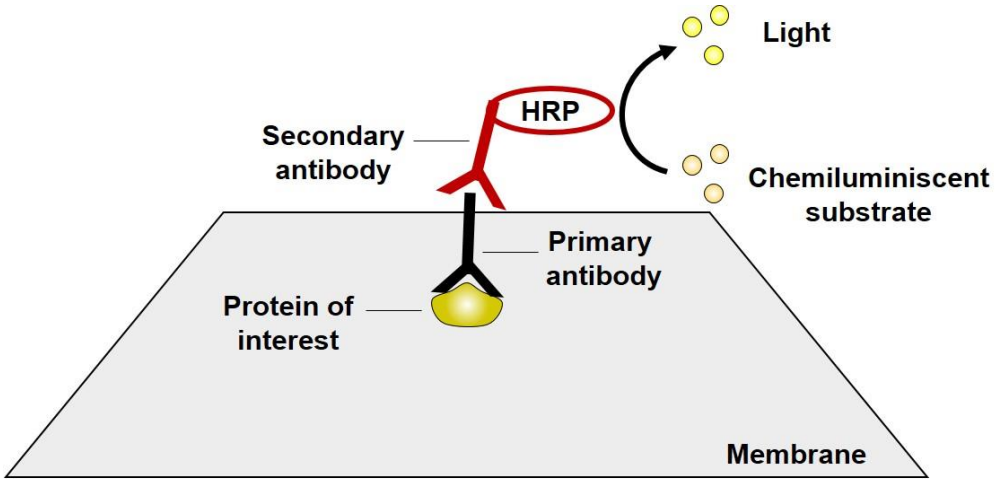


Figure 12. Chemiluminescent detection method in Western blotting. The membrane containing proteins previously separated by mass and charge, is blocked and incubated with a primary antibody recognizing specifically the protein of interest. A secondary antibody labeled with the enzyme horseradish peroxidase (HRP) recognizes the primary antibody. After several washes, a chemiluminescent substrate is added. The HPR when combined with the substrate produces light as byproduct, which is documented with X-ray films.

Western blotting is a technique for protein analysis that enables the detection of specific proteins of interest in the midst of a complex protein mixture through the use of antibodies. First, the proteins on the sample are separated by mass and charge using SDS-PAGE gel electrophoresis. After this, the separated proteins are transferred across an electric field onto a membrane, generally of nitrocellulose or polyvinylidene difluoride (PVDF). Next, the membrane is blocked to prevent nonspecific binding of antibodies to the surface of the membrane, and the protein of interest is detected using an antibody that recognizes it specifically. We used an indirect detection method that amplifies the signal, where a

secondary antibody labeled with the horseradish peroxidase enzyme (HRP) is used to detect the primary antibody attached to the protein (Figure 12). To visualize the protein bands in the membrane, we added a chemiluminescent substrate that, when combined with the enzyme produces light as a byproduct, which we documented with X-ray films. The intensity of the bands correlate with the amount of protein present in the sample. In that way, this technique produces qualitative and semi-quantitative data about the protein of interest.

2.6 TRANSFECTION (Appendix A, B, C and D)

Transfection is the name of a variety of methods used to introduce nucleic acids into a cell to modify it genetically. These methods facilitate the study of gene and protein function and regulation by enhancing or inhibiting expression of specific genes, besides allowing the generation of recombinant proteins in mammalian cells.

The selection of the transfection method depends on the cell type and purpose of the experiments. Optimal characteristics are high transfection efficiency, low cell toxicity, minimal effects on cell physiology, and simplicity. From the variety of transfection methods available, two chemical methods were employed in this work: calcium phosphate and cationic polymer. In principle, these chemical methods use positively charged chemicals that make complexes with the negatively charged nucleic acids. The positively charged complexes are attracted and pass through the negatively charged cell membrane using unknown mechanisms, possibly involving phagocytosis and endocytosis.

The transfection efficiency of these methods varies according to the cell type, and in general terms is low compared with virus-mediated methods. Nevertheless, the chemical methods provide many advantages over the virus-mediated methods such as no size limitation on the packaged nucleic acid, no extra-carrying DNA, and avoiding the use of vectors (91).

2.7 FLUORESCENCE MICROSCOPY (Appendix B and D)

Fluorescence microscopy is a special type of light microscopy. It allows the visualization of fluorescent objects of interest in the specimen under study, providing the advantage of a high contrast between the objects and a dark background. If the objects do not possess natural fluorescence, they can be labeled with a vast selection of fluorescent compounds known as fluorophores. In the present work, our cellular objects or proteins of interest were

labeled with fluorophores (e.g. fluorescent proteins or stains), as described in the respective appendices.

Fluorophores emit light when excited with the right wavelength. Absorption of light energy triggers the movement of an electron towards a higher energy level, going from a ground- to an excited-state. Eventually, the electron relaxes to a lower level and the fluorophore returns to its ground state shedding part of the absorbed energy, in the form of light. The emitted light has a longer wavelength than the absorbed one. The efficiency of the fluorophore, as well as its excitation and emission wavelengths depend largely on its chemical structure (92).

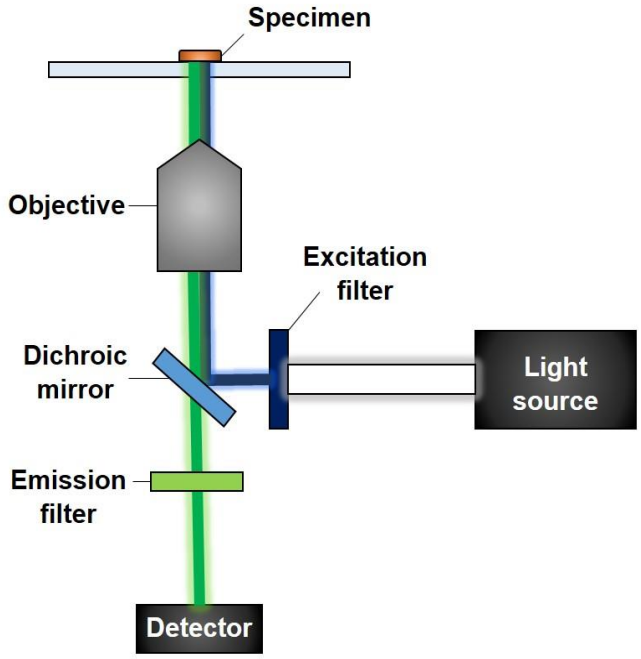


Figure 13. Principle of fluorescence microscopy. From the light source, the excitation light goes through an excitation filter that only lets through light with the desired wavelength. The dichroic mirror reflects this light (blue line) towards the objective, which focuses the light on the sample and collects the emitted light (green line) from it. The dichroic mirror allows the emitted light to pass, which is the only one that can go through the emission filter. The emitted light is directed towards an interface (detector) that allows the visualization of the image.

The difference between the exciting and emitted wavelengths, is known as the Stokes shift. This property allows to sort out effectively the emitted light and generate a visible image in the eyepieces or a camera. This kind of microscope employs incident light. First,

the excitation light goes through an excitation filter that only lets through light with the desired wavelength. A dichroic mirror reflects this light and directs it to the objective, which in turn focusses the light on the sample, and then collects the emitted light from it. As the emitted light has a higher wavelength than the excitation light, this time the dichroic mirror allows it to pass. Subsequently, the emitted light goes through an emission or barrier filter that prevents any other light different than the emitted one to pass (Figure 13). Finally, the emitted light is directed towards an interface, like the eyepieces or a camera, which allows the visualization of the image (92).

2.8 CONFOCAL MICROSCOPY (Appendix D)

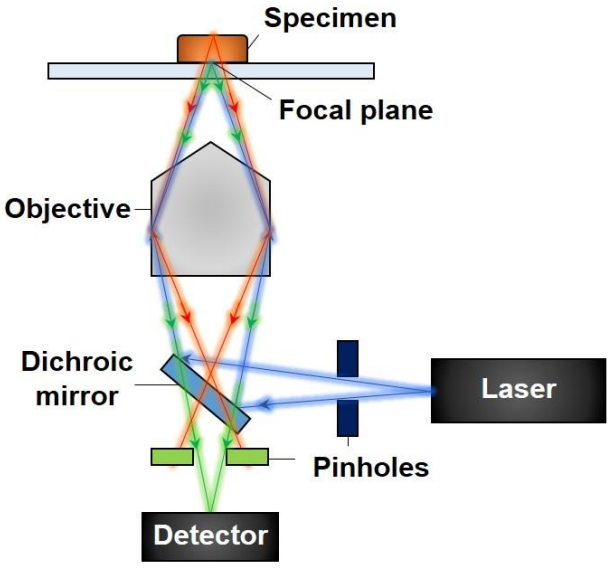


Figure 14. Principle of confocal microscopy. The confocal microscope uses point illumination (e.g. a laser) and small pinholes in a screen, to block the scattered light, with the aim of providing high quality images, with fine detail and without out-of-focus light. Imaging is achieved by scanning serially focal spots in the X-Y plane across the specimen that are collected to a computer, which builds up the image.

Confocal microscopy offers several additional advantages to study our specimens prepared for fluorescence microscopy. In contrast to fluorescence microscopy, it provides higher quality images, with fine detail, without out-of-focus light and, in result, more contrast. The confocal method of image formation creates virtual slices or planes within the specimen, in two dimensions (X and Y). Acquisition of sequential thin virtual slices along the

third dimension (Z-stacks) through the specimen allows the reconstruction of a three-dimensional image in a computer (93).

Confocal microscopy achieves this by illuminating and observing a single diffraction limited spot at a time. In conventional fluorescence microscopy the entire specimen is simultaneously illuminated; even if the brightest and highest intensity is at the focal point of the objective lens, it results in background noise that decreases the quality of the image. The confocal, instead, uses point illumination that often comes from a laser; this means that the excitation beam focuses on a small spot inside the specimen. Additionally, the confocal increases the quality of the image by blocking the scattered light. The conventional fluorescence microscope detects all the resulting fluorescence, including a large unfocused background part. In the confocal, a small pinhole in a screen allows only the emitted light from the desired focal spot to pass through, while the screen blocks any scattered light, which is the out-of-focus fluorescent light (Figure 14). On the other side of the pinhole, the confocal light is detected usually by a photomultiplier tube. Imaging is achieved by scanning serially focal spots in the X-Y plane across the specimen. All these points are collected to a computer that builds the image, which is finally displayed on a video monitor.

3. RESULTS

3.1 USP19: ITS ROLE IN HYPOXIA AND NOVEL INTERACTING PARTNERS

3.1.1 The SIAH E3 ligases interact with and regulate USP19 (Appendix A)

In order to gain insight into the functions of USP19 we performed a yeast-two-hybrid screen to identify some of its interacting partners. We identified ten different USP19 interacting proteins in the screen, of which the E3 ubiquitin ligases seven in absentia homolog 1 and 2 (SIAH1 and SIAH2, respectively) were the most recurrent hits (Table 1 in Appendix A).

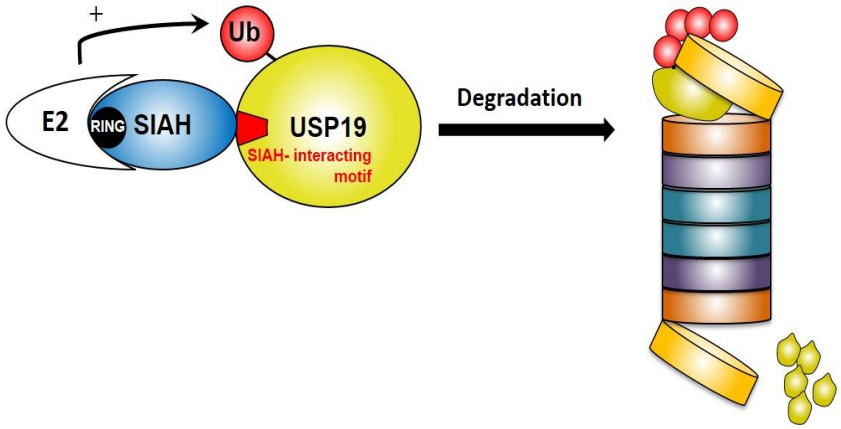


Figure 15. The SIAH E3 ligases interact with and regulate USP19. SIAH1 and SIAH2 promote USP19 ubiquitination and degradation through its E3 ubiquitin ligase activity. A SIAH-interacting motif within USP19 is necessary for this interaction.

The interactions were verified by co-immunoprecipitation of the over-expressed proteins, and the expression of SIAH and USP19 mutants demonstrated that their E3 ligase and DUB catalytic activities, respectively, are not required for the interaction (Figure 2 in Appendix A). We further assessed whether this interaction is important for the regulation of the cellular levels of USP19 and SIAH. Western-blot analysis demonstrated a dramatic decrease of USP19 levels in the presence of SIAH, dependent on the E3 ligase activity. This effect was associated with an increased ubiquitination of the transfected USP19 in the presence of the full-length SIAH ligases, strongly suggesting that USP19 is a target of the E3 activity of the ubiquitin ligases SIAH1 and SIAH2 (Figure 3 in Appendix A and Figure 15).

Several SIAH interacting proteins contain a conserved RPVAxVxPxxR motif that functions as a degradation signal (94). Bioinformatics analysis identified a perfectly conserved VxP core motif encompassing USP19 V468 and P470 flanked by conserved Pro and basic residues. Mutation of the V468 and P470 residues or deletion of the predicted full-length motif (462-473 amino acids) stabilized USP19 in the presence of SIAH, thus confirming its role in the regulation of the protein stability (Figure 4 in Appendix A and Figure 15).

3.1.2 USP19 regulates HIF-1α during hypoxia (Appendix B)

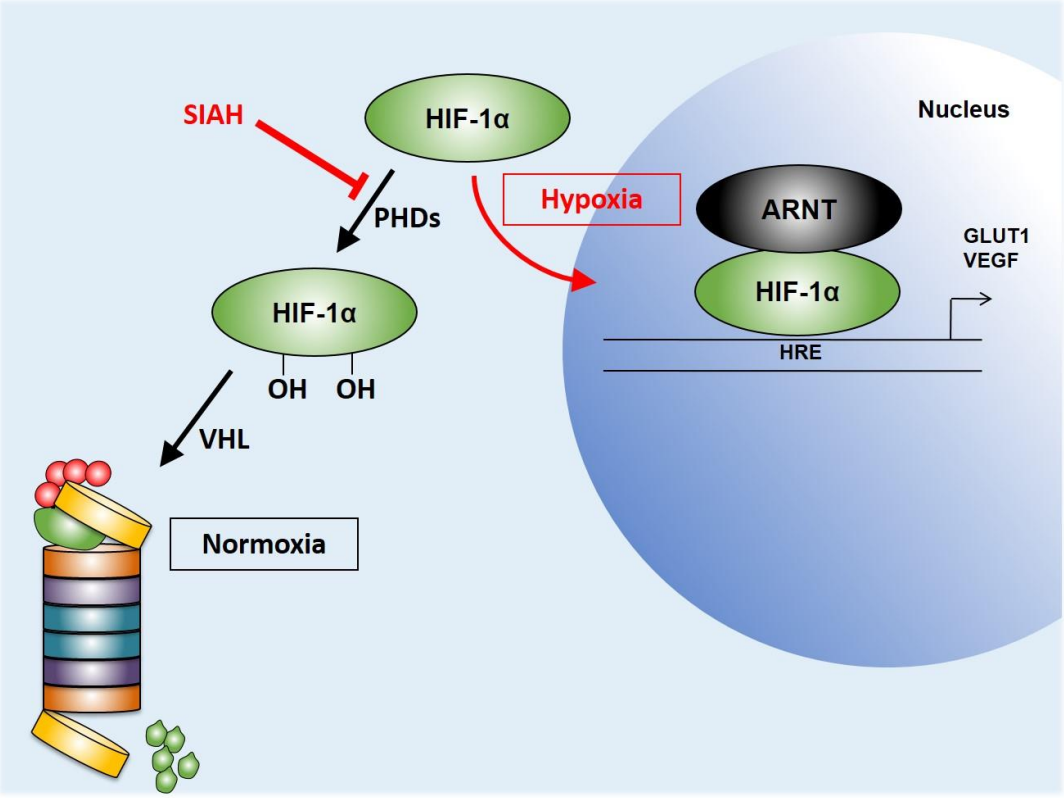


Figure 16. Hypoxia inducible factor (HIF) pathway. Under normoxic conditions HIF-1α is continuously degraded. The PHD enzymes hydroxylate HIF-1α; this modification is required for the binding of the ubiquitin ligase VHL that targets HIF-1α for ubiquitination and proteasomal degradation (black arrows). In hypoxic conditions HIF-1α is stabilized and translocated to the nucleus to dimerize with HIF-1ß and act as a transcription factor of genes involved in angiogenesis, erythropoiesis, glycolysis, iron metabolism, and cell survival. The SIAH E3 ligases stabilize indirectly HIF-1α levels by targeting the PHD enzymes for degradation (red arrows).

The SIAH ligases have been shown to stabilize the levels of the hypoxia inducible factor (HIF-1), a key transcription factor of the hypoxia response (95). HIF-1 regulates the

transcription of at least 70 genes involved in physiological responses to hypoxia like angiogenesis, erythropoiesis and glycolysis. HIF-1 is a heterodimeric protein formed by the short lived HIF-1α and the constitutively expressed HIF-1ß subunits. During normoxia conditions HIF-1α is hydroxylases on Pro 402 and/or 564 by the prolyl hydroxylases 1, 2 and 3 (PHD 1, 2 and 3, respectively). This modification are required for the binding of the Von Hippel-Lindau tumor suppressor (VHL) protein, the recognition component of an E3 ubiquitin ligase that in turn targets HIF-1α for ubiquitination and degradation by the proteasome. In hypoxic conditions, HIF-1α is stabilized and translocated to the nucleus where it dimerizes with HIF-1ß to form HIF-1 that binds to specific DNA sequences in target genes acting as a transcription factor (Figure 16) (96). SIAH modulates indirectly the HIF-1α levels through the targeting of the PHD enzymes for degradation (95).

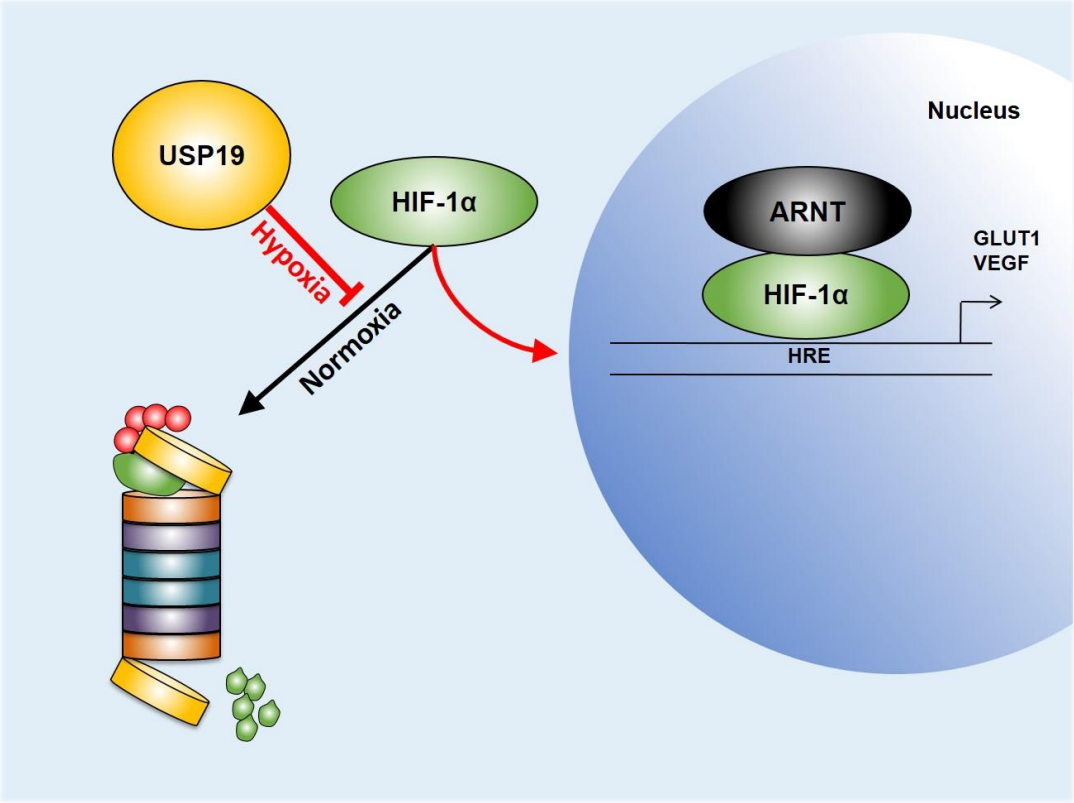


Figure 17. USP19 regulates HIF-1α. USP19 is able to stabilize HIF-1α independently of its catalytic DUB activity (red arrows).

Based on this evidence, we investigated whether USP19 interacts with components of the hypoxia pathway such as HIF-1α, PHD1, PHD2, PHD3 and VHL by co-immunoprecipitation of transiently transfected proteins. We confirmed the interaction of

USP19 with SIAH, and further demonstrated that this DUB interacts with HIF-1α (Figure 1 in Appendix B). The interaction of USP19 with HIF-1α was validated at endogenous levels, inducing the accumulation of detectable levels of HIF-1α by exposing the cells to hypoxia (1% O_2) (Figure 1C in Appendix B). Using deletion mutants, we mapped the interaction of USP19 to the Per-Arnt-Sim (PAS) and basic helix-loop-helix (bHLH) domains of HIF-1α. The bHLH domain near the N terminus is required for the binding to HIF-response element sequences present in HIF target genes, and the PAS domains mediate dimerization to HIF-1ß (Figure 2 in Appendix B).

To test whether USP19 regulates the levels of HIF-1α expression we performed immunofluorescence and western-blot analysis in cells over-expressing the wild type USP19, or mutant variants lacking either the catalytic activity or the transmembrane domain that targets the protein to the endoplasmic reticulum (ER). These set of experiments showed that USP19 is able to stabilize HIF-1α independently of its catalytic DUB activity and location to the ER (Figure 3 and 4 in Appendix B). To confirm the biological role of USP19 in the hypoxia response, we knocked down this DUB in HeLa cells using siRNA. HeLa cells lacking USP19 failed to accumulate HIF-1α and to regulate specific HIF-target genes in hypoxic conditions (Figure 5 in Appendix B and Figure 17).

3.2. USP4 IS ASSOCIATED TO THE PROTEASOME (Appendix C)

To explore the functions of USP4 we did a yeast-two-hybrid screen using as bait its first 318 N-terminal amino acids, which share less sequence similarities with other DUBs than the rest of the enzyme. The interaction with the S9 subunit, located on the lid of the 19S proteasome was the most prominent hit out of 90 identified proteins (Supplementary table S1 in Appendix C).

These results were validated by co-immunoprecipitation. Pull-down assays with the purified proteins fused to GST demonstrated that the interaction is specific and direct experiments (Figure 1 in Appendix C). Furthermore the successful co-immunoprecipitation of USP4 with the core and the base of the proteasome suggest that USP4 interacts with the whole assembled 26S proteasome (Figure 2 in Appendix C and Figure 18).

Co-immunoprecipitation experiments performed with a set of deletion mutants of USP4 and S9 proteins identified the ubiquitin like (UBL) 1 domain of USP4 and the N-terminus of S9 as the regions involved on the interaction (Figure 3 and Figure 4 in Appendix C).

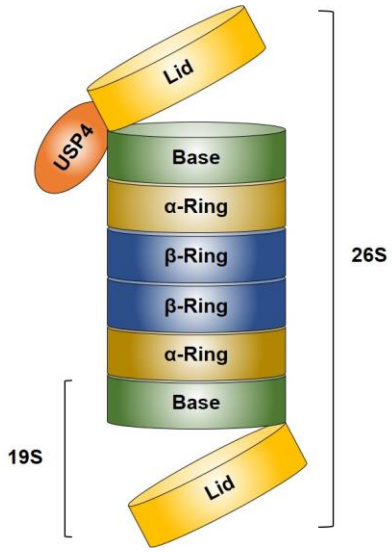


Figure 18. USP4 interacts with the 26S proteasome. USP4 interacts with the N-terminus of the S9 subunit, located in the 19S regulatory particle of the assembled proteasome.

3.3 UCH-L1 IN CELL ADHESION AND BACTERIA INTERNALIZATION (Appendix D)

Many evidences indicate that UCH-L1 regulates membrane proximal events, such as vesicular trafficking in pre-synaptic nerve terminals (97), cell adhesion, spreading (98), motility and invasion (99,100), LFA1-dependent homotypic adhesion in lymphoid cells (101), receptor signaling, and bacteria uptake (102). Several questions still need to be answered to elucidate the biology of UCH-L1 in these events. At present, our group focuses on two different aspects: First, the identification of the UCH-L1 substrates in the context of cell adhesion; and second, the study of the mechanisms by which UCH-L1 enhances bacterial entry. Our preliminary data in both research lines is presented below.

3.3.1 Identification of UCH-L1 substrates in the context of cell-adhesion

Integrins are the best characterized family of cell surface adhesion receptors. They are transmembrane proteins with a large extracellular and a short cytoplasmic domains. Integrins are present as heterodimers containing an α and a β subunits, non-covalently associated. Mammals have 18 α subunits and eight β subunits; which have been found in 24 different combinations (103).

Integrins contribute largely to cell adhesion by associating with extracellular ligands, like proteins in the extracellular matrix (ECM) or counter-receptors on adjacent cells, thus being crucial in the maintenance of tissue integrity and playing important roles in several

biological processes, such as development, wound healing, host defense and angiogenesis (104,105). However, integrins not only provide a physical link between the extracellular space and the cytoskeleton, they also produce sites of signal transduction into the cell (106). Outside-in signaling provides information about the surroundings of the cell and its state in relation to it, determining a variety of cellular responses. Inside-out signaling regulates the conformation of the integrins, having an effect upon the affinity for its ligands (105).

Upon binding of the integrin extracellular domains to any of its specific extracellular ligands, the receptor undergoes a conformational change that initiates the recruitment of approximately 156 components forming a multiprotein complex, and promoting the clustering of integrins (106-108). These multiprotein complexes comprise intracellular signalling and adaptor proteins that connect the integrins to the actin cytoskeleton, and serve both structural and signaling functions. Additional proteins may associate transiently with the adhesion complex to regulate its activity (109). Maturation of integrin clustering gives place to the formation of the structures known as focal adhesion complexes, whose dynamics of assembly and disassembly, which is tightly regulated and cell specific, determines several aspects of adhesion and cell migration (106).

Previously, our group demonstrated that the expression of the functional UCH-L1 in HeLa cells promotes integrin-dependent cell adhesion and spreading, as well as migration. The UCH-L1 expressing cells presented enhanced integrin-signaling activation and increase formation of focal adhesion complexes. This was associated with co-localization and co-immunoprecipitation of the active UCH-L1 with components of the integrin and cadherin adhesion complexes, such as focal adhesion kinase (FAK), a tyrosine kinase recruited at an early stage to focal adhesions integrating many signals to control cell motility (110), p120-catenin, a regulator of the abundance and activity of the adhesion receptors cadherin (111), and vinculin, which interacts indirectly with integrins in focal adhesions, thus stabilizing the complexes (98,112). However, we do not understand yet how UCH-L1 regulates these events and what the significance of the associations described is.

In order to identify the targets of the DUB UCH-L1 in the context of cell migration and adhesion we chose to make use of an inducible gene expression system under the control of tetracycline. This system allows us to turn on and off the expression and modulate the levels of UCH-L1 in transfected cells (113), thus avoiding the variability due to clonal selection that might arise when having different sublines.

We used a tet-on system, built in a stably transfected expression vector. This expression vector possesses a puromycin selection marker (PuroR) and a recombinant tetracycline-controlled transcription factor (rtTA3) downstream the constitutive human ubiquitin C (UBC) promoter. Additionally, this vector carries a tetracycline-inducible promoter (TRE), under which we cloned the catalytically active UCH-L1, or the inactive form UCH-L1$_{C90S}$, both tagged at the N-terminus with the red fluorescent protein mCherry, suitable for microscopy analysis and live cell imaging studies (Figure 19A).

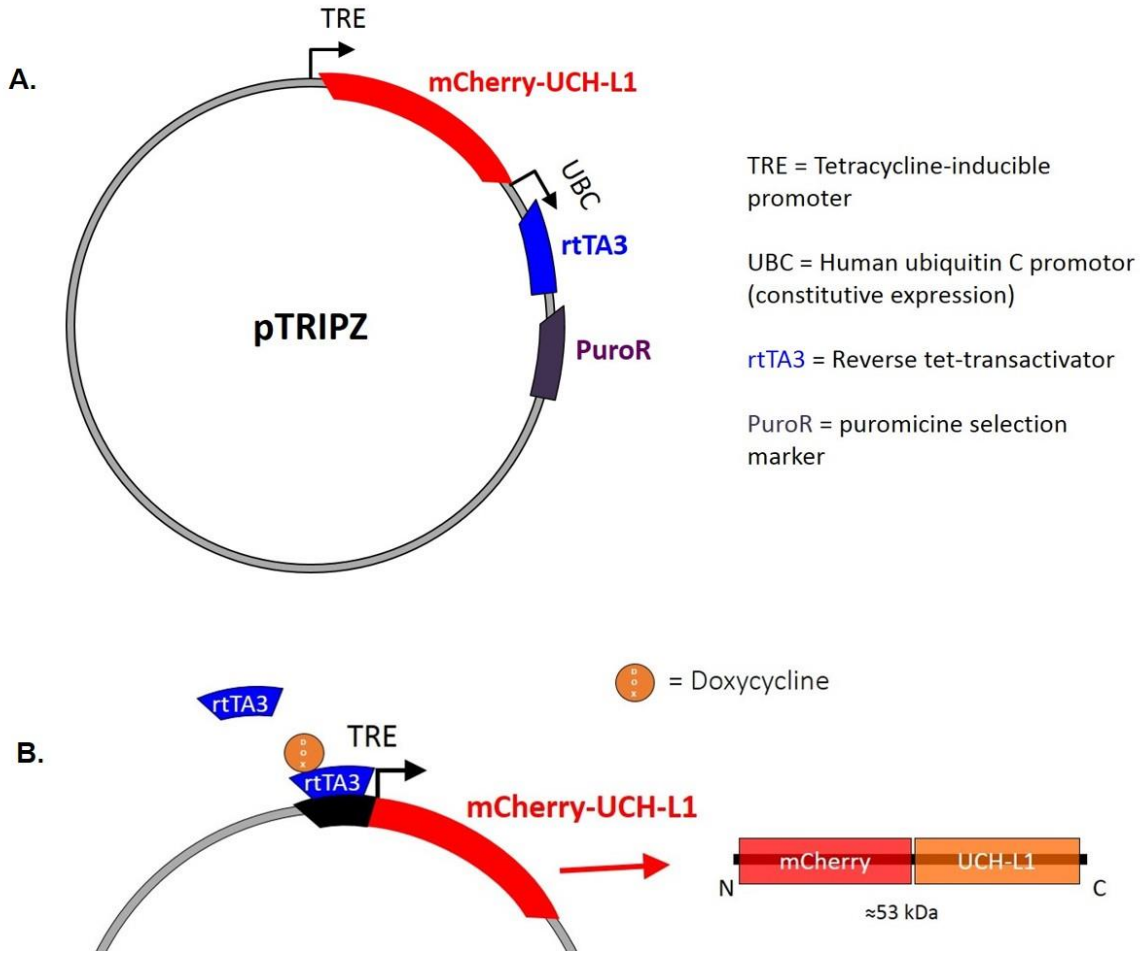


Figure 19. mCherry-UCH-L1 tet-on inducible expression system. A. mCherry-UCH-L1 or mCherry- UCH-L1$_{C90S}$ were cloned downstream a tetracycline-inducible promoter (TRE). The expression vector possesses a puromycin selection marker (PuroR) and a recombinant tetracycline-controlled transcription factor (rtTA3) downstream a constitutive human ubiquitin C promoter (UBC). **B.** The expression of the enzyme is triggered by doxycycline (DOX), which associates directly with the TRE.

In this system, expression of UCH-L1 is triggered by addition of doxycycline (DOX) to the culture medium. This modified form of tetracycline quickly enters into the cells and associates directly with rtTA3, allowing the specific binding of the transcription factor to the TRE, and consequently driving the expression of UCH-L1 (Figure 19B).

To generate stable clones, HeLa cells were transfected with the purified expression vector and kept under puromycin selection for 7 days. Fluorescence microscopy analysis confirmed the expression of the mCherry-UCH-L1 chimeras in cells cultured in the presence of DOX (Figure 21A). To test the performance of the tet-on system, DOX was withdrawn from the medium for 7 days and expression of UCH-L1 was assessed by western blot analysis. As shown in figure 20A, removal of the antibiotic from the medium resulted in complete silencing of the UCH-L1 expression. We also evaluated the catalytic activity of the chimeric proteins, expressed in HeLa cells using the Ub-VS functional probe. This set of experiments demonstrated that only the active mCherry-UCH-L1 could bind the functional probe, resulting in a molecular weight shift of approximately 9 kDa, as assessed by Western-blot analysis (Figure 20B).

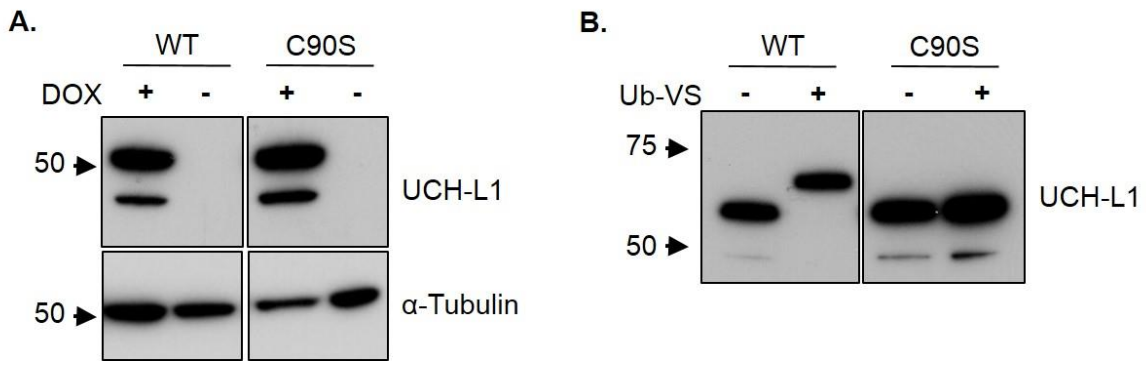


Figure 20. Performance of the mCherry-UCH-L1 and -UCH-L1$_{C90S}$ tet-on systems. A. UCH-L1 expression was assessed by western blotting in lysates of HeLa cells stably transfected with the tet-on inducible mCherry-UCH-L1 (WT) or mCherry-UCH-L1$_{C90S}$ (C90S) plasmids cultured in presence (+) or absence (-) of doxycycline (DOX) **B.** The enzymatic activity of mCherry-UCH-L1 (WT) and the catalytic mutant mCherry-UCH-L1$_{C90S}$ (C90S) was assessed by labelling with the Ub-VS functional probe. Lysates of HeLa cells stably transfected with the tet-on inducible plasmids and cultured in the presence of DOX were incubated for 1 hour at 37°C with (+) or without (-) the Ub-VS probe. Western blots were probed as indicated.

Next, we tested whether cells expressing the active mCherry-UCH-L1 recapitulated the spreading and adhesion phenotypes previously demonstrated in cells stably expressing this DUB (98). Cells were let to adhere onto fibronectin coated surfaces for 1h, and then fixed with 4% paraformaldehyde (PFA) for 20 minutes. Upon permeabilization, the cellular actin

cytoskeleton was stained with FITC-conjugated phalloidin (green), and nuclei were counterstained with DAPI (blue) (Figure 21A). Cell spreading was assessed by measuring the cell area using the ImageJ software. As expected, the area of the cells expressing the catalytically active enzyme (WT, DOX+; n = 87, mean(x) = 417.71) was significantly bigger than that of the control cells not expressing the enzyme (WT, DOX-; n = 96, mean(x) = 266.20; p-value = $4{,}787 \times 10^{-16}$; Figure 21B). Expression of the catalytically inactive mCherry-UCH-L1$_{C90S}$ did not result in a major enhancement of cell spreading. The mean area of these cells was bigger compared to that observed in cells transfected with the wild type mCherry-UCH-L1, irrespectively of the DOX treatment (p-value = 0,8367; Figure 21B). This suggests that the inactive form of the enzyme does not affect cell spreading and that the variation of their cell area might be an effect of clonal selection in these specific transfectants.

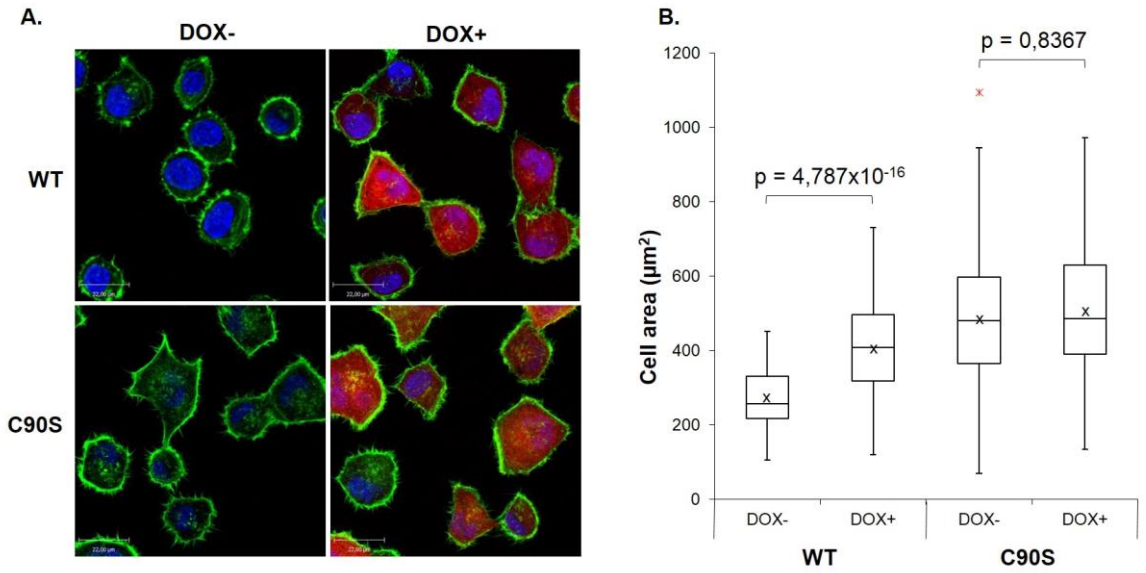


Figure 21. mCherry-UCH-L1 promotes cell spread. A. Confocal images illustrating the morphology of HeLa cells stably transfected with the tet-on inducible mCherry-UCH-L1 (WT) or mCherry-UCH-L1$_{C90S}$ (C90S) plasmids and maintained in presence (DOX+) or absence (DOX-) of doxycycline, cultured for 1 h on fibronectin-coated cover slides. mCherry-UCH-L1 was visualized in red, actin cytoskeleton was stained with FITC-conjugated phalloidin (green), and nuclei were stained with DAPI (blue). **B**. Boxplot representing the cell area measurements from confocal micrographs (as in A) of WT and C90S cells treated (DOX+) or not (DOX-) with doxycycline. The lower and upper limit of the boxes represent the first and third quartile, respectively, while the whiskers are set to represent the lower and higher value within 1.5 time the interquartile range. The horizontal line inside boxes show the medians, while the "x" represent the means. Two-tailed-test performed to compare DOX- and DOX+ treatments show a highly significant difference for WT cells (p-value = 4,787x10-16), and no significant difference for C90S cells (p-value = 0,8367).

We further performed wound healing assays using live cell imaging to confirm that expression of the active mCherry-UCH-L1 promoted cell motility. As shown in figure 22, cells grown in medium supplemented with DOX, thus expressing the active DUB (red, lower left panels), closed the gap with a much faster kinetic compared to cells grown in absence of DOX (upper left panels) or cells expressing the catalytically inactive mCherry-UCH-L1$_{C90S}$ (red, lower right panels).

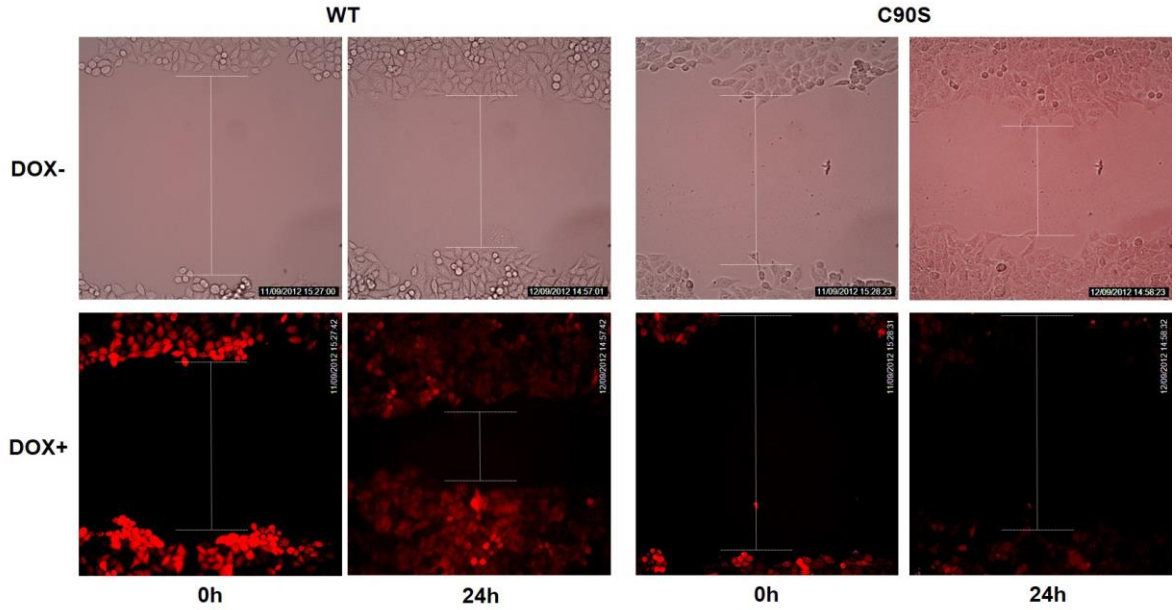


Figure 22. mCherry-UCH-L1 promotes cell motility. Confluent monolayers of HeLa cells stably transfected with the tet-on inducible mCherry-UCH-L1 (WT) or mCherry-UCH-L1$_{C90S}$ (C90S) plasmids and maintained in presence (DOX+) or absence (DOX-) of doxycycline were scratched with a tip to generate a linear wound and photographed at the indicated times (Leica DMI6000, 10X magnification).

Next, we tested whether the mCherry-UCH-L1 chimera maintained the ability to interact with components of the integrin-dependent focal adhesions. To this end, the chimeric DUB was immunoprecipitated from cells grown in the presence (DOX+) or absence (DOX-) of DOX after mild cross-linking (Figure 23). Unexpectedly, we did not see any specific interaction with vinculin in cells expressing either the active or the mutant mCherry-UCH-L1 (upper right panel). Furthermore, a band corresponding to the size of the mCherry-UCH-L1 chimera was also detected in the immunoprecipitates from cells grown in the absence of DOX, possibly due to a leakage of the tet-on system (Figure 23, lower right panel). It is conceivable that the fusion of the mCherry at the N terminus of UCH-L1 may affect the protein folding or may mask sites relevant for protein-protein interactions. To clarify this issue, we produced a new set of HeLa cells stably transfected with a vector expressing the UCH-L1 or

the catalytic inactive UCH-L1$_{C90S}$ tagged at the N-terminus with the FLAG epitope under the doxycycline inducible promoter. Western-blot analysis and labelling with the Ub-VS functional probe confirmed the inducibility of the system (Figure 24A) and the DUB activity of the wild type protein (Figure 24B) in stably transfected HeLa cells.

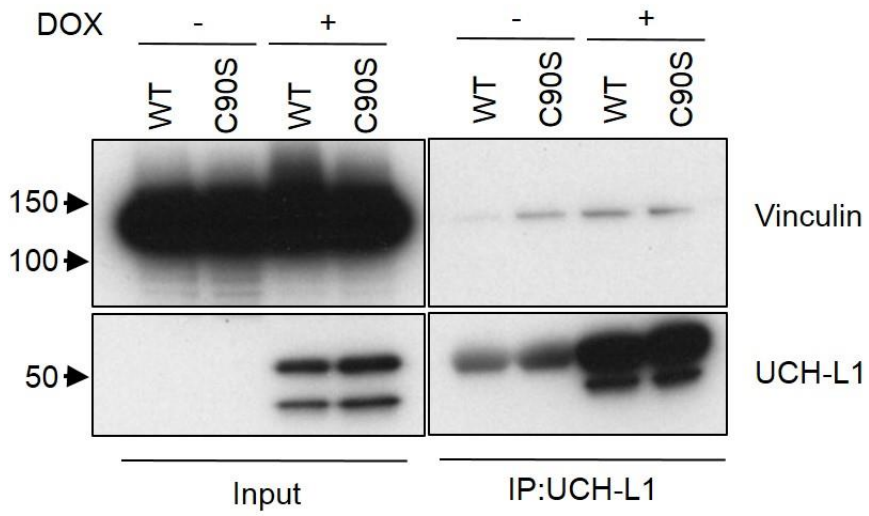


Figure 23. mCherry-UCH-L1 does not interact specifically with vinculin. UCH-L1 was immunoprecipitated from lysates prepared with prior cross-linking. Total cell lysates (input) and the immunoprecipitates (IP) were fractionated in 4-12% SDS-PAGE gels, and western blots were probed with the indicated antibodies.

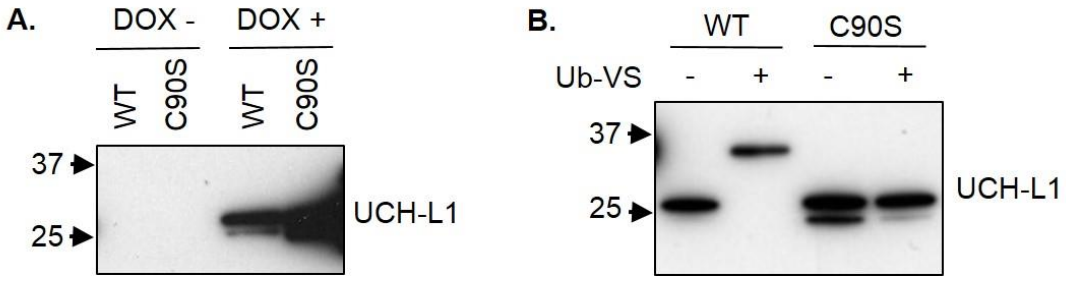


Figure 24. Performance of the FLAG-UCH-L1 and -UCH-L1$_{C90S}$ tet-on systems. A. UCH-L1 expression was assessed by western blotting in lysates of HeLa cells stably transfected with the tet-on inducible FLAG-UCH-L1 (WT) or FLAG-UCH-L1$_{C90S}$ (C90S) plasmids cultured in presence (DOX+) or absence (DOX-) of doxycycline **B.** The enzymatic activity of FLAG-UCH-L1 (WT) and the catalytic mutant FLAG-UCH-L1$_{C90S}$ (C90S) was assessed by labelling with the Ub-VS functional probe. Lysates of HeLa cells stably transfected with the tet-on inducible plasmids and cultured in the presence of DOX were incubated for 1 hour at 37°C with (+) or without (-) the Ub-VS probe. Western blots were probed as indicated.

We repeated the immunoprecipitation experiments described in figure 24 using this new set of cell lines. The results confirmed the capacity of the FLAG-UCH-L1 or FLAG- UCH-L1$_{C90S}$ to interact with vinculin only when expression of the DUB was induced with DOX (Figure 25). Furthermore, we did not detect expression of UCH-L1 in the cells grown in absence of DOX (lower panel), validating the inducible system.

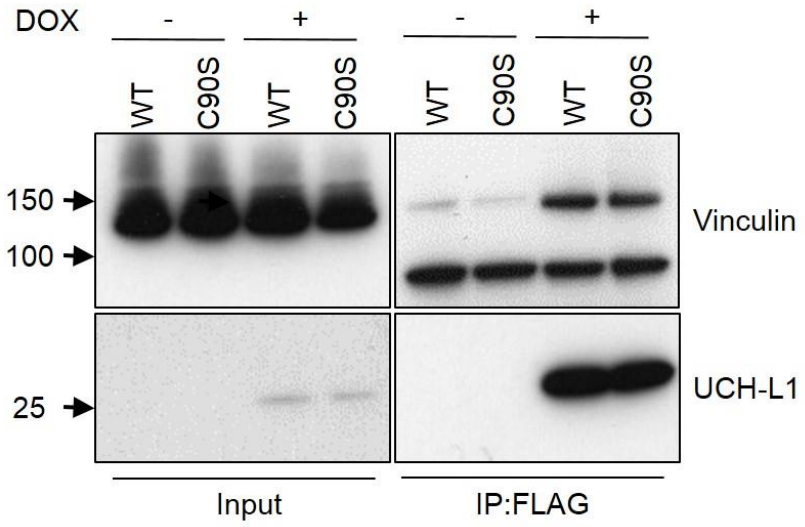


Figure 25. FLAG-UCH-L1 interacts with vinculin. FLAG-UCH-L1 was immunoprecipitated from lysates prepared with prior cross-linking. Total cell lysates (input) and the immunoprecipitates (IP) were fractionated in 4-12% SDS-PAGE gels, and western blots were probed with the indicated antibodies.

3.3.2 Studying the contribution of UCH-L1 to bacterial entry.

Some pathogenic bacteria invade non-phagocytic cells as part of their life cycle. The access to the intracellular space protects bacteria from immune detection, allows them to access deeper tissues and provides an agreeable environment for growth. Although the mechanisms that invasive bacteria use to induce their own uptake are very diverse (114), they have been separated into two groups: the 'zipper' and the 'trigger' mechanisms. Some examples of bacteria employing the 'zipper' mechanism are *Yersinia, Listeria* and *Neisseria*. Surface-exposed proteins of these bacteria interact with surface receptors of the host cell membrane often involved in cell-matrix or cell-cell-adherence. This binding engages the host cell receptor, inducing signaling cascades that result in actin rearrangement and modest membrane extension that leads to bacterial internalization. *Shigella* and *Salmonella* are examples of bacteria that enter using the 'trigger' mechanism. These bacteria inject protein effectors into the host cytoplasm using a specialized secretory apparatus known as type III secretion system. The effectors interact with and modulate heavily the actin

cytoskeleton, promoting the formation of membrane extensions that internalize the bacteria in a process similar to micropinocytosis (115,116).

Our group has previously demonstrated that UCH-L1 promotes the entry of *Listeria monocytogenes* and *Salmonella enterica*. This was associated with an enhanced activation of the actin cytoskeleton (102). However, the mechanisms by which this DUB promotes bacteria entry remains unknown. To inquire into this matter we took advantage of the bacterium *Yersinia pseudotuberculosis*. It is known that this bacterium uses the invasin surface protein to interact with high affinity to β1 integrins, leading to activation of several small GTPases, such as Rac1, which promotes the modulation of the phosphatidylinositol metabolism at the bacterial entry site to induce actin rearrangements, promoting invasion (114). The rational of choosing this bacterium is that we have shown that UCH-L1 promotes activation of the integrin signaling as explained in the previous section (98).

To start, we assessed whether UCH-L1 enhances *Y. pseudotuberculosis* entry, as observed before with *S. enterica* and *L. monocytogenes* (102). HeLa cells stably transfected with the empty vector (CTR), or vector expressing the wild type UCH-L1 or the catalytically inactive UCH-L1$_{C90S}$ were infected with *Y. pseudotuberculosis* at a multiplicity of infection (MOI) of 50:1, and incubated for 1h at 37°C, prior addition of fresh medium supplemented with 100 µg/ml gentamicin. Cells were further incubated for 1h at 37°C to kill non internalized bacteria, and subsequently lysed with 0.5% Sodium deoxycolate in phosphate buffer saline (PBS). Serial dilutions of cell lysate was plated in LB agar plates to evaluate the number of colony-forming units (CFU). As shown in figure 26A, the number of bacteria internalized by HeLa cells expressing the active DUB was higher compared to the number of bacteria recovered in control cells or in cells expressing the UCH-L1$_{C90S}$.

To study the molecular mechanism by which UCH-L1 promotes bacteria invasion, we developed a *Y. pseudotuberculosis* strain expressing the mCherry protein under an L-arabinose inducible promoter. This tool will allow us to follow bacteria entry by fluorescence microscopy and live cell imaging. To assess whether expression of the mCherry protein did not alter the internalization pattern observed with wild type strain, we infected HeLa cells stably expressing the wild type or the inactive UCH-L1 at a MOI of 50:1, and incubated for 1h at 37°C. Cells were washed twice with PBS, fixed in 4% PFA for 20 minutes and extracellular bacteria were finally stained by indirect immunofluorescence using a rabbit anti-LPS antibody, followed by a donkey anti-rabbit Alexa 488 (green) conjugated secondary antibody. Wheat germ agglutinin (WGA) conjugated to Alexa 680 (far red) was used to label the cell membrane. This staining allowed us to identify the internalized

bacteria, which are only mCherry positive from the extracellular bacteria that were both red (mCherry positive) and green (LPS positive) (Figure 26B).

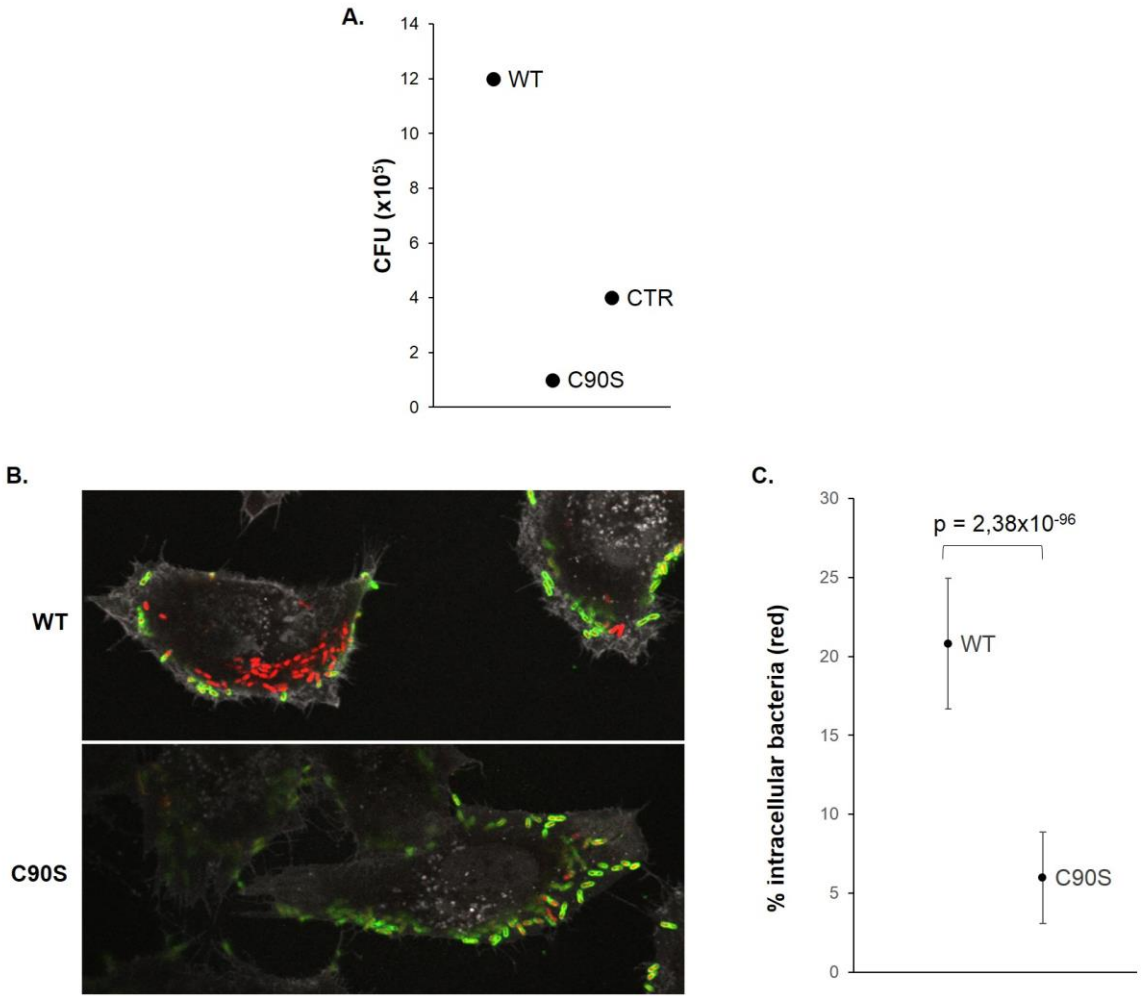


Figure 26. UCH-L1 promotes *Y. pseudotuberculosis* internalization. A. HeLa cells stably transfected with UCH-L1 (WT), UCH-L1$_{C90S}$ (C90S) or the empty vector (CTR) were infected with *Y. pseudotuberculosis* at a MOI of 50:1, and the number of CFU was assessed 1 h post-infection. **B.** HeLa cells infected as in A with *Y. pseudotuberculosis* expressing mCherry were washed and fixed (without permeabilization) 1 h post-infection. Confocal microscopy was used to assess bacteria internalization. Extracellular bacteria were stained using a rabbit anti-LPS antibody (green). WGA was used to label the cell membrane (white). **C.** Quantification of the percentage of intracellular bacteria (red) from ten confocal micrographs as in B (mean ±SD). A Cochran-Mantel-Haenszel test for repeated test of independence shows a highly significant difference of internalized bacteria between the cells expressing the catalytically active (WT) and inactive enzyme (C90S) (p-value = $2,38x10^{-96}$)

As shown in figure 27B, the number of internalized bacteria was significantly higher in cells expressing the wild type UCH-L1 (WT, mean = 20.8), compared with that observed in cells transfected with the inactive UCH-L1$_{C90S}$ (C90S, mean = 5.98; p-value = 2,38x10^{-96}; Figure 26C).

4. DISCUSSION

This thesis summarizes the work performed on three different DUBs. Here, I discussed a mechanism of regulation of USP19, the role of this DUB in the cellular response to hypoxia, the function of USP4 in the proximity of the proteasome, and the function of UCH-L1 in cell adhesion, migration and spreading, and in bacteria internalization. The results described in this thesis do not only illustrate the wide functional diversity of these DUBs, but also give a good understanding of the multi-functionality displayed by many of these enzymes. Last years of research in this topic have shown that although DUBs are substrate-specific, several appear to play roles in the regulation of multiple different pathways or to act on multiple players within the same pathway. This is the case of CYDL, a DUB that negatively regulates NF-κB signaling by deubiquitinating different regulators of this pathway (117,118). It is not well understood yet how the multi-functionality is achieved, but this property, along with the pivotal role of these enzymes, suggest that they have to be tightly controlled. It appears that DUBs can be controlled by a wide variety of mechanisms. For example, their abundance can be regulated through transcriptional control (119,120), their activities can be directed by subcellular localization (121,122), and their functions can be modulated by posttranslational modifications (123) and by conformational changes upon association with interacting proteins (2,44).

Although sequences of individual DUBs vary widely, the members of each family share a high degree of homology in the regions surrounding the conserved catalytic core of the enzymes. The functional diversity and substrate specificity is largely attributed to accessory domains (2). USP19 harbors a p23/CS, a MYND and a TMD domain for which the functions have not been completely elucidated. We found a motif in USP19 that functions as a degron regulating its stability (section 3.1.1). On the other hand, USP4 contains, apart from its DUB domain, a DUSP and two UBL domains. The DUSP domain might be implicated in protein-protein interactions, similarly to how it operates in USP15 (124), the UBL domain, which is localized within the catalytic region has auto-regulatory activities (125), while our findings show that the most N-terminal UBL domain is required for USP4 association with the proteasome (section 3.2).

The catalytic activity of the DUBs constitute other layer of regulation. On one hand, a specificity for the type of ubiquitin chain linkages has been determined for many DUBs: For instance, USPs prefer K48 and K63 chains (45). On the other hand, it seems that at least some DUBs can exert non-catalytic functions. Here we reported a non-catalytic activity for USP19 in the regulation of HIF-1α (section 3.1.2), and previously it was shown to be able to

rescue some ERAD substrates from proteasomal degradation (49). So far these events seem rare, though not unique for USP19. Ubp6, an homolog of USP14, was shown to delay proteasomal degradation independently of its catalytic activity (126), and A20 can inhibit the regulator of the NFκB pathway IκB kinase complex (IKK), by a non-catalytic mechanism (127). The mechanisms used in these non-catalytic functions are still unclear.

The association of DUBs with several proteins is another crucial aspect for their regulation. Thus, the search for interacting partners of DUBs not only helps understanding their functions through the identification of their substrates, but also provides clues about the necessity of their association with some proteins or inclusion in complexes to display a proper activity (46,47). Our interaction studies showed that USP19 levels can be regulated by ubiquitination and proteasomal degradation promoted by the SIAH ligases, and revealed a function for this DUB in stabilizing HIF-1α during cellular response to hypoxia (section 3.1). Although we ignore if the regulation USP19 stability through this pathway is important for all the multiple roles described for this DUB or for any specific one, it is tempting to speculate that this mechanism of regulation might be important for maintaining the adequate levels of USP19 to regulate HIF-1α in hypoxic conditions, since both events are regulated by the SIAH ligases (95). Conversely, we discovered that USP4 is associated with the 26S proteasome (section 3.2). Albeit the purpose of this interaction remains to be evaluated, its location, as well as its incompetence to rescue substrates directed to degradation, suggest that USP4 has a different function than the other DUBs associated to the proteasome, USP14 and UCH37, which counteract the degradation of some proteasomal substrates before they are committed to degradation (35,41). For instance, USP4 could play a role in the stability of the 26S complex. Meanwhile, the interaction of UCH-L1 with components of the focal adhesion complexes appear to be related with the ability of this enzyme to regulate cell adhesion, spreading, migration, resistance to anoikis and bacteria internalization (98). Nevertheless the substrate(s) of UCH-L1 in this context, and the mechanisms used to regulate these events are unknown (section 3.3).

One of the major challenges towards the functional characterization of UCH-L1 has been the identification of its physiological targets. Difficulties encountered up to date to elucidate this issue may depend on the requirement for very transient post-translational modifications or interactions with co-factors to produce a conformational rearrangement of the enzyme, exposing the otherwise inaccessible catalytic site (70). In an effort to identify the substrates of UCH-L1 in the context of cell adhesion and migration we generated a tet-on system, expressing an mCherry tagged UCH-L1 with the purpose of performing biochemical and imaging assays to identify the substrate(s) for this DUB (Section 3.3.1).

Although we could reproduce our previous results showing that the catalytically active UCH-L1 promotes cell spreading and migration (98), the co-immunoprecipitation assays did not worked as expected. Interestingly, expression of a FLAG-tagged UCH-L1, cloned into the same tetracycline inducible vector recapitulated all the phenotypes observed in cells stably expressing this DUB. Thus, it is possible that the mCherry tag, of approximately 28 kDa, may interfere with the folding of UCH-L1 or may mask important sites for protein-protein interaction. It is noteworthy that although the mCherry chimera seems to not interact with focal adhesion components such as vinculin, it stills promotes cell spreading, migration and resistance to anoikis (98), suggesting that those effects may be regulated by focal adhesion independent pathways. The confocal images of cells expressing mCherry-UCH-L1 did not reveal a striking accumulation of the enzyme at the cell membrane during the initial phase of adhesion as reported before (98).

Bacteria use efficient strategies for hijacking the process of endocytosis to induce their internalization into non-phagocytic cells. Previously, our group showed that UCH-L1 promotes internalization of *L. monocytogenes* and *S. enterica*, but the mechanisms involved have not been elucidated. Here, we demonstrated that UCH-L1 also promotes *Y. pseudotuberculosis* internalization (section 3.3.2). All these bacteria employ distinct invasion strategies: both *Y. pseudotuberculosis* and *L. monocytogenes* induce their uptake via zipper mechanisms, using different cell receptors: β1 integrins (*Y. pseudotuberculosis*) and E-cadherin and the Hepatocyte growth factor (HGF) Met (*L. monocytogenes*). On the other hand, *S. enterica* enters via a trigger mechanism (114,116). Hence, since UCH-L1 promotes bacteria invasion regardless of the strategy used by the bacteria, this suggests that this DUB must have a role in actin remodeling, a common event for the two strategies of bacteria invasion. Indeed, our group have previously reported the formation of actin stress fibers in HeLa cells ectopically expressing UCH-L1 (102). Consequently, this DUB may regulate the activity of one or more of the many effectors that promote actin cytoskeleton dynamics.

For a detailed discussion of the findings regarding USP4 and USP19 the reader is referred to the discussion section of the appendices A, B and C.

The diverse biological roles of DUBs regulating several signaling pathways related to diseases such as cancer and neurodegenerative disorders, make them attractive therapeutic targets. Some of the strategies envisioned to target these enzymes are to block the DUB activity through specific inhibitors, and to manipulate signal-transduction pathways in which they are involved or its gene expression (45). The major challenge to

date, has been to ensure the specificity of the therapeutic agents. Most DUBs are cysteine proteases, a class of enzymes difficult to target with small molecules mainly because of the lack of specificity of their inhibitors (128). Additionally, several DUBs share a core domain (45). At present, some cysteine protease inhibitors have been identified. Ubiquitin aldehyde (Uba1) and UbVs are commonly used to study the three-dimensional structure of these enzymes and their catalytic activity (129). Besides having a limited specificity, these compounds exhibit high molecular weights, being not suitable as therapeutic agents (130). Thus, much work is still needed to develop DUB inhibitor molecules to clinic. Recently, the small molecule AP15 was shown to induce tumor cell apoptosis by abrogating the DUB activity of UCH-L5 and USP14, which are associated to the 19S regulatory particle (131). This area will benefit from the progress in understanding the regulatory mechanisms, the substrates, functions and molecular structures of DUBs. To take care of the multi-functional aspect of the DUBs is particularly important to avoid undesired effects since the inhibition of an enzyme could affect its protective effects (130).

5. CONCLUSIONS

This work aimed to gain a better understanding of the biological functions of the deubiquitinating enzymes USP19, USP4 and UCH-L1. We found that:

- USP19 can be regulated through ubiquitination and proteasomal degradation, promoted by the SIAH1 and SIAH2 ubiquitin ligases. The interaction between USP19 and the SIAH ligases is mediated by a SIAH-consensus binding motif.

- USP19 has a role, independent of its catalytic DUB activity, in controlling the key regulator of response to hypoxia, HIF-1α.

- USP4 is a proteasome-interacting DUB, in addition to two previously described ones. It interacts specifically with the S9 subunit, located in the lid of the proteasome.

- The chimera mCherry-UCH-L1 has an impaired function, reproducing only partially our previous findings about the role of UCH-L1 in cell adhesion, spreading and migration.

- UCH-L1 promotes *Y. pseudotuberculosis* internalization.

6. RECOMMENDATIONS AND PERSPECTIVES

The findings presented in this work not only contribute to the functional characterization of the DUBs USP19, USP4 and UCH-L1, but raise new research questions. In the case of USP19 two important remaining questions are: what is the functional role of the interaction between USP19 and SIAH, and, what are the mechanisms through which USP19 regulates HIF-1α (Section 3.1). In the first case one approach could be to explore if the interaction between USP19 and SIAH is required for any of the specific functions linked to this proteins. In this regard, it would be interesting to test if there is a link between SIAH, HIF-1α and USP19 in severe and acute hypoxia conditions. We could learn a lot from studying and understanding the physiological conditions in which USP19 regulates HIF-1α. An additional aspect that we could not solve in this work, and could provide important clues about these mechanisms of regulation, is if USP19 have also a regulatory activity on the SIAH ligases.

Regarding USP4, the main question left is: what is the functional significance of the interaction between the proteasomal subunit S9 and this DUB. The unchanged enzymatic activity of USP4 on fluorogenic substrates when associated with S9 (Section 3.2) (64), together with the localization of this subunit, suggest that USP4 has a different role to the other identified proteasome interacting DUBs, USP14 and UCH37, which display an increase enzymatic activity when associated to the proteasome, and are localized in close proximity to the incoming ubiquitinated substrates (41).

We have not been able to identify the targets of UCH-L1 in cell adhesion and migration (Section 3.3). Although we know that the association of this DUB to some components of the integrin and cadherin signaling pathways is weakened in the absence of the catalytic activity of UCH-L1, as well as the membrane proximal effects observed, our attempts to identify changes in the ubiquitination state of such components due to its DUB activity have failed (98). This suggests that either our assays were not sensitive enough to detect this changes or that the DUB activity is not exerted directly in these proteins but in other one(s) in close relationship to this complexes. Consequently, there is a lot of work to be done in order to dissect the pathways by which UCH-L1 acts to promote cell adhesion, spreading, migration, survival and bacteria internalization. It could be worth to follow the dynamics of some of the focal adhesion, acting remodeling, and microtubule components using live cell imaging, in cells expressing the catalytically active and inactive form of the DUB. The use of technologies like TIRF microscopy could facilitate this task. The integration of this studies to other cell models, where the enzyme is highly expressed such as cells from the brain tissue, gonads, or some cancer cells could provide additional clues on this research problem.

Due to the critical role of ubiquitination, DUBs are an attractive potential therapeutic target and there has been an increasing interest from the pharmaceutical industry to target these enzymes in drug development (45). Although USP19, USP4 and UCH-L1 are implicated in important cellular pathways, many of them cancer-related (Section 1.3), we need to gain a better understanding of their functions before attempting to find clinical applications for them. We still know very little about the functions of USP19, and the roles of USP4 and UCH-L1 are quite controversial. Nevertheless, given the central role of hypoxia in tumor progression and resistance, USP19 could be an interesting target to be exploited in oncology. Likewise, the high expression of UCH-L1 in tumor cells underscore the therapeutic importance of the findings described here. The effects of this enzyme in migration and survival might be associated with malignant transformation and tumor progression. On the other hand, the mechanisms used by UCH-L1 to promote bacteria internalization could be exploited to limit bacterial infection, or to use attenuated strains of bacteria to deliver vectors or enzymes in tumor cells.

REFERENCES

1. Pickart CM. Back to the future with ubiquitin. Cell. January 23, 2004;116(2):181-190.

2. Komander D, Clague MJ, Urbé S. Breaking the chains: structure and function of the deubiquitinases. Nat Rev Mol Cell Biol. August 2009;10(8):550-563.

3. Simoni R, Hill R, Vaughan M. The use of isotope tracers to study intermediary metabolism: Rudolf Schoenheimer. Journal of Biological Chemistry. January 1, 2002.

4. Turk V. Special issue: Proteolysis 50 years after the discovery of lysosome in honor of Christian de Duve. Biochim. Biophys. Acta. January 1, 2012;1824(1):1-2.

5. Ciechanover A. Intracellular protein degradation: from a vague idea thru the lysosome and the ubiquitin-proteasome system and onto human diseases and drug targeting. Cell Death Differ. September 2005;12(9):1178-1190.

6. Ciechanover A, Elias S, Heller H, Ferber S, Hershko A. Characterization of the heat-stable polypeptide of the ATP-dependent proteolytic system from reticulocytes. J. Biol. Chem. August 25, 1980;255(16):7525-7528.

7. Hershko A, Ciechanover A, Heller H, Haas AL, Rose IA. Proposed role of ATP in protein breakdown: conjugation of protein with multiple chains of the polypeptide of ATP-dependent proteolysis. Proc. Natl. Acad. Sci. U.S.A. April 1980;77(4):1783-1786.

8. Hershko A, Ciechanover A, Rose IA. Resolution of the ATP-dependent proteolytic system from reticulocytes: a component that interacts with ATP. Proc. Natl. Acad. Sci. U.S.A. July 1979;76(7):3107-3110.

9. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature. October 21, 2004;431(7011):931-945.

10. Jensen ON. Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry. Curr Opin Chem Biol. February 2004;8(1):33-41.

11. Hochstrasser M. Evolution and function of ubiquitin-like protein-conjugation systems. Nature Cell Biology. August 2000;2(8):E153-7.

12. Jentsch S, Pyrowolakis G. Ubiquitin and its kin: how close are the family ties? Trends Cell Biol. August 2000;10(8):335-342.

13. Pickart CM, Eddins MJ. Ubiquitin: structures, functions, mechanisms. Biochim. Biophys. Acta. November 29, 2004;1695(1-3):55-72.

14. Kerscher O, Felberbaum R, Hochstrasser M. Modification of proteins by ubiquitin and ubiquitin-like proteins. Annual review of cell and developmental biology. January 1, 2006;22:159-80.

15. Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. J. Biol. Chem. July 10, 1983;258(13):8206-8214.

16. Pickart CM. Mechanisms underlying ubiquitination. Annu. Rev. Biochem. 2001;70:503-533.

17. Schulman BA, Harper JW. Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. Nat. Rev. Mol. Cell Biol. May 2009;10(5):319-331.

18. van Wijk SJL, Timmers HTM. The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. FASEB J. April 2010;24(4):981-993.

19. Li W, Bengtson MH, Ulbrich A, Matsuda A, Reddy VA, Orth A, et al Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PLoS ONE. 2008;3(1):e1487.

20. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. April 2002;82(2):373-428.

21. Welchman RL, Gordon C, Mayer RJ. Ubiquitin and ubiquitin-like proteins as multifunctional signals. Nat. Rev. Mol. Cell Biol. August 2005;6(8):599-609.

22. Hunter T. The age of crosstalk: phosphorylation, ubiquitination, and beyond. Mol. Cell. December 14, 2007;28(5):730-738.

23. Deshaies RJ, Joazeiro CAP. RING domain E3 ubiquitin ligases. Annu. Rev. Biochem. 2009;78:399-434.

24. Metzger MB, Hristova VA, Weissman AM. HECT and RING finger families of E3 ubiquitin ligases at a glance. J. Cell. Sci. February 1, 2012;125(Pt 3):531-537.

25. Dye BT, Schulman BA. Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. Annu Rev Biophys Biomol Struct. 2007;36:131-150.

26. Dikic I, Robertson M. Ubiquitin ligases and beyond. BMC Biol. 2012;10(1):22.

27. Husnjak K, Dikic I. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annu. Rev. Biochem. 2012;81:291-322.

28. Peng J, Schwartz D, Elias JE, Thoreen CC, Cheng D, Marsischky G, et al A proteomics approach to understanding protein ubiquitination. Nat Biotechnol. August 2003;21(8):921-926.

29. Rieser E, Cordier SM, Walczak H. Linear ubiquitination: a newly discovered regulator of cell signalling. Trends in Biochemical Sciences. February 2013;38(2):94-102.

30. Ikeda F, Dikic I. Atypical ubiquitin chains: new molecular signals. “Protein Modifications: Beyond the Usual Suspects” review series. EMBO Rep. June 2008;9(6):536-542.

31. Hicke L. Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. March 2001;2(3):195-201.

32. Pickart CM, Fushman D. Polyubiquitin chains: polymeric protein signals. Curr Opin Chem Biol. December 2004;8(6):610-616.

33. Xu P, Duong DM, Seyfried NT, Cheng D, Xie Y, Robert J, et al Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell. April 3, 2009;137(1):133-145.

34. Groll M, Ditzel L, Löwe J, Stock D, Bochtler M, Bartunik HD, et al Structure of 20S proteasome from yeast at 2.4 A resolution. Nature. April 3, 1997;386(6624):463-471.

35. Finley D. Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. 2009;78:477-513.

36. Pickart CM, Cohen RE. Proteasomes and their kin: proteases in the machine age. Nat. Rev. Mol. Cell Biol. March 2004;5(3):177-187.

37. Lasker K, Förster F, Bohn S, Walzthoeni T, Villa E, Unverdorben P, et al Molecular architecture of the 26S proteasome holocomplex determined by an integrative approach. Proc Natl Acad Sci USA. January 31, 2012;109(5):1380-1387.

38. Sauer RT, Baker TA. AAA+ proteases: ATP-fueled machines of protein destruction. Annu. Rev. Biochem. June 7, 2011;80:587-612.

39. Tomko RJ, Funakoshi M, Schneider K, Wang J, Hochstrasser M. Heterohexameric ring arrangement of the eukaryotic proteasomal ATPases: implications for proteasome structure and assembly. Mol. Cell. May 14, 2010;38(3):393-403.

40. Smith DM, Chang S, Park S, Finley D, Cheng Y, Goldberg AL. Docking of the proteasomal ATPases" carboxyl termini in the 20S proteasome"s alpha ring opens the gate for substrate entry. Mol. Cell. September 7, 2007;27(5):731-744.

41. Lander GC, Estrin E, Matyskiela ME, Bashore C, Nogales E, Martin A. Complete subunit architecture of the proteasome regulatory particle. Nature. February 9, 2012;482(7384):186-191.

42. Yao T, Cohen RE. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature. September 26, 2002;419(6905):403-407.

43. Amerik AY, Hochstrasser M. Mechanism and function of deubiquitinating enzymes. Biochim. Biophys. Acta. November 29, 2004;1695(1-3):189-207.

44. Reyes-Turcu FE, Ventii KH, Wilkinson KD. Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes. Annu. Rev. Biochem. 2009;78:363-397.

45. Fraile JM, Quesada V, Rodríguez D, Freije JMP, López-Otín C. Deubiquitinases in cancer: new functions and therapeutic options. Oncogene. May 10, 2012;31(19):2373-2388.

46. Sowa ME, Bennett EJ, Gygi SP, Harper JW. Defining the human deubiquitinating enzyme interaction landscape. Cell. July 23, 2009;138(2):389-403.

47. Ventii KH, Wilkinson KD. Protein partners of deubiquitinating enzymes. Biochem. J. September 1, 2008;414(2):161-175.

48. Nijman SMB, Luna-Vargas MPA, Velds A, Brummelkamp TR, Dirac AMG, Sixma TK, et al A genomic and functional inventory of deubiquitinating enzymes. Cell. December 2, 2005;123(5):773-786.

49. Hassink GC, Zhao B, Sompallae R, Altun M, Gastaldello S, Zinin NV, et al The ER-resident ubiquitin-specific protease 19 participates in the UPR and rescues ERAD substrates. EMBO Rep. July 2009;10(7):755-761.

50. Iphöfer A, Kummer A, Nimtz M, Ritter A, Arnold T, Frank R, et al Profiling ubiquitin linkage specificities of deubiquitinating enzymes with branched ubiquitin isopeptide probes. Chembiochem. July 9, 2012;13(10):1416-1420.

51. Combaret L, Adegoke OAJ, Bedard N, Baracos V, Attaix D, Wing SS. USP19 is a ubiquitin-specific protease regulated in rat skeletal muscle during catabolic states. Am. J. Physiol. Endocrinol. Metab. April 2005;288(4):E693-700.

52. Lu Y, Adegoke OAJ, Nepveu A, Nakayama KI, Bedard N, Cheng D, et al USP19 deubiquitinating enzyme supports cell proliferation by stabilizing KPC1, a ubiquitin ligase for p27Kip1. Mol. Cell. Biol. January 2009;29(2):547-558.

53. Pichlmair A, Kandasamy K, Alvisi G, Mulhern O, Sacco R, Habjan M, et al Viral immune modulators perturb the human molecular network by common and unique strategies. Nature. July 26, 2012;487(7408):486-490.

54. Mei Y, Hahn AA, Hu S, Yang X. The USP19 deubiquitinase regulates the stability of c-IAP1 and c-IAP2. J. Biol. Chem. October 14, 2011;286(41):35380-35387.

55. Matsuoka S, Ballif BA, Smogorzewska A, McDonald ER, Hurov KE, Luo J, et al ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage. Science. May 25, 2007;316(5828):1160-1166.

56. Luna-Vargas MPA, Faesen AC, van Dijk WJ, Rape M, Fish A, Sixma TK. Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain. EMBO Rep. Nature Publishing Group; March 18, 2011;12(4):365-372.

57. Zhao B, Schlesiger C, Masucci MG, Lindsten K. The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. August 2009;13:1886-1895.

58. Fan Y, Yu Y, Mao R, Tan X, Xu G, Zhang H, et al USP4 targets TAK1 to downregulate TNFα-induced NF-κB activation. October 1, 2011;18(10):1547-60.

59. Xiao N, Li H, Luo J, Wang R, Chen H, Chen J, et al Ubiquitin-specific protease 4 (USP4) targets TRAF2 and TRAF6 for deubiquitination and inhibits TNFα-induced cancer cell migration. Biochem J. January 16, 2012;441(3):979-986.

60. Zhou F, Zhang X, van Dam H, Dijke ten P, Huang H, Zhang L. Ubiquitin-specific protease 4 mitigates Toll-like/interleukin-1 receptor signaling and regulates innate immune activation. J. Biol. Chem. March 30, 2012;287(14):11002-10.

61. Zhang X, Berger FG, Yang J, Lu X. USP4 inhibits p53 through deubiquitinating and stabilizing ARF-BP1. The EMBO Journal. Nature Publishing Group; April 26, 2011;30(11):2177-2189.

62. Zhang L, Zhou F, Drabsch Y, Gao R, Snaar-Jagalska BE, Mickanin C, et al USP4 is regulated by AKT phosphorylation and directly deubiquitylates TGF-β type I receptor. Nature Cell Biology. Nature Publishing Group; June 17, 2012;14(7):717-726.

63. Gray DA, Inazawa J, Gupta K, Wong A, Ueda R, Takahashi T. Elevated expression of Unph, a proto-oncogene at 3p21.3, in human lung tumors. Oncogene. June 1, 1995;10(11):2179-83.

64. Zhao B, Schlesiger C, Masucci MG, Lindsten K. The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. Journal of Cellular and Molecular Medicine. November 26, 2009;13(8b):1886-1895.

65. Frederick A, Rolfe M, Chiu MI. The human UNP locus at 3p21.31 encodes two tissue-selective, cytoplasmic isoforms with deubiquitinating activity that have reduced expression in small cell lung carcinoma cell lines. Oncogene. January 15, 1998;16(2):153-65.

66. Wilkinson KD, Lee KM, Deshpande S, Duerksen-Hughes P, Boss JM, Pohl J. The neuron-specific protein PGP 9.5 is a ubiquitin carboxyl-terminal hydrolase. Science. November 3, 1989;246(4930):670-673.

67. Larsen CN, Price JS, Wilkinson KD. Substrate binding and catalysis by ubiquitin C-terminal hydrolases: identification of two active site residues. Biochemistry. May 28, 1996;35(21):6735-6744.

68. Walters BJ, Campbell SL, Chen PC, Taylor AP, Schroeder DG, Dobrunz LE, et al Differential effects of Usp14 and Uch-L1 on the ubiquitin proteasome system and synaptic activity. Mol Cell Neurosci. December 2008;39(4):539-548.

69. Osaka H. Ubiquitin carboxy-terminal hydrolase L1 binds to and stabilizes monoubiquitin in neuron. Human Molecular Genetics. July 1, 2003;12(16):1945-1958.

70. Das C, Hoang QQ, Kreinbring CA, Luchansky SJ, Meray RK, Ray SS, et al Structural basis for conformational plasticity of the Parkinson's disease-associated ubiquitin hydrolase UCH-L1. Proc. Natl. Acad. Sci. U.S.A. March 21, 2006;103(12):4675-4680.

71. Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT. The UCH-L1 gene encodes two opposing enzymatic activities that affect alpha-synuclein degradation and Parkinson's disease susceptibility. Cell. October 18, 2002;111(2):209-218.

72. Bradbury JM, Thompson RJ. Immunoassay of the neuronal and neuroendocrine marker PGP 9.5 in human tissues. J. Neurochem. February 1985;44(2):651-653.

73. Sakurai M, Sekiguchi M, Zushida K, Yamada K, Nagamine S, Kabuta T, et al Reduction in memory in passive avoidance learning, exploratory behaviour and synaptic plasticity in mice with a spontaneous deletion in the ubiquitin C-terminal hydrolase L1 gene. Eur. J. Neurosci. February 2008;27(3):691-701.

74. Saigoh K, Wang YL, Suh JG, Yamanishi T, Sakai Y, Kiyosawa H, et al Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. Nat. Genet. September 1999;23(1):47-51.

75. Leroy E, Boyer R, Auburger G, Leube B, Ulm G, Mezey E, et al The ubiquitin pathway in Parkinson's disease. Nature. October 1, 1998;395(6701):451-452.

76. Maraganore DM, Farrer MJ, Hardy JA, Lincoln SJ, McDonnell SK, Rocca WA. Case-control study of the ubiquitin carboxy-terminal hydrolase L1 gene in Parkinson's disease. Neurology. November 10, 1999;53(8):1858-1860.

77. Löwe J, McDermott H, Landon M, Mayer RJ, Wilkinson KD. Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases. J. Pathol. June 1990;161(2):153-160.

78. Karim R, Tummers B, Meyers C, Biryukov JL, Alam S, Backendorf C, et al Human Papillomavirus (HPV) Upregulates the Cellular Deubiquitinase UCHL1 to Suppress the Keratinocyte's Innate Immune Response. PLoS Pathog. May 2013;9(5):e1003384.

79. Hurst-Kennedy J, Chin L, Li L. Ubiquitin C-terminal hydrolase l1 in tumorigenesis. Biochem Res Int. 2012;2012:123706.

80. Jang MJ, Baek SH, Kim JH. UCH-L1 promotes cancer metastasis in prostate cancer cells through EMT induction. Cancer Lett. March 28, 2011;302(2):128-135.

81. Kim HJ, Kim YM, Lim S, Nam YK, Jeong J, Kim HJ, et al Ubiquitin C-terminal hydrolase-L1 is a key regulator of tumor cell invasion and metastasis. Oncogene. January 8, 2009;28(1):117-127.

82. Li L, Tao Q, Jin H, van Hasselt A, Poon FF, Wang X, et al The tumor suppressor UCHL1 forms a complex with p53/MDM2/ARF to promote p53 signaling and is frequently silenced in nasopharyngeal carcinoma. Clinical cancer research : an official journal of the American Association for Cancer Research. June 1, 2010;16(11):2949-58.

83. Xiang T, Li L, Yin X, Yuan C, Tan C, Su X, et al The ubiquitin peptidase UCHL1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is frequently silenced in breast cancer. PLoS ONE. January 1, 2012;7(1):e29783.

84. Young KH. Yeast two-hybrid: so many interactions, (in) so little time... Biol. Reprod. February 1998;58(2):302-311.

85. Berggård T, Linse S, James P. Methods for the detection and analysis of protein-protein interactions. Proteomics. August 2007;7(16):2833-2842.

86. Shoemaker BA, Panchenko AR. Deciphering Protein–Protein Interactions. Part I. Experimental Techniques and Databases. PLoS Comput Biol. 2007;3(3):e42.

87. Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. Microbiol. Rev. March 1995;59(1):94-123.

88. Klockenbusch C, Kast J. Optimization of Formaldehyde Cross-Linking for Protein Interaction Analysis of Non-Tagged Integrin β1. Journal of Biomedicine and Biotechnology. January 1, 2010;2010:1-13.

89. Sutherland B, Toews J, Kast J. Utility of formaldehyde cross-linking and mass spectrometry in the study of protein-protein interactions. Journal of mass spectrometry : JMS. June 1, 2008;43(6):699-715.

90. Einarson M, Pugacheva E. Identification of protein-protein interactions with glutathione-S-transferase (GST) fusion proteins. Cold Spring Harbor. January 1, 2007.

91. Kim TK, Eberwine JH. Mammalian cell transfection: the present and the future. Anal Bioanal Chem. June 13, 2010;397(8):3173-3178.

92. Lichtman JW, Conchello J. Fluorescence microscopy. Nature Methods. December 1, 2005;2(12):910-919.

93. Nwaneshiudu A, Kuschal C, Sakamoto FH, Anderson RR, Schwarzenberger K, Young RC. Introduction to Confocal Microscopy. Journal of Investigative Dermatology. December 1, 2012;132(12):e3.

94. House CM, Frew IJ, Huang H, Wiche G, Traficante N, Nice E, et al A binding motif for Siah ubiquitin ligase. Proc. Natl. Acad. Sci. U.S.A. March 18, 2003;100(6):3101-3106.

95. Nakayama K, Ronai Z. Siah: new players in the cellular response to hypoxia. Cell Cycle. November 2004;3(11):1345-1347.

96. Lahiri S, Roy A, Baby SM, Hoshi T, Semenza GL, Prabhakar NR. Oxygen sensing in the body. Prog. Biophys. Mol. Biol. July 2006;91(3):249-286.

97. Chen F, Sugiura Y, Myers KG, Liu Y, Lin W. Ubiquitin carboxyl-terminal hydrolase L1 is required for maintaining the structure and function of the neuromuscular junction. Proc. Natl. Acad. Sci. U.S.A. January 26, 2010;107(4):1636-1641.

98. Frisan T, Coppotelli G, Dryselius R, Masucci MG. Ubiquitin C-terminal hydrolase-L1 interacts with adhesion complexes and promotes cell migration, survival, and anchorage independent growth. FASEB J. December 2012;26(12):5060-5070.

99. Buus R, Faronato M, Hammond DE, Urbé S, Clague MJ. Deubiquitinase activities required for hepatocyte growth factor-induced scattering of epithelial cells. Current biology : CB. September 15, 2009;19(17):1463-6.

100. Kim JH, Jung EJ, Lee HS, Kim MA, Kim WH. Comparative analysis of DNA methylation between primary and metastatic gastric carcinoma. Oncology reports. May 1, 2009;21(5):1251-9.

101. Rolén U, Freda E, Xie J, Pfirrmann T, Frisan T, Masucci MG. The ubiquitin C-terminal hydrolase UCH-L1 regulates B-cell proliferation and integrin activation. Journal of Cellular and Molecular Medicine. August 1, 2009;13(8B):1666-78.

102. Bassères E, Coppotelli G, Pfirrmann T, Andersen JB, Masucci M, Frisan T. The ubiquitin C-terminal hydrolase UCH-L1 promotes bacterial invasion by altering the dynamics of the actin cytoskeleton. Cellular Microbiology. November 1, 2010;12(11):1622-33.

103. Arnaout MA, Mahalingam B, Xiong J. Integrin structure, allostery, and bidirectional signaling. Annual review of cell and developmental biology. January 1, 2005;21:381-410.

104. Buckley CD, Rainger GE, Bradfield PF, Nash GB, Simmons DL. Cell adhesion: more than just glue (review). Molecular membrane biology. January 1, 1998;15(4):167-76.

105. Harburger DS, Calderwood DA. Integrin signalling at a glance. J. Cell. Sci. January 15, 2009;122(Pt 2):159-63.

106. Carragher NO, Frame MC. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. Trends Cell Biol. May 2004;14(5):241-249.

107. Zaidel-Bar R, Itzkovitz S, Ma'ayan A, Iyengar R, Geiger B. Functional atlas of the integrin adhesome. Nature Cell Biology. August 1, 2007;9(8):858-67.

108. Parsons JT, Horwitz AR, Schwartz MA. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nature Reviews Molecular Cell Biology. September 1, 2010;11(9):633-643.

109. Huttenlocher A, Horwitz AR. Integrins in Cell Migration. Cold Spring Harbor Perspectives in Biology. September 1, 2011;3(9):a005074-a005074.

110. Guan JL. Role of focal adhesion kinase in integrin signaling. The international journal of biochemistry & cell biology. August 1, 1997;29(8-9):1085-96.

111. Reynolds AB, Roczniak-Ferguson A. Emerging roles for p120-catenin in cell adhesion and cancer. Oncogene. October 18, 2004;23(48):7947-56.

112. Peng X, Nelson ES, Maiers JL, DeMali KA. New insights into vinculin function and regulation. International review of cell and molecular biology. January 1, 2011;287:191-231.

113. Berens C, Hillen W. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. Eur J Biochem. August 2003;270(15):3109-3121.

114. Pizarro-Cerdá J, Cossart P. Bacterial Adhesion and Entry into Host Cells. Cell. February 1, 2006;124(4):715-727.

115. Veiga E, Cossart P. The role of clathrin-dependent endocytosis in bacterial internalization. Trends Cell Biol. October 1, 2006;16(10):499-504.

116. Cossart P, Sansonetti PJ. Bacterial invasion: the paradigms of enteroinvasive pathogens. Science. April 9, 2004;304(5668):242-8.

117. Burrows JF, Johnston JA. Regulation of cellular responses by deubiquitinating enzymes: an update. Frontiers in bioscience (Landmark edition). January 1, 2012;17:1184-200.

118. Glittenberg M, Ligoxygakis P. CYLD: a multifunctional deubiquitinase. Fly. January 1, 2007;1(6):330-2.

119. Baek K. Cytokine-regulated protein degradation by the ubiquitination system. Current protein & peptide science. April 1, 2006;7(2):171-7.

120. Yoshida H, Jono H, Kai H, Li J. 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. December 9, 2005;280(49):41111-21.

121. Nakamura N, Hirose S. Regulation of mitochondrial morphology by USP30, a deubiquitinating enzyme present in the mitochondrial outer membrane. Molecular biology of the cell. May 1, 2008;19(5):1903-11.

122. Endo A, Matsumoto M, Inada T, Yamamoto A, Nakayama KI, Kitamura N, et al Nucleolar structure and function are regulated by the deubiquitylating enzyme USP36. J. Cell. Sci. March 1, 2009;122(Pt 5):678-86.

123. Kessler BM, Edelmann MJ. PTMs in conversation: activity and function of deubiquitinating enzymes regulated via post-translational modifications. Cell Biochem. Biophys. June 2011;60(1-2):21-38.

124. de Jong RN, Ab E, Diercks T, Truffault V, Daniëls M, Kaptein R, et al Solution structure of the human ubiquitin-specific protease 15 DUSP domain. J. Biol. Chem. February 24, 2006;281(8):5026-31.

125. Luna-Vargas MPA, Faesen AC, van Dijk WJ, Rape M, Fish A, Sixma TK. Ubiquitin-specific protease 4 is inhibited by its ubiquitin-like domain. EMBO Rep. Nature Publishing Group; March 18, 2011;12(4):365-372.

126. Hanna J, Hathaway NA, Tone Y, Crosas B, Elsasser S, Kirkpatrick DS, et al Deubiquitinating Enzyme Ubp6 Functions Noncatalytically to Delay Proteasomal Degradation. Cell. October 6, 2006;127(1):99-111.

127. Skaug B, Chen J, Fenghe du, He J, Ma A, Chen Z. Direct, Noncatalytic Mechanism of IKK Inhibition by A20. Mol. Cell. November 1, 2011;44(4):559-571.

128. Turk B. Targeting proteases: successes, failures and future prospects. Nature reviews. Drug discovery. September 1, 2006;5(9):785-99.

129. Love KR, Catic A, Schlieker C, Ploegh HL. Mechanisms, biology and inhibitors of deubiquitinating enzymes. Nat Chem Biol. November 2007;3(11):697-705.

130. Lim K, Baek K. Deubiquitinating enzymes as therapeutic targets in cancer. Current pharmaceutical design. January 1, 2013;19(22):4039-52.

131. D'Arcy P, Brnjic S, Olofsson MH, Fryknäs M, Lindsten K, de Cesare M, et al Inhibition of proteasome deubiquitinating activity as a new cancer therapy. Nature medicine. December 1, 2011;17(12):1636-40.

APPENDIX A

Paper I: Velasco K, Zhao B, Callegari S, Altun M, Liu H, et al. (2013) **An N-terminal SIAH-interacting motif regulates the stability of the ubiquitin specific protease (USP)-19**. Biochem. Biophys. Res. Commun. 433 (4): 390-395.

Abstract
The Ubiquitin Specific Protease-19 (USP19) regulates cell cycle progression and is involved in the cellular response to different types of stress, including the unfolded protein response (UPR), hypoxia and muscle atrophy. Using the unique N-terminal domain as bait in a yeast-two hybrid screen we have identified the ubiquitin ligases Seven In Absentia Homolog (SIAH)-1 and SIAH2 as binding partners of USP19. The interaction is mediated by a SIAH-consensus binding motif and promotes USP19 ubiquitylation and proteasome-dependent degradation. These findings identify USP19 as a common substrate of the SIAH ubiquitin ligases.

This manuscript was published in the journal *Biochemical and Biophysical Research Communications (*doi: 10.1016/j.bbrc.2013.02.094*).*

Link to the article:
http://www.sciencedirect.com/science/article/pii/S0006291X13003951?via=ihub

APPENDIX B

Paper II: Altun M, Zhao B, Velasco K, Liu H, Hassink G, et al. (2012) **Ubiquitin-specific protease 19 (USP19) regulates hypoxia-inducible factor 1α (HIF-1α) during hypoxia**. J Biol Chem 278: 1962-1969.

Abstract

A proper cellular adaptation to low oxygen levels is essential for processes such as development, growth, metabolism, and angiogenesis. The response to decrease in oxygen supply, referred to as hypoxia, is also involved in numerous human diseases including cancer, inflammatory conditions, and vascular disease. The hypoxia-inducible factor 1-α (HIF-1α), a key player in the hypoxic response, is kept under stringent regulation. At normoxia, the levels are kept low as a consequence of the efficient degradation by the ubiquitin-proteasome system, and in response to hypoxia, the degradation is blocked and the accumulating HIF-1α promotes a transcriptional response essential for proper adaptation and survival. Here we show that the ubiquitin-specific protease-19 (USP19) interacts with components of the hypoxia pathway including HIF-1α and rescues it from degradation independent of its catalytic activity. In the absence of USP19, cells fail to mount an appropriate response to hypoxia, indicating an important role for this enzyme in normal or pathological conditions.

This manuscript was published in the *Journal of Biological Chemistry* (doi: 10.1074/jbc.M111.305615).

Link to the article:
http://www.jbc.org/cgi/pmidlookup?view=long&pmid=22128162

APPENDIX C

Paper III: Zhao B, Velasco K, Sompallae R, Pfirrmann T, Massuci M, Lindsten K. (2012) **The ubiquitin specific protease-4 (USP4) interacts with the S9/Rpn6 subunit of the proteasome**. Biochem Biophys Res Commun 427 (3): 490-496.

Abstract
The proteasome is the major non-lysosomal proteolytic machine in cells that, through degradation of ubiquitylated substrates, regulates virtually all cellular functions. Numerous accessory proteins influence the activity of the proteasome by recruiting or deubiquitylating proteasomal substrates, or by maintaining the integrity of the complex. Here we show that the ubiquitin specific protease (USP)-4, a deubiquitylating enzyme with specificity for both Lys48 and Lys63 ubiquitin chains, interacts with the S9/Rpn6 subunit of the proteasome via an internal ubiquitin-like (UBL) domain. S9/Rpn6 acts as a molecular clamp that holds together the proteasomal core and regulatory sub-complexes. Thus, the interaction with USP4 may regulate the structure and function of the proteasome or the turnover of specific proteasomal substrates.

This manuscript was published in the journal *Biochemical and Biophysical Research Communications* (doi: 10.1016/j.bbrc.2012.09.075).

Link to the article:
http://www.sciencedirect.com/science/article/pii/S0006291X12018220?via=ihub

APPENDIX D

MATERIAL AND METHODS: UCH-L1 IN CELL ADHESION AND MIGRATION

Cell lines, bacteria strains and plasmids

HeLa cells [American Type Culture Collection (ATCC), Teddington, UK] were cultured in Iscove´s modified Dulbecco´s medium (IMDM, Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 10 µg/ml ciprofloxacin (Sigma-Aldrich; complete medium).

Tetracycline regulated mCherry-UCH-L1 and mCherry-UCHL1$_{C90S}$ expression vectors were produced by cloning the mCherry coding sequence into the BamHI and EcoRI sites of the pRSFDuet1 vector (Millipore, Billerica, MA, USA). The UCH-L1 and C90S sequences were amplified using the primers 5'-ATTCCAATTGATGCAGCTCAAGCCGATGGAGATC-3' and 5'-GCAGTCGACTTAGGCTGCCTTGCAGAGAGCCAC-3' and cloned in frame with the mCherry gene into the EcoRI and SalI sites. The chimeras were then amplified with the primers 5'-ATTCACCGGTATGGTGAGCAAGGGCGAGGAG-3' and 5'GCAACGCGTTTAGGCTGCCTTGCAGAGAGCCAC-3', and cloned into the AgeI and MluI sites of the tet-on inducible lentiviral pTripZ vector (Open Biosystems, Lafayette, CO, USA). For the FLAG-UCH-L1 construct the primers used were 5'-TTAACCGGTATGGATTACAAGGATGACGATGA-3' and 5'-TTAACGCGTTTAGGCTGCCTTGCAGA-3'. All restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Semiconfluent monolayers of cells grown in a 12-well plate were transfected with 1 µg of the indicated expression vectors using the JetPEI transfection kit (Polyplus Transfection, Illkirch, France), according to the manufacturer´s instructions, and further selected in complete medium in the presence of 1 µg/ml puromycin. Expression of the mCherry-UCH-L1 and -UCHL1$_{C90S}$ proteins was induced by adding 1µg/ml doxycycline to the culture medium.

HeLa stably transfected with the pCEP4-UCH-L1, or pCEP4-UCHL1$_{C90S}$ plasmids (99) were cultured in complete medium in the presence of 300 µg/ml hygromycin.

Yersinia pseudotuberculosis was cultured in LB medium. Bacteria were transformed with the pMW165 plasmid carrying the mCherry gene under an L-arabinose inducible promoter, obtained from Dr. Dirk Bumman (Biozentrum, University of Basel, Basel, Switzerland). The mCherry tagged *Y. pseudotuberculosis* was cultured in LB medium supplemented with 100 µg/ml ampicillin and 20mM L-arabinose.

Ub deconjugase activity assay

Deconjugase activity was determined using the Ub-VS (vinyl-sulfone) functional probe (Boston Biochem, Cambridge, MA, USA). The cells expressing the protein to be tested were lysed for 30 min at 4°C in 80 µl of buffer containing 50 mM Tris (pH 7.4), 0.5% Nonidet P-40, 5mM $MgCl_2$, 250 mM sucrose, 1 mM DTT, and 1 mM ATP, and the cell lysates were clarified by centrifugation (14,000 rpm, 4°C, 30 min). Cell lysates (20 µg) were incubated with 1 µg of Ub-VS probe for 1h at 37°C, and enzyme activity was assessed in Western blots as a shift of the UCH-L1 specific band corresponding to the size of the probe.

Immunofluorescence

Cells ($2x10^5$) in IMDM containing 1% FBS were seeded on 13 mm cover slides previously coated with 1 µg/ml fibronectin (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C, and let adhere for 1 h. The slides were then washed once in PBS and fixed with 4% PFA (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. After permeabilization and blocking with 0.2% Triton X-100 and 3% BSA in PBS for 30 min, the slides were incubated with 1 µg/ml TRITC-phalloidin (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 1 h at 37°C. The nuclei were counterstained with DAPI (Vectashield; Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was visualized by confocal microscopy (LSM510 META; Zeiss, Oberkochen, Germany). The cell area was estimated from micrographs using the ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA), and the figures were made using Volocity Image Analysis Software (PerkinElmer, Inc., Waltham, MA, USA). Data were analyzed with the R statistical package (3.0.2 Version). The Shapiro-Wilk test was used to analyze the normal distribution of the variables. The Student´s t-test was used to compare the means between groups in each experiment.

For the bacteria internalization assays 1.5 $x10^5$ cells/well were seeded on 13 mm cover slides in a 12 well plate in complete medium. Next day, after adding the bacteria at a MOI of 50:1, plates were centrifuged 5 min at 1500 rpm and incubated 1 h. The slides were then washed in PBS, fixed with 4% PFA for 15 min and blocked with 3% BSA in PBS for 30 min. The slides were stained with rabbit anti *Y. pseudotuberculosis* LPS (1:500, provided by Maria Fällman, Umeå University, Umeå, Sweden) and WGA conjugated to Alexa 680 (1:500, Molecular Probes). The number of bacteria internalized was quantified from micrographs taken by confocal microscopy using ImageJ. Data were analyzed using the Cochran-Mantel-Haenszel (CMH) test. The test was performed using the Excel spreadsheet written by John Mc Donald (http://udel.edu/~mcdonald/statcmh.html.).

Wound healing assay

$10x10^3$ cells/well were seeded on a 12-well plate and grown to confluence. The monolayers were then scratched with a pipette tip to generate a linear wound and washed to remove cell debris. The monolayers were left in complete medium and photographed every 30 min during 24 h (Leica DMI6000, Leica Microsystems GmbH, Wetzlar, Germany).

Immunoprecipitation

$10x10^6$ cells were plated onto 10 cm dishes in complete IMDM supplemented with 1% FBS, and let adhere for 1 h at 37°C. Protein cross-linking was performed by addition of PFA (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 1% for 20 min at room temperature, and the reaction was stopped by adding 0.125 M glycine for 5 min. The cells were collected by gentle scraping, washed twice with cold PBS, and lysed in 2 ml lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, 0.01% SDS, 1 mM EDTA, protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM N-ethylmaleimide (NEM), 10 mM iodoacetamide, and 1 mM DTT (Sigma-Aldrich, St. Louis, MO, USA) for 30 min on ice. The cell lysates were clarified, and protein concentration was measured with a detergent-compatible protein assay in microliter plates (Biorad, Hercules, CA, USA). UCH-L1 was immunoprecipitated from equal amounts of proteins by incubation in gentle rotation at 4°C with either FLAG-beads (Sigma-Aldrich, St. Louis, MO, USA) for 5 h, or an antibody recognizing UCH-L1 (PGP9.5; Biomol International) for 3 hours and GammaBind G Sepharose (GE Healthcare Piscataway, NJ, USA) for 2 additional hours. The beads were washed 3 times in lysis buffer, re-suspended in 2X Laemmli buffer, and boiled 10 min.

Western blot

Proteins were fractionated by SDS-polyacrylamide gel electrophoresis using precast 4-12% gradient gels (Invitrogene, Carlsbad, CA, USA), transferred to PVDF membranes (Millipore, Darmstad, Germany), and probed with 1:1000 dilution of the indicated antibodies, followed by the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare Piscataway, NJ, USA). The blots were developed by enhanced chemiluminescence (GE Healthcare Piscataway, NJ, USA) according to the manufacturer's instructions. The following antibodies were used: rabbit polyclonal anti-UCH-L1 (PGP9.5; Biomol International), mouse monoclonal anti-vinculin (Millipore, Darmstad, Germany) and anti-tubulin (Oncogene, Cambridge, MA, USA).

Gentamicin assay

Gentamicin assays were performed in six-well tissue culture plates. $0.5x10^5$ cells per well were plated 1 day prior to the assay. Cells were washed once with serum-free medium without antibiotics and bacterial suspension was added in serum-free medium without antibiotics at a MOI of 50:1. Cells were incubated with the bacterial suspension at 37°C for 1 h. The bacteria-containing medium was removed and cells were further incubated with medium containing 100 μg/ml gentamicin for 1 h at 37°C. Cells were washed once in PBS and lysed by adding 0.2% Triton X-100. Subsequently, the cell lysates were incubated for 24 h at 37°C onto LB agar plates and incubated for 24 h at 37°C. The number of viable bacteria released from the cells was assessed as the number of CFU.