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Role of calpain in USP1 stability regulation and genome integrity maintenance

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ROLE OF CALPAIN IN USP1 STABILITY REGULATION AND GENOME INTEGRITY MAINTENANCE

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LIST OF ABBREVIATIONS

ALP: alkaline phosphatase

APAF1: apoptotic protease activating factor 1

APC/C: anaphase-promoting complex/cyclosome

ATG: autophagy-related gene

ATM: Ataxia Telangiectasia mutated

ATR: Ataxia Telangiectasia and RAD3-related protein

ATRIP: ATR-interacting protein

BACH1/BRIP1: BRCA1-associated C-terminal helicase 1, BRCA1-interacting protein 1

bHLH: basic-helix-loop-helix

BRCA1: breast cancer type 1

BRCA2: breast cancer type 2

CAPN1: calpain 1 or micro-μ-calpain

CAPN2: calpain 2 or milli-m-calpain

CAPNS1: calpain small subunit 1

CAST: calpastatin

Cdk5: cyclin-dependent kinase 5

CHK1: checkpoint kinase 1

CMA: chaperone-mediated autophagy

CPD: cyclobutane-pyrimidine dimer

DCAF: DDB1 and Cul4-associated factor

DDB1: damage-specific DNA-binding protein 1

DDR: DNA damage response

DSB: double strand break

DUB: deubiquitinating enzyme

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ERK: extracellular signal-regulated kinase

ESCRT: endosomal sorting complex required for transport

FA: Fanconi anemia

FAK: focal adhesion kinase

FAN1: FANCD2/FANCI-associated nuclease 1

FANCD2: Fanconi anemia complementation group D2 protein

FANCD2-L: monoubiqutinated form of FANCD2

FoxO: forkhead box

GAS2: growt arrest-specific 2

Gas2DN: Gas2 dominant negative

HR: homologous recombination

HSC70: heat shock cognate protein of 70 KDa

ICL: interstrand crosslink

ID: inhibitors of DNA binding

IκBα: inhibitor of κB-α

IKK: inhibitor of NF-κB kinase

JAMM: JAB1/MPN/Mov34 metallo-enzyme

JNK: c-Jun N-terminal kinase

LAMP: lysosome-associated membrane protein

LC3: microtubule-associated protein light chain 3

LGMD2A: limb-girdle muscular dystrophy type 2A

MAP3K1: mitogen-activated protein kinase kinase kinase 1

MEF: mouse embryonic fibroblast

MMC: mitomycin C

NER: nucleotide excision repair

NF-κB: nuclear factor-κB

NHEJ: non-homologous end joining

OUT: ovarian tumour protease

PALB2: partner and localized of BRCA2

PCNA: proliferating cell nuclear antigen

PD: Parkinson's disease

PE: phosphatidylethanolamine

PHD: plant homeodomain

PIP: phosphatidylinositol

PIP2: phosphatidylinositol-4,5-bisphosphate

PIP-box: PCNA-interacting protein

PKA: protein kinase A

PKCι: protein kinase Cι

PP2A: protein phosphatase 2A

RANKL: receptor activator of NF-κB ligand

RFC: replication factor C

RPA: replication protein A

SCF: Skp1-Cullin-F-box protein complex

TLS: translesion synthesis

TNF: tumour necrosis factor

TRAF2: TNF receptor-associated factor 2

UAF1: USP1-associated factor 1

Ub: ubiquitin

UBA: ubiquitin-associated domain

UBC: ubiquitin-conjugating enzyme

UBD: ubiquitin-binding domain

UBE2T: ubiquitin-conjugating enzyme E2T

UBM: ubiquitin-binding motif

UBZ: ubiquitin-binding zinc finger

UCH: ubiquitin C-terminal hydrolase

UIM: ubiquitin interacting motif

ULP: ubiquitin-like protein

USP1: ubiquitin-specific peptidase 1

UV: ultraviolet

XPV: Xeroderma pigmentosum

ZnF-UBP: zinc finger ubiquitin-specific protease domain

THESIS ABSTRACT

The calpains are a family of calcium-regulated intracellular cysteine proteases, among which the best studied isoforms, micro-calpain (CAPN1) and milli-calpain (CAPN2), are heterodimers consisting of a catalytic subunit and a common regulatory subunit, CAPNS1, required for function. Calpain is involved in many processes important for cancer biology, such as autophagy, indeed in calpain-depleted cells autophagy is impaired, with a subsequent increase in apoptosis sensitivity. Calpain is also important in all the stages of the stress response.

A proteomic approach was employed for the identification of novel CAPNS1 interacting proteins. Proteins immunoprecipitating with endogenous CAPNS1 in HT1080 cell lysates were analyzed by Mass Spectrometry. We identified novel partners among which the deubiquitinating enzyme USP1, a key regulator of the DNA damage response and genome integrity maintenance via its specific action on FANCD2, involved in DNA repair and protection from chromosome instability, and PCNA, involved in the regulation of translesion DNA synthesis (TLS), that bypasses DNA lesions with low stringency basepairing requirements.

We performed co-immunoprecipitation assays in lysates of 293T cells and confirmed that the interaction was specific. Furthermore, we observed that calpain is able to bind a USP1 C-terminal deleted mutant, suggesting that USP1 first 523 aminoacids were sufficient for the binding.

To understand what is the biological effect exerted by calpain upon USP1, we depleted calpain activity in a series of cell lines, and followed the fate of endogenous USP1. We transfected CAPNS1 specific siRNAs, or treated cells with a specific inhibitor of calpain, and we observed a strong decrease in USP1 protein levels. This effect should be at a post-transcriptional level, since any significant change in USP1 mRNA levels was detected. We also obtained the same result by transfecting a siRNA specific for *CAPN1*, the gene encoding for the catalytic subunit micro-calpain.

Moreover, we studied the role of calpain in the PCNA-mediated switch between high fidelity replication and TLS upon UV irradiation. In mouse embryonic fibroblasts knockout for CAPNS1, USP1 downregulation is coupled to an increase in PCNA monoubiquitination. Moreover, CAPNS1-depleted U2OS cells showed an increase in the percentage of nuclei containing PCNA-induced foci upon UV irradiation.

Since we demonstrated that calpain can modulate an important regulator of DNA damage response such as USP1, we investigated whether calpain could have a role in genome integrity maintenance. CAPNS1-depleted cells showed a reduced rescue in DNA repair compared to control cells, suggesting that increased levels in PCNA monoubiquitination could lead to an increased amount of errore-prone TLS.

Calpain plays an important role in autophagy, so we asked whether USP1 degradation in absence of calpain activity could involve autophagic pathways. We first blocked macroautophagy by silencing *ATG5* gene, and we observed that USP1 was downregulated, suggesting that the depletion of ATG5 could lead to an increased activity of other degradation pathways. To impaire chaperone-mediated autophagy (CMA), we depleted a protein important for autophagosome formation, LAMP-2A. Also in this case we observed a decrease in USP1 protein levels, thus suggesting that USP1 could be alternatively degraded by different pathways. However, we observed that USP1 is stabilized upon inhibition of lysosomal enzymes, suggesting that USP1 may be degraded in the lysosome.

To better understand the mechanism by which calpain affect USP1 stability we search for an effect of calpain upon USP1 co-factor and activator UAF1/WDR48. CAPNS1-depleted cells showed UAF1/WDR48 downregulation, but UAF1/WDR48 overexpression only partially rescued USP1 protein levels in this cells. Furthermore, we provided evidences that calpain regulation of p35/p25 activator of Cdk5 can affect Cdh1 phosphorylation and thus APC/C-Cdh1 activity, leading to a regulation of USP1 stabilization.

In conclusion, we identified USP1 as a novel interactor of calpain, and we found that calpain is important for USP1 stability, since in its absence USP1 is downregulated. The importance of this novel regulation is strengthened by the recent findings that unveiled a role of USP1 in maintenance of a mesenchymal stem cell program in osteosarcoma, and thus placing calpain in a crucial regulatory position for cancer development.

AIM OF THE PhD THESIS

During my PhD program I have been working at Laboratorio Nazionale CIB. The main subject of interest in our group is calpain function, since the observation that the product of *GAS2*, one of genes specifically induced at growth arrest, is an inhibitor of milli-calpain and its overexpression sensitizes cells to apoptosis in a p53-dependent manner (Benetti et al., 2001). This observation indicates a pro-survival role for calpain in cancer cells treated with chemotherapeutics, and suggest that targeting calpain might be useful for the improvement of therapeutic protocols in cancer treatment. Calpain can be considered a biomodulator, indeed its proteolytic action produces large fragments that keep intact functional domains, thus modulating the function of substrate proteins. Indeed, calpain is involved in the regulation of several cellular processes, such as cellular migration, autophagy, cell death and survival, that are involved in tumorigenesis. As recently demonstrated at Laboratorio Nazionale CIB, in cells lacking calpain activity autophagy is less active, with a subsequent increase of apoptosis sensitivity (Demarchi et al., 2006). Moreover, I have been involved in the study of the role of calpain in oncogene-induced or genotoxic stress-induced senescence, a process that could be considered a barrier for cancer initiation. Indeed, we found that calpain small subunit 1 (CAPNS1) depletion impairs cellular senescence in BJ human fibroblasts upon Ras^{v12} induction and in HT1080 human fibrosarcoma cells upon treatment with sub-lethal doses of doxorubicin. Moreover, calpain depletion is coupled to a reduced level of H2AX phosphorylation in several cellular systems upon genotoxic stress. The perturbation of H2AX phosphorylation dynamics due to calpain depletion can reflect an increase in DNA damage or a defect in DNA repair. These results are contained in the following paper:

Demarchi F., Cataldo F., Bertoli C., Schneider C.; DNA damage response links calpain to cellular senescence. Cell Cycle 9:4, 755-760; February 15, 2010.

The observation that calpain is involved in senescence induction and DNA damage response, together with other observations obtained in the past years in our laboratory, such as that calpain is rapidly activated following radiation exposure and DNA damage, and its loss sensitizes cells to nutrient depletion and genotoxic stress (Demarchi et al., 2005; Demarchi et al., 2006), and that calpain depletion is coupled to ROS increase and FoxO transcription factors hyperactivation (Bertoli et al., 2009), led us to deeply investigate calpain involvement in stress response. To perform this task, we utilized a proteomic approach to identify new calpain interactors that could help us to clarify calpain role in DNA damage and stress response. We have identified the deubiquitinating enzyme USP1 (Ubiquitin-Specific Peptidase 1) as a novel CAPNS1 interactor through mass spectrometry analysis. USP1 is a key regulator of the DNA damage response and

genome integrity maintenance via its specific action on the DNA replication processivity factor PCNA (Huang et al., 2006) and FANCD2, a component of the Fanconi complex, involved in DNA repair and protection from chromosome instability (Nijman et al., 2005). The aim of this PhD thesis is to unravel the biological significance of the interaction between calpain and USP1, and in particular the work is divided into three parts:

1. The biochemical characterization of calpain/USP1 interaction
2. The study of the role of calpain in USP1 regulation
3. The investigation of the role of calpain in the regulation of DNA damage response

The unpublished results presented in this work will demonstrate that we identified USP1 as a novel interactor of calpain, and that calpain is important for USP1 stability and for some cellular processes that required USP1 activity such as the regulation of DNA damage response.

1. INTRODUCTION

1.1 THE CALPAIN SYSTEM

1.1.1 The calpain family, general aspects

Calpains are a well conserved family of calcium-dependent intracellular cysteine proteases, involved in the regulation of several cellular processes, such as signal transduction, cell proliferation, cell cycle progression, differentiation, apoptosis, membrane fusion and platelet activation (Suzuki et al., 2004). Deregulation of calpain activity is implicated in the development of diseases such as neuronal degeneration, Alzheimer, cancer, multiple sclerosis (Zatz and Starling, 2005).

Calpains are found in almost all eukaryotes and a few bacteria. They were originally defined as cytosolic proteases, showing calcium-dependent proteolytic activity at a neutral pH. Like other intracellular proteases, such as proteasome proteases and caspases, calpain activity is strictly regulated; however, unlike proteasome or lysosomal proteases, calpains act for proteolytic processing rather than for degradation (calpains cleave their substrates at one or few sites, usually keeping functional domains intact). The consequence of calpain-mediated proteolysis is the transformation of substrates proteins into new functional states, and this transformation affects various cellular functions. Therefore calpain is considered as an intracellular “modulator protease” (Sorimachi et al., 2011).

The name “calpain” was chosen to underline calcium dependence and the homology with the cysteine-protease papain. Indeed, calpains belong to the papain superfamily of cysteine proteases, but their similarities to papains and cysteine cathepsins are less significant than the similarities between different calpains (Sorimachi et al., 2011).

Currently there are 14 known human calpain isoform genes, characterized by the presence of a protease domain similar to that found in the most studied members of the family, micro(μ)-calpain and milli(m)-calpain (Figure 1.1), which were named on the basis of calcium concentration required for their activity *in vitro*.

1.1.2 Calpain structure and regulation

Conventional calpains, micro(μ)-calpain (or calpain 1) and milli(m)-calpain (or calpain 2), require calcium and neutral pH for proteolytic activity. Both micro- and milli-calpain are heterodimers consisting of a large catalytic subunit of 80 KDa molecular weight, and a small regulatory subunit of 28 KDa, calpain small subunit 1 (or calpain 4). The catalytic subunit differs between micro- and

milli-calpain: calpain 1, encoded by *CAPN1* gene, and calpain 2, encoded by *CAPN2* gene, respectively (Figure 1.1). The regulatory small subunit, encoded by *CAPNS1* gene, is common to both isoforms and is required for function (Goll et al., 2003).

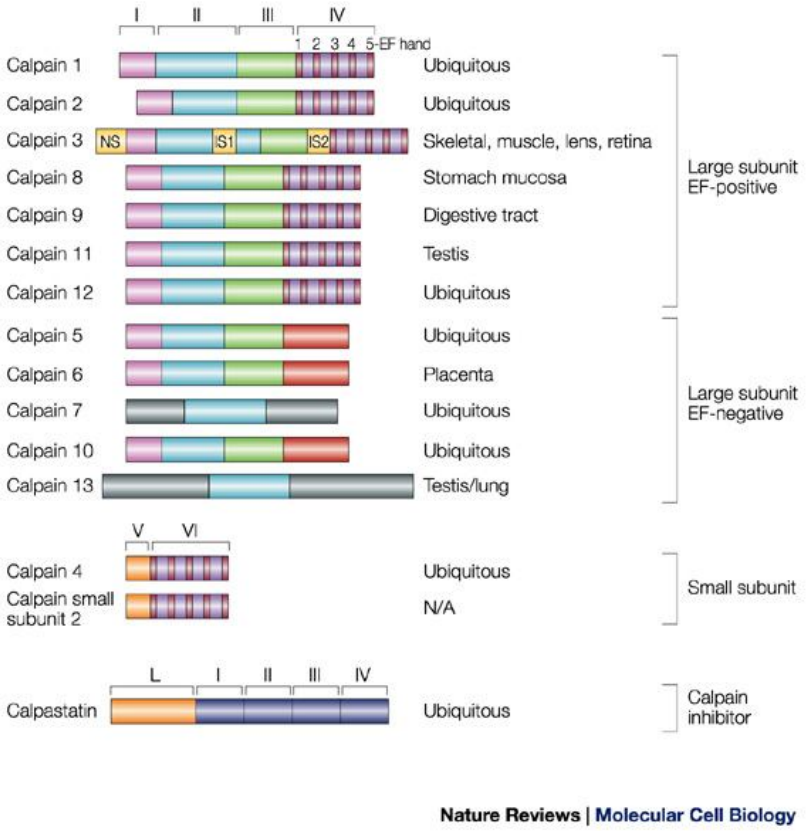

Figure 1.1 Schematic representation of the main structural domains of the mammalian calpain protein family and their endogenous inhibitor calpastatin (Frame et al., 2002).

Human CAPN1 (micro-calpain) and CAPN2 (milli-calpain) are 62,4% identical at the aminoacidic sequence level; accordingly, they have almost the same substrate and inhibitor specificities, and both are almost ubiquitously expressed. They differ for the *in vitro* calcium concentration required for proteolytic activity (micromolar and millimolar respectively) (Sorimachi et al., 2011).

The large subunits consist of four functional domains (DI to DIV). DI is a N-terminal short alpha-helix region, that is autolysed when calpains are activated by calcium, resulting in functionality at lower calcium concentrations, different substrate specificity and, in some cases, further subunit dissociation. Thus, this autolysis event constitutes one of the intrinsic regulatory mechanisms critical for calpain activity (Sorimachi, 2011). DII is the conserved protease domain, and is divided in two subdomains, IIa and IIb, which upon calcium binding form the catalytic cleft. This domain is rich in cysteine residues and is characterized by the catalytic triade Cys105-His262-Asn286. The distance and alignment of these residues in the inactive molecule don’t permit the activation,

indicating that a structural change is needed to allow calpain activation (Franco and Huttenlocher, 2005). Calpain is structurally inactive in the absence of calcium, and this is essential because calpain resides in the cytosol, where it is in direct contact with a large number of proteins and its activity must be strictly regulated (Sorimachi et al., 2011). DIII contains the C2 domain (a structural domain that is involved in membrane targeting), and is involved in structural changes during calcium binding. The carboxy-terminal DIV contains five EF-hands (a EF-hand unit consist of two helix linked by a calcium-binding loop), among which the fifth is needed for heterodimerization (Maki et al., 2002). Calpain small subunit 1 consist of two domains, DV and DVI, among which DVI contains EF-hands and together with DIV of large subunits helps heterodimerization (Figure 1.2).

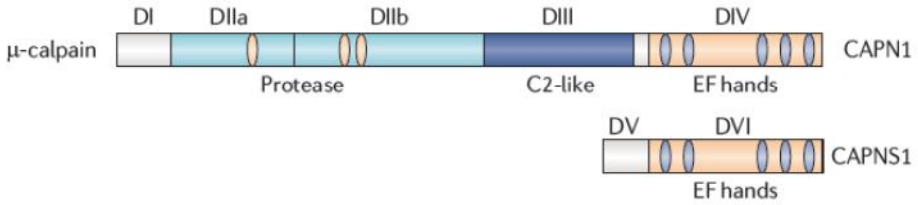

Figure 1.2 Schematic structure of μ-calpain heterodimer. CAPN1 (the large 80 KDa catalytic subunit of calpain 1) contains domains DIIa and DIIb (protease domains), as well as DIII and DIV (EF hand domains; shown by the purple ovals). CAPNS1 (the small 28 KDa regulatory subunit) contains domains DV and DVI. CAPNS1 associates with CAPN1 to form a heterodimer; an additional small subunit, CAPNS2, has also been described (not shown). The C2-like domain of the calpains has superficial similarity to the C2 domains of other enzymes, and influences calcium-induced activation of calpain. The enzymatic catalytic triad residues (orange ovals) of the calpains are located within the protease domain. (Storr et al., 2011).

The protease domain DII is conserved among calpain isoforms, but divergences exist between the others domains, thus not all calpain isoforms depend on calcium or require regulatory subunit for function. Within calpain family there are isoforms that are ubiquitously expressed, such as micro- and milli-calpain, and isoforms that are expressed in a tissue-specific manner, such as calpain 9, expressed in the digestive tract.

A deficiency in CAPNS1 triggers downregulation of CAPN1 and CAPN2 proteins, suggesting that the regulatory subunit is required for the stability of both catalytic subunits *in vivo*, probably acting as an intramolecular chaperone. CAPN2 (milli-calpain large subunit) shows full proteolytic activity *in vitro* after a long incubation; while in cells unfolded large calpains subunits are degraded before forming active conformations. Disruption of mouse *Capn1* or *Capn2* genes produces different phenotypes: *Capn1* -/- mice are normal and fertile (Azam et al., 2001), whereas *Capn2* -/- mice die

before blastocyst stage (Dutt et al., 2006), suggesting that micro- and milli-calpain differ in their function or expression levels at least during development. Cells from *Capns1* -/- mice were used to demonstrate the role of conventional calpains in specific cellular events. CAPNS1 is required for calcium-dependent repair of wounded plasma membranes (Mellgren et al., 2007) and, as demonstrated in our laboratory, for the induction of senescence (Demarchi et al., 2010) and macroautophagy (Demarchi et al., 2006). A recent study demonstrated that the calpain-calpastatin system play an essential role in embryogenesis by regulating the survival of placenta trophoblast. This result was obtained by the study of milli-calpain knockout mice, that died around embryonic day 15. The death was preceded by cell death associated with caspase activation and DNA fragmentation in placental trophoblast, that suggests that milli-calpain contributes to trophoblast survival through suppression of caspase activation. Moreover, by means of double-knockout mice it was shown that micro- and milli-calpain share at least in part similar *in vivo* functions under the control of calpastatin (Takano et al., 2011).

Calpain activity is finely regulated in the cell, indeed calcium concentration needed for function is higher than that normally found in cells. Thus, calpain activation is promoted by the reduction of calcium requirements, through mechanisms such as autolysis of DI domain (Imajoh et al., 1986), and interaction with membrane phospholipids (Cong et al., 1989). It was been shown that calcium induces an autoproteolysis event in which occurs the sequential formation of two forms of active calpain with molecular masses of 78 KDa and 75 KDa, respectively. Furthermore, when calpain binds to phospholipid vesicles, it undergoes autoproteolysis which mainly accumulates the 78 KDa species. Calpain calcium-induced conformational change is the triggering event that leads to the appearance of the active 78 KDa calpain, the predominant form of calpain at the membrane level (Melloni et al., 1996).

Several phospholipids enhance m-calpain autolysis at the plasma membrane, including phosphatidylinositol (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP2). Both μ-calpain and m-calpain can be phosphorylated by protein kinase Cι (PKCι), and this phosphorylation has been associated with increased cell migration and invasion of lung cancer cells. ERK directly phosphorylates m-calpain on a specific serine residue, influencing cell adhesion and motility, and this might decrease the calcium concentration that is required for m-calpain activation. In addition, protein kinase A (PKA) reduces m-calpain activity. Localization of m-calpain at the plasma membrane, through PIP2 anchorage, is important for the activation of the protease. The subcellular localization of calpains is complex and variable, thus indicating a dynamic regulation, and constitutes an important factor for their functions modulation (Franco and Huttenlocher, 2005). Moreover, calpain activity is negatively regulated by calpastatin (Wendt et al., 2004) and Gas2

(Benetti et al., 2001). Calpastatin is the ubiquitously expressed endogenous inhibitor of μ-calpain and m-calpain. It consists of an N-terminal L domain that contains an N-terminal XL region, and four repetitive inhibitory domains (I-IV), each one containing A, B and C regions. The structure of calpastatin allows it to reversibly inhibit up to four calpain heterodimers. The inhibitory action of calpastatin requires calcium-induced structural changes in calpain to allow the A, B and C regions within each inhibitory domain of calpastatin to bind to calpain, and region B to block the active site of calpain. Calpastatin forms intracellular storage aggregates, and is released into the cytosol following calcium influx to allow interaction with calpain. A single gene (*CAST*) encodes for calpastatin and it has multiple promoters that generate distinct isoforms with N-terminal variation. Both type I and type II calpastatin contain the L domain but have different N-terminal sequences generated from tandem promoters that are associated with exons 1xa and 1xb. Type III calpastatin is the product of a promoter that is associated with the untranslated exon 1u and results in a protein in which the XL region is absent from the L domain (Figure 1.3).

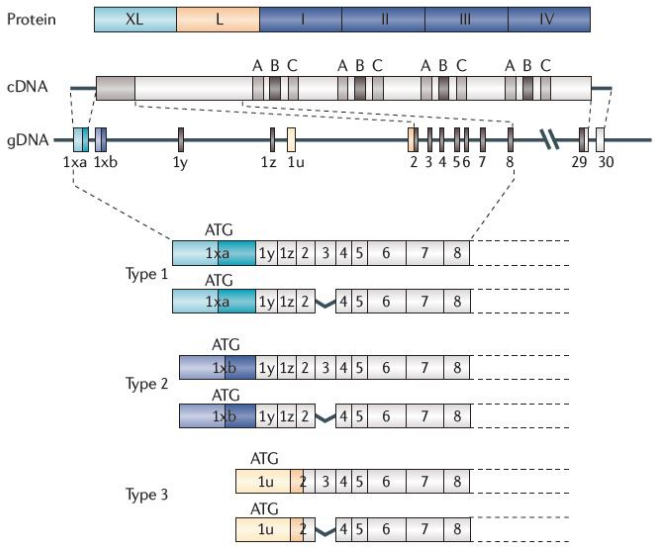

Figure 1.3 Schematic structure of calpastatin. The generic cDNA that encodes calpastatin contains the protein L domain with the XL region that is encoded at the amino-terminus, and the inhibitory domains I-IV (which each contain the subdomains A-C). The 5' (L domain) and 3' exons of the calpastatin gene are shown on the genomic DNA. The exons that are incorporated into transcripts through the initiation of transcription from putative promoters immediately 5' to exons 1xa, 1xb and 1u are indicated. Below are shown the 5' ends of the resulting transcripts that result in multiple protein variants that arise from differential splicing (Storr et al., 2011).

Type IV calpastatin is a testis-specific isoform that is generated from a promoter between exons 14 and 15 and lacks the L domain and the inhibitory domain I. These promoters can be differentially regulated in a tissue-specific manner and in response to agonists. In addition to the use of

alternative promoters, calpastatin transcripts are alternatively spliced in the L domain, and this allows numerous calpastatin polypeptides to be generated from one gene. The cellular consequence of multiple calpastatin splicing variants has not been fully elucidated; however, the absence of exon 6 has been shown to promote the formation of intracellular storage aggregates (Storr et al., 2011). Post-translational modifications of calpastatin are an important event for the regulation of this protein, indeed it was demonstrated that calpastatin derived from rat skeletal muscle and from rat brain is subjected to *in vitro* phosphorylation by PKC and PKA, and this modification can modulate calpastatin specificity (Salamino et al., 1994) or inhibitory efficiency (Salamino et al., 1997). More recently, it was shown that in human neuroblastoma cells PKA triggers calpastatin phosphorylation, and this event lead to aggregation of calpastatin, while calcium influx lead to disaggregation, through a protein phosphatase activity. This reversible phosphorylation event regulates the amount of soluble calpastatin available to bind and inhibit calpain (Averna et al., 2001).

A gene product with a specific calpain inhibitory activity has been characterized at Laboratorio Nazionale CIB: the growth arrest specific gene 2 (*Gas2*) (Benetti et al., 2001). The growth arrest specific genes have been identified as specifically induced during the G0 arrest phase of the cell cycle in NIH3T3 fibroblasts, indeed their expression was induced upon prolonged starvation in low serum (Schneider et al., 1988). Gas2 is a component of the microfilament system and colocalizes with actin fibers (Brancolini et al., 1992), and it has been shown to bind domain III and IV of milli-calpain through its amino-terminal domain, while its carboxyl-terminal region exerts the inhibitory function. The isolated amino-terminal region has been demonstrated to act as a dominant negative form of Gas2 (Gas2DN), being able to bind, but not to inhibit, calpain activity and to rescue *in vivo* the effects of Gas2 on calpain function (Benetti et al., 2001).

1.1.3 Calpain physiological functions

Calpain is involved in a number of processes essential for cellular biology, among which the best characterized is cellular migration. Initial research showed a role of calpain in the regulation of integrin-mediated cell adhesion through the observation that m-calpain localized to integrin-associated focal adhesion structures and cleaved the focal adhesion protein talin (Beckerle et al., 1987). Subsequently it was demonstrated that this event was influenced by MAP3K1 (MEKK1) through the downstream activation of calpain (Cuevas et al., 2003). Studies in which calpain activity is suppressed by a combination of gene ablation, pharmacological inhibition and RNA interference (RNAi), further showed the involvement of m-calpain in the regulation of focal adhesion turnover and cell migration in tumour-derived cell models (Mamoune et al., 2003). Beyond the cleavage of focal adhesion kinase (FAK) and talin, calpain can cleave numerous other

substrates to promote cellular motility, such as paxillin (Yamaguchi et al., 1994), fodrin (Sato et al., 2004), ezrin (Wang et al., 2005), vinculin (Serrano and Devine, 2004), and alpha-actinin (Selliah et al., 1996). Recently, calpain-mediated cleavage of paxillin has been implicated in negative regulation of focal adhesion dynamics and reduced cellular migration (Cortesio et al., 2011). Other studies showed alternative pro-migratory mechanisms for calpain, such as regulation of membrane protrusion at the leading edge of migrating cells, that seems to be dependent on the proteolytic cleavage of proteins that modify the actin cytoskeleton, such as cortactin (Perrin et al., 2006) (Figure 1.4).

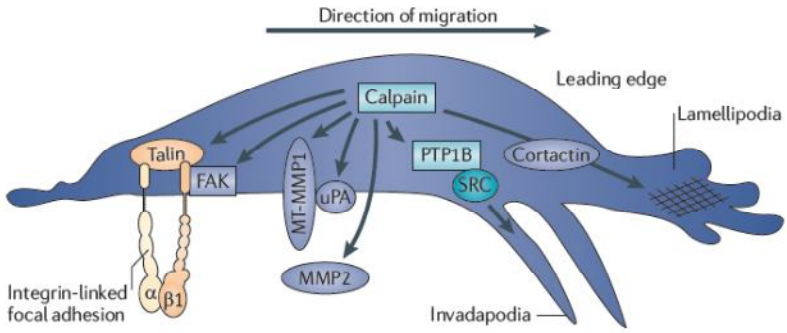

Figure 1.4 Role of calpain in cell migration. It is likely that no single calpain-mediated proteolytic event promotes cell migration in isolation but rather that several events coordinate the calpain-mediated proteolysis of other substrates and other distinct signal transduction pathways to promote cell motility (Storr et al., 2011).

Calpain can promote both apoptosis and cell survival in dependence of cellular context, including the type of apoptotic stimuli, expression and cellular localization of the protein (Tan et al., 2006a).
Calpain activity has been implicated in the pro-survival activities of both the tumour suppressor protein p53 and nuclear factor-κB (NF-κB). Calpain is able to cleave wild-type p53, regulating protein stability to prevent p53-dependent apoptosis (Gonen et al., 1997; Kubbutat and Vousden, 1997; Pariat et al., 1997; Atencio et al., 2000). Growth arrest-specific protein 2 (GAS2), a protein which is cleaved during apoptosis to allow rearrangement of the actin cytoskeleton, can physically associate with calpain to prevent p53 cleavage resulting in enhanced p53 stability (Benetti et al., 2001). Moreover, calpain can promote survival through the activation of NF-κB, by cleaving of its inhibitor IκBα. Calpain-mediated IκBα cleavage can occur in response to tumour necrosis factor (TNF) (Han et al., 1999), and activation of the epidermal growth factor receptor (EGFR) family member ERBB2 in breast cancer (Pianetti et al., 2001). Moreover, μ-calpain can regulate receptor activator of NF-κB ligand (RANKL)-supported osteoclastogenesis by activating NF-κB (Lee et al., 2005). Proteolysis of IκBα has been reported to be m-calpain, μ-calpain or calpain 3-mediated, and can be inhibited by overexpression of calpastatin.

Calpain is responsible for the proteolysis of various substrates that can sensitize cells to apoptosis, such as the transcription factor MYC (Small et al., 2002), and calpain activity can also affect the cell cycle, through mechanisms that include cleavage of cyclin E to a more active low-molecular-mass form in breast cancer (Wang et al., 2003), and altered cellular location of m-calpain during mitosis (Schollmeyer, 1988). In addition, calpain can interfere with the interaction between protein phosphatase 2A (PP2A) and AKT to prevent forkhead box O (FOXO)-mediated cell death (Bertoli et al., 2009). Interestingly, PP2A can also negatively regulate calpain during cell migration (Xu and Deng, 2006).

On the other hand calpain shows a pro-apoptotic activity. Indeed several studies have shown that calpains cooperate with the caspase machinery in the induction of apoptosis. Evidences indicate that caspase 7, caspase 9, caspase 10 and caspase 12 are all subject to calpain-mediated cleavage. Caspase 10 and caspase 7 are both activated by calpain cleavage, whereas caspase 9 is inactivated (Chua et al., 2000). Calpain can facilitate apoptosis through the cleavage of various members of the apoptosis regulating BCL-2 family, including promoting apoptosis through BAX and BID-mediated cytochrome c release, and cleavage of BCL-2 to allow BAX translocation into the mitochondria (Wood et al., 1998).

Calpain is able to activate several other substrates that are involved in promoting apoptosis, such as CDK5 (Lin et al., 2006), APAF1 (Fettucciari et al., 2006), JNK (Tan et al., 2006b), JUN and FOS (Pariat et al., 2000).

Calpain may act as a barrier against cancer progression, since it was been demonstrated that regulates β-catenin signalling functions (Benetti et al., 2005). β-catenin is a protein involved both in cell adhesion and in the Wnt pathway, thus it is an essential regulator of embryogenesis and tumorigenesis. β-catenin levels are kept in control by the adenomatous polyposis coli-axin-glycogen synthase kinase 3β complex, which targets it for proteasomal degradation. Calcium release from internal stores leads to nuclear export and calpain-mediated degradation of β-catenin in the cytoplasm. Calpain plays an important role in the control of β-catenin levels and functions, since calpain knockdown induces an increase in the free transcriptional competent fraction of endogenous β-catenin. The known calpain inhibitors, Gas2 and Calpastatin, can control levels, function and localization of β-catenin through endogenous calpain regulation. Indeed, Gas2 dominant negative (Gas2DN), by counteracting the action of both Gas2 and calpastatin, is able to enhance calpain effect on β-catenin, independently of the classical glycogen synthase kinase 3β and proteasome pathway. Indeed, Gas2DN can increase the activity of calpain and induce degradation of stabilized or mutated β-catenin. Interestingly, in a cellular model where the classical proteasome pathway is

impaired, such as in colon cancer cells, Gas2DN leads to a significant reduction in proliferation and anchorage-independent growth (Benetti et al., 2005).

Furthermore, calpain has a role in autophagy, a process in which damaged proteins and organelles are sequestered into autophagosomes which fuse with lysosomes and are degraded to maintain cellular homeostasis. Autophagy acts as a double-edged sword in oncology. On the one hand it is implicated in tumour cell survival in which defective apoptotic pathways prevail, on the other hand it is implicated in tumour suppression (Mathew et al., 2007; Ravikumar et al., 2010) (Figure 1.5).

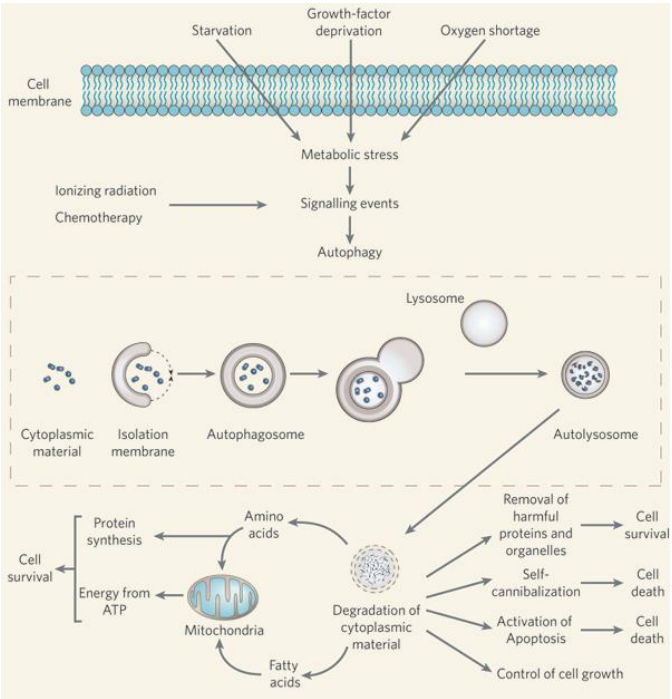

Figure 1.5 Schematic representation of the autophagic process. Both metabolic stress and cancer therapies activate signalling pathways that stimulate autophagy. The process involves the sequestration of cytoplasmic material by a membrane-bound vesicle called the autophagosome, which then fuses with a lysosome to form an autolysosome. The degradation of cytoplasmic material within the autolysosome can promote cell survival either by generating free fatty acids and aminoacids, which can be recycled to maintain energy production and protein synthesis, or by removing harmful proteins and organelles. It can also promote cell death independently (presumably through self-cannibalization) or together with apoptosis. Furthermore, the turnover of proteins and organelles by autophagy may contribute to the control of cell growth (Levine, 2007).

Macroautophagy is a process responsible for the degradation of the majority of intracellular proteins in mammalian cells. Cytosolic proteins, organelles and portion of the nucleus can be degraded by autophagy to maintain cellular homeostasis and remove damaged or unwanted products (Klionsky, 2005). This process requires subsequent steps: first of all cytoplasmic proteins or organelles are enclosed by a double-membrane autophagosome, next the outer membrane of the autophagosome

fuse with a lysosome, and this lead to the destruction of the cargo and the inner membrane by hydrolytic enzymes (Figure 1.5). The autophagic pathway has been dissected at the molecular level in yeast, where 27 autophagic genes (*ATG* genes) were identified. Calpain-mediated cleavage of autophagy-related 5 (ATG5) provides a link between autophagy and apoptosis. Indeed, ATG5 is involved in the formation of autophagosomes that encapsulate proteins and organelles for lysosomal degradation, and cleavage of ATG5 promotes the translocation of truncated ATG5 to mitochondria where it associates with BCL-XL and results in cytochrome c release, possibly by blocking the function of BCL-XL (Yousefi et al., 2006). Studies performed in this laboratory (LNCIB) demonstrated that the lack of calpain leads to a strong increase in apoptotic cell death (Demarchi et al., 2005), and subsequently that calpain was required for macroautophagy (Demarchi et al., 2006) in two systems lacking calpain activity, such as mouse embryonic fibroblasts (MEFs) derived from CAPNS1 knockout mice and CAPNS1-silenced human cells. The measurement of autophagic activity was achieved in this cells by means of different approaches, such as the analysis of endogenous and exogenous LC3 levels. LC3 (MAP-LC3, microtubule-associated protein light chain 3), the mammalian orthologue of yeast Atg8, is the first protein identified on the autophagosome membrane and requires several post-translational modifications before targeting to autophagosomes. The C-terminal region of LC3 is cleaved after its synthesis, and the resulting processed form, called LC3-I, is conjugated to phosphatidylethanolamine (PE). LC3 PE-conjugated form, called LC3-II, is tightly associated with the autophagosome. LC3 autophagosomal localization is induced by different autophagic stimuli, and for this reason LC3 is considered one of the best markers for monitoring the autophagic process (Kabeya et al., 2000).

The data obtained by this work indicate that in CAPNS1-deficient cells the autophagic program is not efficiently activated. As a result of this defect, CAPNS1-deficient cells are more sensitive to apoptosis induced by several autophagic stimuli, including ceramide, etoposide, and starvation (Demarchi et al., 2006).

1.1.4 The autophagic pathways

In mammalian cells, three different mechanisms contribute to the degradation of intracellular components inside lysosomes. Two of these mechanisms, macroautophagy and microautophagy, are processes that allow the simultaneous sequestration of multiple cytosolic constituents (soluble proteins and organelles) and their degradation in the lysosomal lumen. In contrast, chaperone-mediated autophagy (CMA) allows the lysosomal degradation of specific cytosolic proteins on a molecule-by-molecule basis. The selectivity of this pathway is achieved by means of the recognition of a pentapeptide amino acid motif (KFERQ consensus sequence) in the CMA

substrates by a cytosolic chaperone (Heat Shock Cognate protein of 70 KDa, HSC70). The substrate-chaperone complex is targeted to the lysosomal surface, where it interacts with the lysosome-associated membrane protein (LAMP) type 2A, a lysosomal membrane receptor for this pathway. After unfolding, the substrate translocates into the lysosomal lumen, assisted by a luminal chaperone (lys-hsc70), where it is rapidly degraded (Figure 1.6).

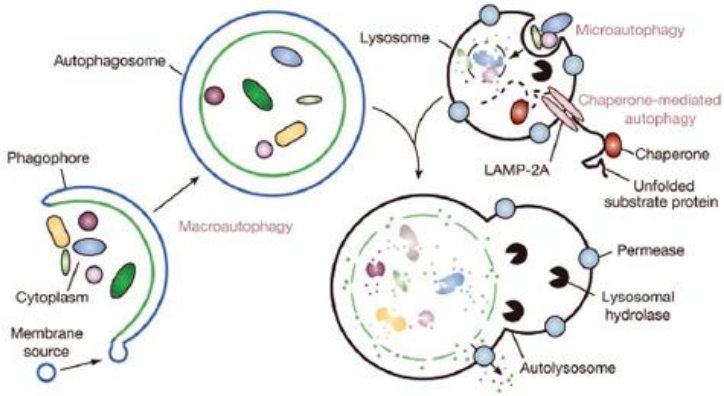

Figure 1.6 Schematic representation of different autophagic pathways: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA).

Binding of substrate proteins to LAMP-2A is a limiting step for CMA. Levels of LAMP-2A at the lysosomal membrane are tightly controlled and constitute a regulatory mechanism for CMA. Up-regulation of CMA occurs during prolonged nutritional stress (starvation), exposure to toxic compounds, and mild oxidative stress, suggesting a role for this pathway in the selective removal of abnormal or damaged proteins under these conditions. In addition, a role for CMA in antigen presentation has recently been described (Zhou et al., 2005). CMA activity decreases during aging, and a blockage of this pathway by mutant forms of synuclein also occurs in familial forms of Parkinson's disease (PD). However, because of the complexity of the phenotypes associated with both aging and PD, the direct consequences of blockage of CMA in these systems are difficult to understand. The *LAMP-2* gene undergoes alternative splicing that gives rise to at least three different splicing variants (LAMP-2A, LAMP-2B, and LAMP-2C). These LAMP-2 variants are present in different intracellular locations and probably have both common and isoform-specific functions. LAMP-2A is the only isoform known to participate in CMA, whereas a role for LAMP-2B in macroautophagy has been proposed. As in Danon disease, a human vacuolopathy resulting from mutations in the *LAMP-2* gene, *LAMP-2* knockout mice (lacking all three LAMP-2 variants) present a severe phenotype with major alterations in lysosomal biogenesis and autophagy. The selective blocking of the expression of LAMP-2A in cultured fibroblasts without affecting the other

two LAMP-2 isoforms, indicate the existence of cross-talk between different forms of autophagy, because macroautophagy is up-regulated in cells with impaired CMA. Despite this compensatory mechanism, cells with reduced CMA activity are more sensitive to many stressors, supporting an essential role for CMA as part of the cellular response to stress (Massey et al., 2006).

1.1.5 Calpain in human diseases

Although *Capn1*-knockout mice show no evident phenotype, *Capn2*- and *Capns1*-knockout mice are embryonic lethal, thus suggesting that calpain is essential for embryogenesis. Whereas defects in ubiquitous calpains may be lethal, defects in tissue-specific calpains may cause tissue-specific symptoms, such as muscular dystrophy with defective CAPN3 or *Capn3* gene. On the other hand, conventional calpains could be over-activated in muscular distrophies, cardiomyopathies, traumatic ischemia and lissencephaly, probably because calcium homeostasis is compromised in these diseases. Conventional calpains inhibitors are currently used to prevent the progression of such diseases (Sorimachi, 2011).

Although calpain physiological functions and mechanisms of activation remains to be fully elucidated, experimental studies have demonstrated clear roles of calpain in important cellular processes. Moreover calpain activity is implicated in pathology of several diseases, including cancer, neurotoxicity and limb-girdle muscular dystrophy type 2A (LGMD2A). Indeed, in 1995 CAPN3 (p94) mutations were shown to be responsible for limb-girdle muscular dystrophy type 2A (LGMD2A), also called calpainopathy (Richard et al., 1995).

Calcium sensitivity is functionally important for calpain, as physiologic concentrations of calcium range from 100-1000 nM (Chan and Mattson, 1999) and rise to 5-10 µM during excitotoxic conditions (Hyrc et al., 1997); therefore, it is expected that µ-calpain would be affected by small changes in calcium concentrations, while m-calpain is likely activated by intracellular signaling via phosphorylation by protein kinase A (PKA). Following calcium stimulation, the 80 KDa subunit is autocatalytically processed to a 76 KDa fragment, and the 28 KDa regulatory subunit is processed to 18 KDa (Goll et al., 1992). Alterations in calcium homeostasis lead to persistent, pathologic activation of calpain in a number of neurodegenerative diseases. Pathologic activation of calpain results in the cleavage of a number of neuronal substrates that negatively affect neuronal structure and function, leading to inhibition of essential neuronal survival mechanisms, in the acute neurodegenerative diseases such as cerebral ischemia and in the chronic neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, multiple sclerosis, prion-related encephalopathy, and amylotrophic lateral sclerosis (Vosler et al., 2008).

Aberrant expression of calpain has been implicated in tumorigenesis. For instance, increased expression of micro-calpain is observed in schwannomas and meningiomas (Kimura et al., 1998), CAPN1 mRNA in renal carcinoma (Braun et al., 1999), and m-calpain in colorectal adenocarcinomas (Lakshmikuttyamma et al., 2004). Decreased expression of calpastatin has been observed in endometrial cancers (Rios-Doria et al., 2003).

Other members of calpain family are involved in cancer biology, indeed increased expression of calpain 6 was observed in uterine sarcomas and carcinosarcomas (Lee et al., 2007) and in uterine cervical neoplasia (Lee et al., 2008); decreased expression of calpain 3 in melanoma (Moretti et al., 2009) and of CAPN9 in gastric cancer (Yoshikawa et al., 2000). Moreover, alteration of CAPN10 expression levels have been linked to laryngeal (Moreno-Luna et al., 2011), colorectal (Frances et al., 2007) and pancreatic cancer (Fong et al., 2010).

1.2 UBIQUITINATION AND DEUBIQUITINATING ENZYMES

1.2.1 The ubiquitination pathway

Ubiquitination (or ubiquitylation) is a reversible post-translational modification of cellular proteins, in which a 76-amino acid polypeptide, ubiquitin (Ub), is primarily attached to the ε-amino group of lysines in target proteins. The selective degradation of many short-lived proteins in eukaryotic cells is carried out by the ubiquitin-proteasome system (Hershko and Ciechanover, 1998). Moreover, ubiquitin can act as a signal to target proteins to specific localization in the cell, so ubiquitination is a major player in regulating several cellular processes, including cell division, differentiation, signal transduction, and protein trafficking. Aberrations in the ubiquitination system are implicated in pathogenesis of some diseases, malignancies, neurodegenerative disorders, and pathologies of the inflammatory immune response (Mukhopadhyay and Riezman, 2007).

The process of marking a protein with ubiquitin consists of a series of steps:

1. Activation of ubiquitin in a two-step reaction by an E1 ubiquitin-activating enzyme in a process requiring ATP as energy source. The initial step consists in the production of a ubiquitin-adenylate intermediate. In the second step, ubiquitin is transferred to the E1 active site cysteine residue, with release of AMP. This step results in a thioester linkage between the C-terminal carboxyl group of ubiquitin and the E1 cysteine sulfhydryl group.
2. Transfer of ubiquitin from E1 to the active cysteine of a ubiquitin-conjugating enzyme (UBC) E2 via a trans-thio-esterification reaction. Mammalian genomes contain 30-40 UBCs.

3. The final step of the ubiquitination creates an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin. Generally, this step requires the activity of one of the hundreds of E3 ubiquitin-protein ligases (often termed simply ubiquitin ligase). E3 enzymes function as the substrate recognition molecules of the system and are capable of interaction with both E2 and substrate (Figure 1.7).

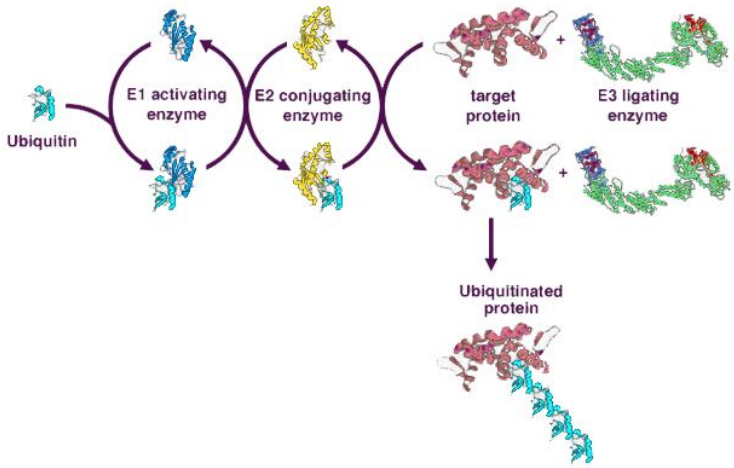

Figure 1.7 Steps of the ubiquitination process.

In the ubiquitination cascade, E1 enzymes can bind with dozens of E2s, which can bind with hundreds of E3s in a hierarchical way. Other ubiquitin-like proteins (ULPs) are also modified via the E1-E2-E3 cascade (Kirisako et al., 2006). The anaphase-promoting complex (APC) and the SCF complex (Skp1-Cullin-F-box protein complex) are two examples of multi-subunit E3s involved in recognition and ubiquitination of specific target proteins for degradation by the proteasome. Following addition of a single ubiquitin moiety to a protein substrate (monoubiquitination), further ubiquitin molecules can be added to the first, obtaining a polyubiquitin chain. Moreover, some substrates are modified by addition of ubiquitin molecules to multiple lysine residues in a process termed multiubiquitination. Ubiquitin has seven lysine residues that may serve as points of ubiquitination, they are: K48, K63, K6, K11, K27, K29 and K33. These different linkages may define unique signals that are recognized by ubiquitin-binding proteins, through recognition by ubiquitin interacting motifs (UIMs) that bind to ubiquitin.

Historically, the original type of ubiquitin chains identified were those linked via lysine 48, however, more recent work has uncovered a wide variety of linkages involving all possible lysine residues and in addition chains assembled on the N-terminus of a ubiquitin molecule ("linear chains") (Xu and Peng, 2006).

The most studied polyubiquitin chains (lysine 48-linked) target proteins for destruction via the proteasome, a process known as proteolysis. At least four ubiquitin molecules must be attached to lysine residues on the target protein in order for it to be recognised by the 26S proteasome subunit. The proteasome is a complex, barrel-shaped structure with two chambers, within which proteolysis occurs. Proteins are rapidly degraded into small peptides (usually 3-24 amino acid residues). Ubiquitin molecules are cleaved off the protein immediately prior to destruction and are recycled for further use.

Lysine 63-linked chains direct the localization of proteins. Monoubiquitination of proteins also targets the localization of proteins (Thrower et al., 2000). Ubiquitin can also mark trans-membrane proteins (for example receptors) for removal from membranes with consequent release within the cell where they can fulfill several signaling roles.

1.2.2 The deubiquitinating enzymes

Deubiquitinating enzymes (DUBs) are proteases that cleave ubiquitin removing monoubiquitination or modifying polyubiquitin chains on target proteins. The human genome encodes for 79 deubiquitinating enzymes (DUBs) that act oppositely to E3 ligases (Nijman et al., 2005) and can be divided into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumour proteases (OTUs), Josephins and JAB1/MPN/Mov34 metallo-enzymes (JAMM). UCH, USP, OTU and Josephin families are cysteine-proteases, while JAB1/MNP/Mov34 family members are zinc-dependent metallo-proteases. A common characteristic of DUBs is the abundance of ubiquitin-binding domains, including zinc finger ubiquitin-specific protease domains (ZnF-UBPs), ubiquitin-interacting domains (UIMs), and ubiquitin-associated domains (UBAs), that bind ubiquitin at low affinity (Komander et al., 2009).

Like other proteases, DUBs regulation is important to avoid unwanted cleavage of non-substrate proteins. A regulatory mechanism is the conformational change induced by the substrate binding. For example, USP deubiquitinating enzymes undergo conformational changes upon binding to ubiquitin, that include: alignment of the catalytic triad in an active conformation in USP7, displacement of active site loops that would otherwise block the binding of the C-terminal tail of ubiquitin in USP14, or movement of the finger domain in order to accommodate the globular body of ubiquitin in USP8. USP5 (more commonly known as isopeptidase T or IsoT) hydrolyzes unanchored polyubiquitin chains *in vitro* and *in vivo*. A free ubiquitin carboxyl-terminus is required at the proximal end of the chains (the end where the chain would be attached to a target protein) to achieve efficient catalysis. Binding of free ubiquitin to the N-terminal ZnF-UBP domain induces an

allosteric conformational change that lead to an increased catalytic rate for ubiquitin chain processing.

Other regulation mechanisms include the binding with a scaffold or adapter protein, transcriptional regulation or post-translational covalent modifications, and subcellular localization.

There is a strong correlation between phosphorylation and ubiquitination (Hunter, 2007). For example, inducible phosphorylation of CYLD inhibits its capacity to deubiquinate TNF receptor-associated factor 2 (TRAF2), and suppress NF-κB signalling, and USP8 constitutive phosphorylation in interphase can inhibit its isopeptidase activity. Some DUBs can be subjected to post-translational catalytic cleavages, such as USP1 that undergoes an auto-proteolytic event (Huang et al., 2006). Several alternative examples of allosteric activation by interacting proteins have been described. The WD40 domain containing protein USP1-associated factor 1 (UAF1; also known as WDR48 or p80) is found in a heterodimeric complex with at least three USPs (USP1, USP12 and USP46). *In vitro* analysis shows a major increase in the catalytic rate of all three DUBs caused by WD40 domain-dependent binding of UAF1, but not for several other USP family members. Interestingly, WD40 domains are highly enriched in a global survey of proteins that interact with 75 DUBs. Other DUBs require the incorporation into large macromolecular complexes, such as 26S proteasome subunit. Such large complexes can simply act as scaffolds to substate presentation, or could directly regulate catalytic activity, for example through allosteric interaction (Komander et al., 2009).

The localization of a protein is often linked to its activation. Indeed, several DUBs, such as USP8 and CYLD, have been shown to undergo epidermal growth factor (EGF)-dependent translocation to endosomes or association with a phosphotyrosine associated protein interaction network. Subcellular localization will determine the spectrum of substrates available for processing. Only USP19 and USP30 have predicted transmembrane domains. USP30 has been localized to mitochondria and implicated in the regulation of mitochondrial morphology. Several DUBs show nuclear accumulation, and USP36 specifically localizes to the nucleolus and regulates its structure and function.

DUB activities can be grouped in three categories. First, ubiquitin is transcribed from several genes (in mammals there are four ubiquitin genes, two of which encoding polyubiquitin, Ubb and Ubc, and the other two encoding fusions with ribosomal proteins) (Kimura and Tanaka, 2010) as a linear fusion of multiple ubiquitin molecules or with ribosomal proteins, thus DUBs activity is required for the generation of free ubiquitin molecules. Second, DUBs can remove ubiquitin from post-translationally modified proteins, leading to protein stabilization by rescue from proteasomal degradation. On the other hand, once a protein is targeted to proteasomal degradation DUBs can act

by recycling ubiquitin molecules, contributing to ubiquitin homeostasis. Third, DUBs can modify polyubiquitin chains (Komander et al., 2009) (Figure 1.8).

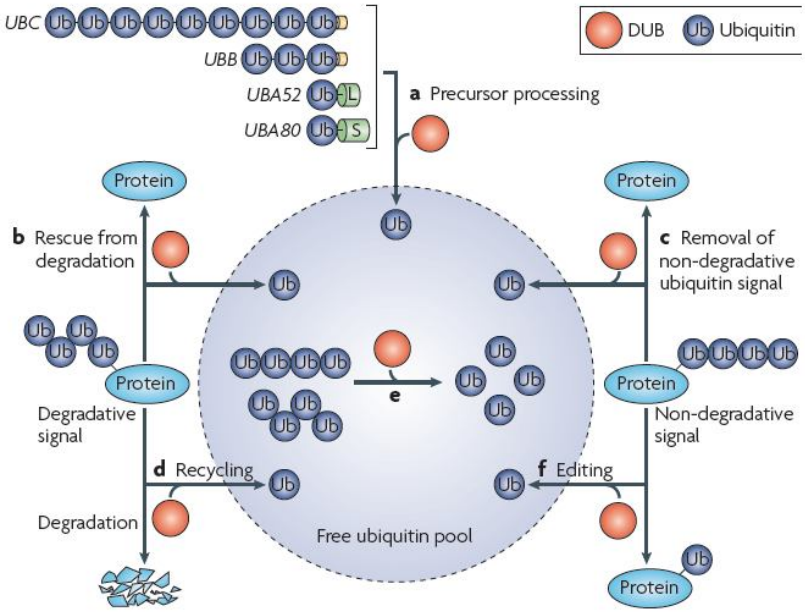

Figure 1.8 General roles of DUBs. (a) Ubiquitin is encoded by four genes and is transcribed and translated as a linear fusion consisting of multiple copies of ubiquitin or ubiquitin fused to the amino terminus of two ribosomal proteins, 40S ribosomal protein L40 (L) and 60S ribosomal protein S27a (S). Generation of free ubiquitin from these precursors is a key function of deubiquitinases. (b) Deubiquitination can rescue proteins from degradation.
(c) Deubiquitination can remove a non-degradative ubiquitin signal. (d) DUBs have a crucial role in maintaining ubiquitin homeostasis and preventing degradation of ubiquitin together with substrates of the 26S proteasome and lysosomal pathways (recycling of ubiquitin). (e) Disassembly of ubiquitin chains generated by the removal from substrates ensures that recycled ubiquitin re-enters the free ubiquitin pool. (f) Some DUBs might function to modify ubiquitin chains and thereby help to exchange one type of ubiquitin signal for another (Komander et al., 2009).

DUBs activity *in vitro* is often very low, suggesting that the regulation can be due to the cellular context; moreover, the substrate binding site can be occluded and the catalytic triad of some DUBs seems to be in an inactive conformation, that could indicate that a conformational change is necessary for activation.

One of physiological functions of DUBs is the regulation of protein stability, since deubiquitination can preserve proteins from proteasomal or lysosomal degradation and increase their stability. Indeed, Lys 63-linked polyubiquitin chains or multiple monoubiquitination establish sorting of targeted proteins to the lysosome through engagement with the ESCRT machinery, whereas Lys 48-linked polyubiquitin chains and probably most other ubiquitin linkages can target the protein for proteasomal degradation. If the accumulation of a target protein results deleterious for the cell, for example if the protein is the product of a proto-oncogene, the specific DUB might be an attractive

target for pharmaceutical treatment, with the aim of substrate destabilization. This is the case of USP28 that stabilizes the proto-oncogene MYC, or USP7 that stabilizes p53 and its E3 ubiquitin ligase MDM2.

Many DUBs could be found in association with E3 ligases that have an intrinsic tendency to self-ubiquitinate, and reciprocally E3 ligases can stabilize DUBs through ubiquitination (Li et al., 2002). E3 ligases-DUBs interaction can allow a fine regulation of the ubiquitination of a common substrate.

Another function of DUBs is the regulation of homeostasis and recycle of ubiquitin. When a protein is targeted for degradation, ubiquitin molecules are recycled, and seems that proteasomal degradation of ubiquitinated substrates is linked to the recycling activity of the JAMM/MPN+ DUB POH1 in mammals, one of three 26S proteasomal DUBs. POH1 is part of the structural complex of 19S proteasome lid, that recognize proteasomal substrates and regulates entry into the proteolytic centre of 26S complex. Like other JAMM/MPN+ proteins, POH1 shows specificity for Lys63-linked polyubiquitin chains, but can also cleave the first isopeptide bond that links the substrate and the proximal ubiquitin. The other two proteasomal DUBs, UCH37 and USP14, have a redundant function, indeed ubiquitinated proteins accumulate only when both are knocked down, indicating a defect in the ubiquitin-proteasome system. Nevertheless, the proteolytic activity of the 26S proteasome towards a peptide substrate is unimpaired.

Moreover, deubiquitination can act to negatively regulate ubiquitin signaling. The identification of more than 15 UBDs (ubiquitin binding domains) has strengthened the emerging concept that reversible ubiquitination might have key signalling roles independent of protein degradation, like phosphorylation. Dynamic ubiquitination of histones regulates chromatin structure, and the role of ubiquitin in DNA damage repair pathways is also firmly established. Condensation of chromosomes in metaphase is accompanied by deubiquitination of histones H2A and H2B113. Four enzymes (MYSM1, USP3, USP16 and USP22) have been proposed to directly remove ubiquitin from ubiquitinated histone H2A. MYSM1, USP16 and USP22 might share a common role in the transcriptional control of potentially distinct gene cohorts, and USP22, USP16 and USP3 DUB activities have been implicated in cell cycle progression. USP3, which can remove ubiquitin from both H2A and H2B histones, might indirectly influence the cell cycle through effects on DNA damage repair pathways, leading to DNA damage checkpoint activation. Monoubiquitination of Fanconi anemia complementation group D2 protein (FANCD2) and proliferating cell nuclear antigen (PCNA) promotes DNA repair through chromatin association and the enhanced recruitment of enzymes involved in the translesion synthesis repair pathway, respectively. Both of these proteins are substrates of USP1, but only deubiquitylation of FANCD2 is necessary for DNA repair,

reflecting the need for a dynamic population of FANCD2. The major inflammatory pathway leading to activation of the transcription factor NF-κB is regulated by reversible ubiquitination. Here, the role of two DUBs, CYLD and A20, is fairly well understood. CYLD was originally identified as a tumour suppressor and is mutated in cylindromatosis, a condition resulting in the development of benign tumours on the scalp. CYLD DUB activity was shown to negatively regulate NF-κB activation by processing Lys 63-linked polyubiquitin chains on TRAF2, which are required for activation of inhibitor of NF-κB kinase (IKK) by TRAF2. As CYLD gene expression is upregulated by NF-κB, it provides a conduit for negative-feedback regulation of this pathway. Furthermore, CYLD has been linked to a wide variety of substrates and cellular processes, including cell cycle progression. The NF-κB pathway is under strong control by reversible ubiquitination, and regulatory roles for another DUB, Cezanne, have also been shown (Komander et al., 2009).

1.2.3 The ubiquitin specific protease (USP) family

USP family is the largest family of DUBs in humans, since it is predicted to count over 50 members. Structures of six USP domain deubiquitinating enzymes indicate that the USP domain fold is highly conserved despite low sequence similarity. The USP structure consists of three sub-domains, comparable to the finger, the palm and the thumb of a right hand (Figure 1.9).

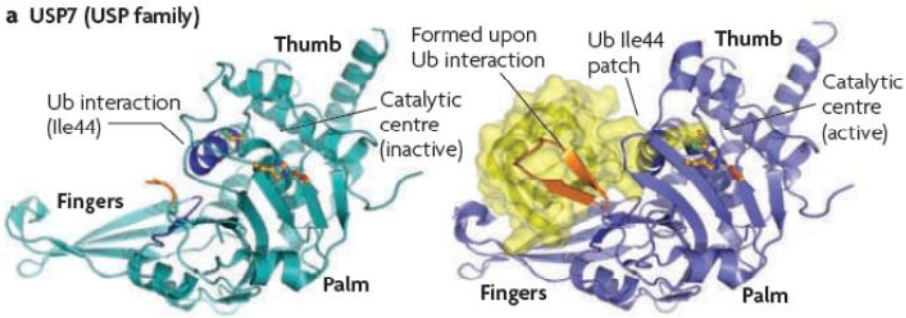

Figure 1.9 Inactive (green) and active (blue) structures of the catalytic domains of ubiquitin-specific protease 7 (USP7), part of the USP family, with ubiquitin (Ub) shown in yellow. The Thumb, Palm and Fingers subdomains of the USP domain are indicated (Komander et al., 2009).

Only CYLD, a deubiquitinating enzyme implicated in the human benign tumor syndrome cylindromatosis, lacks the finger subdomain. Structures of USP domains bound to ubiquitin or a ubiquitin based inhibitor show that the C-terminus of ubiquitin sits in a cleft located between the thumb and the palm subdomains, while the globular portion of ubiquitin interacts with the finger. Most USPs contain a core catalytic domain with insertions and terminal extensions bearing additional protein interactions domains. USP domains not bound to a substrate are in an inactive

catalytic configuration, and they undergo conformational changes when ubiquitin binds (Komander et al., 2009).

1.3 USP1-REGULATED PATHWAYS

1.3.1 The Fanconi Anemia pathway

Fanconi anemia (FA) is a rare autosomal or X-linked recessive disease characterized by chromosomal instability and cancer susceptibility. FA is a genetically heterogeneous disease and has been traditionally classified using cell fusion experiments that correct sensitivity to DNA cross-linking agents such as MMC (complementation). The majority of human FA genes have been identified by complementation analysis and cell fusion experiments. In this process, cell lines derived from FA patients are fused; those fusions that do not complement the sensitivity to crosslinking agents both belong to the same complementation group (Joenje and Patel, 2001). Each distinct complementation group is indicated by a letter prefixed with *FANC*. Complementation analysis was performed by transfection of a cell line from each complementation group with a cDNA library and selected with media containing a crosslinking agent. The plasmid containing the cDNA was recovered from clones and sequenced to reveal the identity of the putative Fanconi gene (Strathdee and Buchwald, 1992).

To date at least 15 complementation groups (A, B, C, D1, D2, E, F, G, I, J, L, M, N, O, P) have been identified (Crossan and Patel, 2012). Complementation groups A, C, and G are the most prevalent accounting for approximately 60%, 15%, and 10% of patients, respectively. The genes for all the complementation groups have been cloned, and the proteins have been demonstrated to cooperate in a common biochemical pathway, the Fanconi anemia pathway. Disruption of the FA pathway results in the common clinical and cellular phenotype observed in FA. All Fanconi anemia complementation groups except FA-B are inherited autosomally. Fanconi anemia frequency is estimated to be 1-5 per million and the frequency of heterozygous carriers to be 1 in 300. Clinically, FA is characterized by childhood onset aplastic anemia, increased cancer/leukemia susceptibility and developmental defects. Typically, FA patients develop bone marrow failure leading to aplastic anemia during the first decade of life and at least 20% develop malignancies. Most commonly, these include acute myelogenous leukemia and myelodysplastic syndrome, but also head and neck squamous cell carcinoma, gynecological squamous cell carcinoma, esophageal carcinoma, and liver, brain, skin and renal tumors. FA subtypes FA-D1 and FA-N are associated with increased predisposition to medulloblastoma, Wilms' tumor and acute leukemia in early childhood, and are clinically different from the other FA subtypes. Common developmental defects observed in FA are

short stature, developmental disability and abnormalities of the skin, upper extremities, head, eyes, kidneys and ears; in addition, male FA patients often present abnormal gonads.

Cells derived from FA patients are hypersensitive to treatment with a class of DNA damaging agents, called interstrand crosslinkers. These compounds, such as cisplatin, mitomycin C (MMC), melphalan and diepoxybutane, act by covalently binding opposite strands of DNA together. These lesions, called interstrand crosslinks (ICLs) are extremely toxic to cells, as they totally block both DNA transcription and DNA replication. The failure of a cell to repair such lesions ultimately results in chromosomal breaks and complex radial structure formation (Auerbach and Wolman, 1976; Jacquemont and Taniguchi, 2007).

Complementation group	Gene alias	Chromosome location	Molecular function
FANCA		16q24.3	Core complex
FANCB		Xp22.2	Core complex
FANCC		9q22.32	Core complex
FANCD1	BRCA1	13q13.1	Homologous recombination
FANCD2		3p25.3	Core complex substrate, exonuclease activity
FANCE		6p21.31	Interacts with FANCD2
FANCF		11p14.3	Core complex
FANCG		9p13.3	Core complex
FANCI		15q26.1	Core complex substrate
FANCJ	BRIP1	17q23.2	G-quadreplex resolution
FANCL		2p16.1	E3 ubiquitin ligase
FANCN	PALB2	16p12.2	Homologous recombination
FANCM		14q21.2	Helicase/translocase
FANCO	RAD51C	17q22	Homologous recombination, RAD51 paralogue
FANCP	SLX4	16p13.3	Holliday junction resolvase

Table 1.1 Fanconi anemia complementation groups (Crossan and Patel, 2012)

All FA proteins are required for cellular resistance to DNA crosslinking agents and are considered to cooperate in a common pathway (the FA pathway) that regulates the sensing, signaling and/or repair of interstrand DNA crosslinks (Figure 1.10).

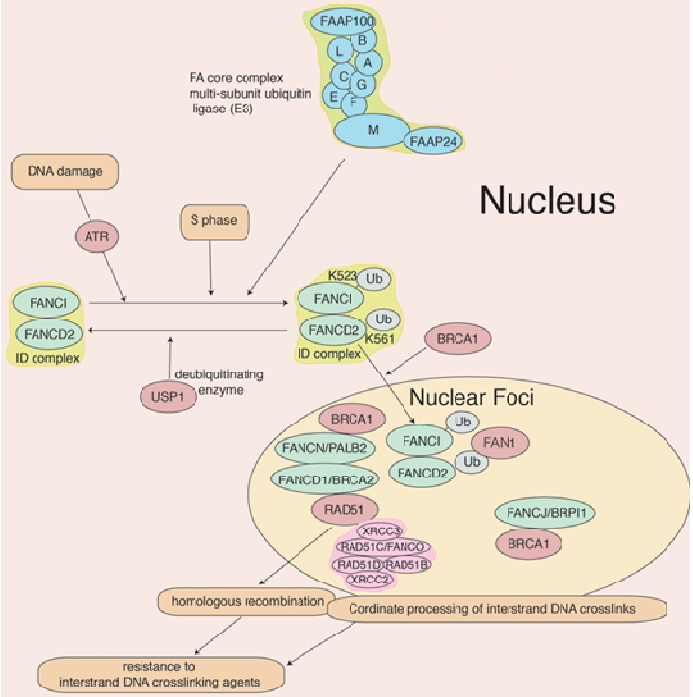

Figure 1.10 Scheme of the Fanconi Anemia pathway.

FA proteins are closely related to the breast/ovarian cancer susceptibility genes products BRCA1 and BRCA2, and to their partner proteins. *FANCD1* (FA-D1) is identical to *BRCA2* and *FANCN* is in fact *PALB2* (partner and localizer of BRCA2), a crucial regulator of the BRCA2 protein. Additionally, *FANCJ* is identical to *BACH1/BRIP1*, a DNA helicase that interacts directly with BRCA1. Furthermore, FANCD2, FANCD1/BRCA2, FANCN/PALB2 and BRCA1 colocalize in nuclear foci at the site of DNA damage and BRCA1 itself is required for efficient nuclear foci formation of FANCD2. In light of these interplays between FA and BRCA proteins, the FA pathway is also called the "FA-BRCA pathway" or "FA-BRCA network" (Jacquemont and Taniguchi, 2007).

Ubiquitination plays a crucial role in the FA pathway. Indeed eight FA proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM), a FANCM-interacting protein called FAAP24 and a factor called FAAP100, form a nuclear protein complex (the FA core complex) that is able to monoubiquitinate FANCD2 and FANCI following DNA damage (Garcia-Higuera et al., 2001). Monoubiquitinated FANCD2 and FANCI localize to chromatin and form foci at the presumed sites of DNA damage (Wang et al., 2004; Sims et al., 2007; Smogorzewska et al., 2007). The core complex, through FANCL, acts as an E3 ubiquitin ligase monoubiquitinating FANCD2 and FANCI following DNA damage (Meetei et al., 2003). Mutations in components of the core complex, often found in human FA patients, result in destabilization of this multi-protein assembly and loss of the E3 ligase activity (Gurtan et al., 2006). FANCD2 and FANCI are paralogues that

form a heterodimer distinct from the core complex (Joo et al., 2011). FANCI becomes phosphorylated and monoubiquitinated in response to DNA damage; however, only the phosphorylation modification is necessary to promote the monoubiquitination of FANCD2 and maintain cellular resistance to crosslinking agents (Ishiai et al., 2008).

FANCL contains a PHD (plant homeodomain) finger/RING finger domain exhibiting auto-ubiquitin ligase activity *in vitro*. FANCL associated through its PHD/RING finger domain with UBE2T, a ubiquitin conjugating enzyme (E2) that is also required for *in vivo* FANCD2 monoubiquitination.

During DNA replication, in the S phase of the cell cycle, DNA polymerases copy the DNA at the level of several replication forks. Lesions in the chromatin structure, such as chemically-induced crosslinks, lead to a block of the replication fork, that is no more able to proceed. The Fanconi anemia pathway is activated in response to the stalled replication forks in the S phase of the cell cycle. Cell cycle synchronization studies reveal that FANCD2 monoubiquitination occurs predominantly in S phase. FANCD2 is monoubiquitinated at the G1/S boundary, remains monoubiquitinated throughout S phase, and becomes deubiquitinated at the end of S phase as the synchronized cell population enters G2 (Taniguchi et al., 2002). During S phase, monoubiquitinated FANCD2 protein specifically colocalizes with replication foci containing BRCA1, RAD51 (Taniguchi et al., 2002; Hussain et al., 2004), and PCNA proteins (Howlett et al., 2009). These foci are believed to represent sites of homologous repair. The FA core complex associates with chromatin during S phase, and is released in mitosis (Mi and Kupfer, 2005). Replication fork arrest leads to the local generation of single-strand DNA, perhaps through helicase unwinding of the DNA duplex. Single-strand DNA binds RPA (Replication Protein A) and then ATR (Ataxia Telangiectasia and RAD3-related protein) and ATRIP (ATR-interacting protein). After recruitment to the stalled fork, ATR phosphorylates multiple substrates, leading to additional cell cycle checkpoint and DNA repair responses (Zou and Elledge, 2003). One principal substrate of ATR is CHK1 (Checkpoint Kinase 1). Recent studies implicate ATR/CHK1 in the activation of the FA pathway. Indeed, agents that activate ATR kinase, such as hydroxyurea, crosslinkers, or ultraviolet light are potent activators of FANCD2 monoubiquitination (Gregory et al., 2003). Moreover, cells from patients with Seckel syndrome express a mutant ATR and fail to monoubiquitinate FANCD2 following DNA damage. Similarly, siRNA-mediated silencing of ATR expression abrogates FANCD2 monoubiquitination (Andreassen et al., 2004). Knockdown of ATR also results in a chromosome breakage phenotype and cellular hypersensitivity to MMC, consistent with an upstream role for ATR in the FA pathway (Andreassen et al., 2004). Moreover, disruption of the

CHK1 kinase either by siRNA knockdown or by specific inhibitors of CHK1 kinase leads to DNA crosslinker sensitivity and a disruption of the FA pathway.

Taken together, these observations support a model in which ATR and CHK1, in response to DNA replication fork stalling, modulate the FA pathway. This may occur through the direct phosphorylation of one or more subunits of FA core complex, for example phosphorylation of FANCM may promote the DNA translocation of FA core complex and facilitate the identification of DNA crosslinks, leading to subsequent activation of the rest of the FA pathway, which in turn may be involved in the processing of the lesion that has given rise to the failure in DNA replication.

Recently, a novel nuclease associated to the FA pathway has been identified, FAN1. FAN1 contains a conserved nuclease domain at its C-terminus, which confers to the protein endonuclease and 5' to 3' exonuclease activity. FAN1 shows preference for a 5'-flap DNA substrate, the opposite polarity to the two endonucleases previously implicated in ICL processing, MUS81/EME1 and XPF/ERCC1, which preferentially cleave 3'-flaps. FAN1 is implicated in resolving ICLs, for has been demonstrated that its depletion leads to an increase in cellular sensitivity to crosslinking agents, that can be rescued by the reintroduction of a wild-type FAN1, but not upon reintroducing a mutant in the nuclease domain, suggesting that the enzymatic activity of this protein is required for the correct repair of this kind of DNA lesions. FAN1 can be considered downstream of the FA pathway, because FAN1 contains a ubiquitin-binding zinc finger (UBZ) domain at its N terminus. This domain is supposed to interact with monoubiquitinated FANCD2/FANCI, because the binding is disrupted when the UBZ domain is mutated. Upon ICL-induced DNA damage, FAN1 is recruited to nuclear foci by monoubiquitinated FANCD2, while a mutant of FANCD2 that cannot be ubiquitinated (K561R) fails to complete this event. When at the level of the stalled replication fork, FAN1 performs nucleolytic incisions and promote the unhooking of the crosslink, allowing the bypass of the lesion by translesion synthesis (TLS) polymerases (see paragraph 1.3.3). Given its exonuclease activity FAN1 may be involved also in later steps of the process, such as DNA excision and homologous recombination (HR) (Huang and D'Andrea, 2010).

Deubiquitination is very important for the regulation of the FA pathway. USP1 is not a FA gene *per se*, since no human FA patients harboring mutations in the USP1 gene have been identified. Disruption of the *Usp1* gene in chicken cells (DT40) results in crosslinker hypersensitivity, and the chicken *Usp1* and *Fancl* genes are epistatic for crosslink repair (Oestergaard et al., 2007). In rare cases, disruption of a ubiquitin E3 ligase results in a similar outcome to disruption of a corresponding DUB (Nijman et al., 2005), suggesting that coupled ubiquitination and deubiquitination may be essential for the function of some pathways. In 2009, D'Andrea and colleagues disrupted the murine *Usp1* gene. Interestingly, *Usp1* -/- mice had a strong similarity to

FA mice (small size, infertility, mitomycin C hypersensitivity, and chromosome instability). In addition, *Usp1* -/- mice exhibited a higher rate of perinatal lethality and absence of male germ cells. This results lead to suppose that USP1 is required for FANCD2 foci assembly and contributes to homologous recombination (HR) repair, suggesting a regulatory role of USP1 in the FA pathway (Kim et al., 2009).

1.3.2 USP1 is the deubiquitinating enzyme for FANCD2/FANCI

In a recent work the group of René Bernards identified USP1 as the negative regulator of FANCD2 monoubiquitination. As explained previously (see paragraph 1.3.1), upon monoubiquitination on Lys561, FANCD2 relocalizes to nuclear DNA damage foci, where it binds with BRCA1, RAD51 and FANCD1/BRCA2. These foci seems to mark the sites in which DNA damage-induced double strand breaks (DSBs) are repaired through homologous recombination (HR). FANCD2 is also monoubiquitinated during S phase, and this event is required for normal progression through this cell cycle phase. The monoubiquitinated form of FANCD2 (FANCD2-L) disappears when cells exit S phase and is transiently present in cells that have been exposed to DNA damage. Both forms of FANCD2 are stable and not subject to proteasomal degradation, indicating that the monoubiquitination does not serve to target FANCD2-L for degradation. It is more likely that FANCD2 function can be regulated by a DUB. Bernards' group constructed a library of shRNA (short hairpin RNA) targeting 55 human DUBs, and they showed that only upon silencing of USP1 the monoubiquitinated form of FANCD2 (FANCD2-L) increased in a comparable amount that after mytomicin C (MMC) treatment. So they concluded that USP1 is a negative regulator of FANCD2 monoubiquitination and that the catalytic activity of USP1 is required for this function. Moreover, they dissected the expression of USP1 during the cell cycle, and they demonstrated that USP1 protein levels were high in G1/S synchronized cells and remained high until FANCD2-L has disappeared in late S phase. They showed that USP1 is subjected to polyubiquitination and consequently degraded by proteasome, and that USP1 has a nuclear localization and physically interacts with FANCD2. Subsequently they asked whether USP1 inhibition, with the consequent increase in the levels of FANCD2-L form, can interfere in the DNA repair mechanisms, and they observed that upon USP1 silencing there is a protection effect from DNA damaging agents such as mitomycin C. In conclusion this work places USP1 in a central role in the DNA repair network by limiting FANCD2 activity and in the regulation of DNA repair mediated by FA/BRCA pathway (Nijman et al., 2005).

As anticipated, USP1 has a positive regulatory effect on the Fanconi anemia pathway-mediated DNA repair (Kim et al., 2009). This effect could be due to different reasons. First, it is possible that

deubiquitinating FANCD2 and FANCI removes these proteins from chromatin, making them available for additional repair sites. Indeed, it was shown that the crosslink sensitivity in the absence of USP1 could not be rescued by overexpression of FANCD2 alone (Kim et al, 2009); however, the rescue of sensitivity with both FANCI and FANCD2 have yet to be investigated. Second, removal of FANCI and FANCD2 from chromatin may allow the completion of late DNA repair stages (Cotto-Rios et al., 2011b).

1.3.3 PCNA and the translesion synthesis

Replication of the genome and packaging of the replicated DNA into chromatin during S phase is achieved by a dynamic complex of proteins that are present at the level of replication forks. Proliferating Cell Nuclear Antigen (PCNA) is a sliding clamp that goes through the DNA molecule, and plays a central role in orchestrating the association of replication factors during DNA replication, and during recognition and repair of DNA damage (Kirchmaier, 2011). Post-translational modifications of PCNA play an important role in coordinating DNA replication and DNA damage tolerance processes. PCNA is a homotrimeric protein complex that forms a ring around double-stranded DNA. During normal replication, this sliding clamp functions as a processivity factor by binding the replicative DNA polymerases to the DNA template. Upon replication fork stalling, PCNA is ubiquitinated to promote DNA damage tolerance. PCNA interacts with numerous proteins to facilitate DNA replication and repair, epigenetic processes and sister chromatid cohesion. At the replication fork, PCNA forms ring-shaped trimers around DNA in which each monomer is arranged in a head to tail orientation within the ring (Figure 1.11).

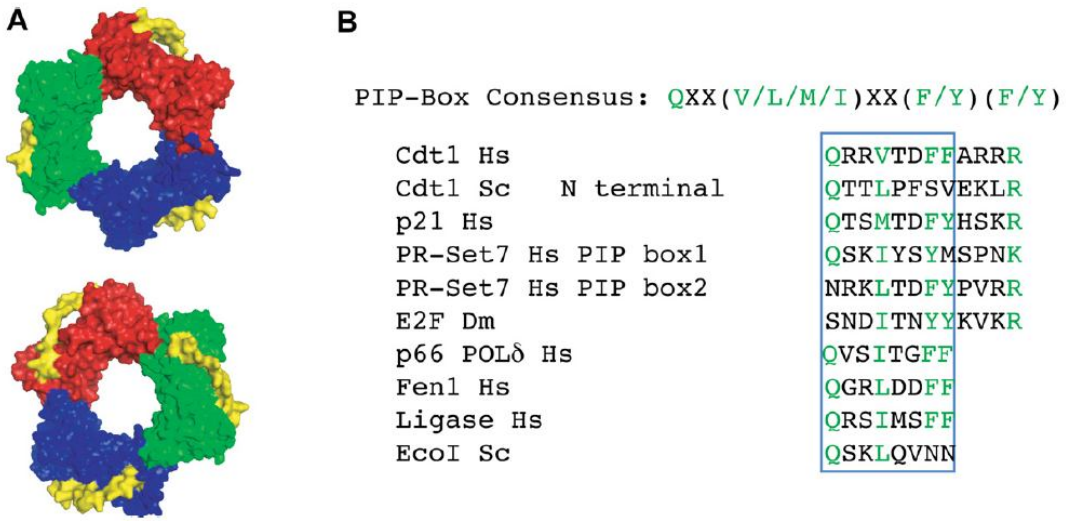

Figure 1.11 PCNA structure. (a) Interdomain connecting loops (yellow) within PCNA homotrimer (monomers are shown in green, red and blue). (b) PCNA-interacting protein (PIP) box consensus sequence. PIP boxes constitute a hydrophobic domain that binds within a hydrophobic pocket beneath the interdomain connecting loop on PCNA.

Additional residues outside of the PIP box on binding partners can also contribute to interactions with PCNA (Kirchmaier, 2011).

During DNA replication and repair, PCNA is loaded onto DNA at the 3' ends of primer-template junctions by the clamp-loading complex, replication factor C (RFC) (O'Donnell and Kuriyan, 2006). Once loaded onto DNA, PCNA acts a sliding clamp that interacts with and enhances the processivity of the DNA polymerases Polε and Polδ on the leading and lagging strands of DNA (Burgers and Gerik, 1998). Many PCNA binding proteins interact with a hydrophobic pocket and the interdomain connecting loop on PCNA through a conserved PCNA binding motif known as a PIP (PCNA-interacting protein) box (Moldovan et al., 2007). PIP boxes fold into a hydrophobic domain that binds a hydrophobic pocket beneath the interdomain connecting loop on PCNA. Other residues outside of the PIP box may also affect the strength of interactions with unmodified PCNA as well as with different modified forms of PCNA. In addition to Polδ and Polε, PCNA acts as a binding platform for several other proteins directly involved in DNA synthesis, such as the PIP box-containing Flap endonuclease I, FEN-1, DNA ligase I, and several alternative polymerases and other proteins involved in DNA repair. PCNA also facilitates the establishment of sister chromatid cohesion during S phase through recruiting the PIP box-containing acetyltransferase Eco1 to chromatin. Competition between PIP box proteins for binding to PCNA likely contributes to complex formation and stability, although the relative affinity of most proteins containing PIP boxes for PCNA remains to be determined.

The addition and removal of post-translational modifications to PCNA affect protein-protein interactions with PCNA during responses to DNA damage. PCNA is differentially modified with ubiquitin-related molecules in response to different forms of DNA damage to promote DNA repair through several alternate repair pathways. When the DNA replication machinery becomes stalled upon encountering a lesion, activation of DNA damage responses that permit the continuation of DNA replication through an "error prone pathway" can occur. In this pathway, termed translesion DNA synthesis (TLS), alternative low fidelity DNA polymerases are utilized to replicate the damaged DNA template. This kind of replication employes specialized, damage-tolerant DNA polymerases. However, their tolerance for distorted templates makes these enzymes less accurate, even on undamaged DNA. As a consequence, TLS is generally an error-prone process that may cause damage-induced mutations (Pages and Fuchs, 2002). Therefore, all organisms have control systems that keep the TLS polymerases in check and prevent their unrestrained activity in the absence of damage. Translesion synthesis is an evolutionarily conserved process that allows the replication machinery to bypass DNA lesions using a low-fidelity DNA polymerase. Unlike the

high-fidelity DNA polymerases, low-fidelity or TLS polymerases are non-processive, lack any proofreading capability, and contain larger active sites capable of accommodating distorted bases and base pair mismatches. The high-fidelity replicative polymerases such as Polα, Polδ and Polε belong to the classical B-family of DNA polymerases, while many TLS polymerases including Polη, Polκ, Polι and Rev1 belong to the Y-family (Figure 1.12).

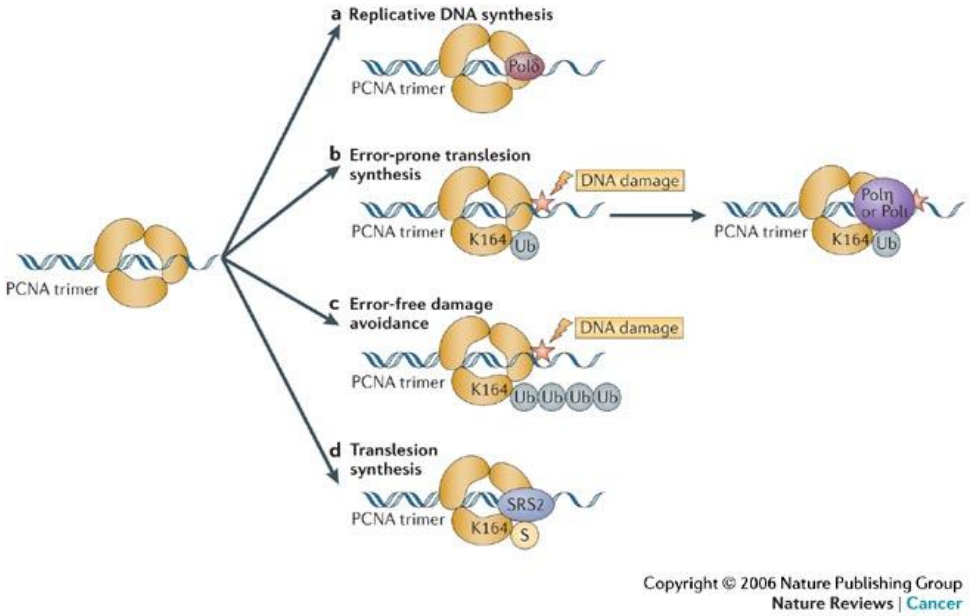

Figure 1.12 During DNA replication, induced or spontaneous lesions will result in stalled replication forks. It is essential for cells bypassing the lesions. This can occur by either an error-prone or error-free damage-tolerance pathway. The error-prone pathway involves low-fidelity translesion synthesis (TLS) polymerases, which incorporate any base opposite the lesion so that replication can continue. The recruitment of the determined polymerase is dependent on the modification status of PCNA. During DNA replication, unmodified PCNA functions as a processivity factor for replicative polymerases (POLδ) (a). When DNA damage occurs replication is stalled, and PCNA is monoubiquitylated. Monoubiquitylation activates TLS by recruiting Y-family polymerases (Pol η) to the ubiquitin (Ub) moiety (b). By contrast, the error-free pathway (c) requires the assembly of Lys63-linked ubiquitin chains on Lys164 of PCNA, which decreases the use of TLS polymerases. In yeast, Lys164 of PCNA has also been shown to undergo sumoylation (d). PCNA is sumoylated in S-phase independently of DNA damage, and results in the recruitment of the helicase SRS2. This mechanism seems to set the course for TLS as it blocks homologous recombination (Hoeller et al., 2006).

These two families of DNA polymerases share the same basic “fingers”, “thumb” and “palm” structure, but many Y-family TLS polymerases also contain a “little finger” domain that confers additional flexibility to the active site. The process of TLS requires the exchange of one polymerase for another, an event that is thought to occur in a step-wise fashion involving at least two polymerase-switching events. In the first switch, the stalled replicative DNA polymerase is replaced by a TLS polymerase capable of replicating over the DNA lesion. The TLS response is then

extended by either the same or another TLS polymerase. This extension step is necessary to allow the lesion to escape detection by the 3'-5' exonuclease proofreading activity of the replicative DNA polymerase *in vitro*, and extension can range from 5-60 nucleotides, depending on the lesion and polymerase involved. Ultimately, a final switch restores a replicative DNA polymerase to the DNA template and processive DNA replication resumes. TLS polymerases are often considered error-prone, as they display a higher frequency of misincorporation on undamaged templates than their replicative counterparts *in vitro*. Furthermore, replication past certain lesions is often mutagenic, and necessarily so in certain cases, for example at abasic sites. Consistent with this, genetic studies in *S. cerevisiae* have shown that loss of Rev1 or either of two subunits of Polζ, Rev3 or Rev7, results in decreased mutagenesis induced by DNA damage *in vivo*. However, several TLS polymerases have been shown to display proper base pairing opposite specific lesions. For example, Polη preferentially inserts two "A" opposite a thymine dimer, a common UV photoproduct, and Polκ has been shown to accurately bypass benzopyrene-induced guanine adducts. Thus, translesion synthesis can be either mutagenic or accurate, depending on the lesion and which TLS polymerase is used. Underscoring the potential significance of TLS processes, mutation of the XPV gene, which encodes Polη in humans, results in a variant form of *Xeroderma pigmentosum* (XPV). Patients with XPV are hypersensitive to UV damage and are predisposed to cancer. The cell's inability to appropriately substitute another TLS polymerase for Polη supports the idea that at least some of these specialized DNA polymerases are not functionally redundant but are specific for a particular type of DNA damage or lesion. Much less is known about template switching, an error-free form of DNA damage tolerance that is genetically distinct from translesion synthesis. Evidence for this pathway is primarily based on epistasis studies in yeast showing that its loss results in increased mutagenesis, presumably due to an increased reliance on the more error-prone TLS for lesion bypass. As the name implies, template switching is hypothesized to mediate lesion bypass by temporarily replacing the lesion-containing DNA template with an undamaged template, namely the newly synthesized daughter strand of the sister duplex. Two models for template switching have been proposed, one involving fork reversal using the nascent sister strand, and the other involving invasion of the sister duplex by a single-stranded gap in a manner reminiscent of homologous recombination (Chang and Cimprich, 2009).

Monoubiquitination may alter protein-protein interactions, and in the case of PCNA, occurs in response to specific forms of DNA damage. Alternatively, this modification may then be converted to different forms of polyubiquitin chains, depending on the type of linkages found between ubiquitin molecules. PCNA can be targeted for degradation (polyubiquitination) and misregulation of turnover of PCNA leads to genome instability. K63 polyubiquitin chains do not trigger

proteolysis of PCNA, but rather act as signals signifying the presence of specific forms of DNA damage.

Chromatin-associated PCNA is ubiquitinated at K164 in response to damage in the template DNA strand caused by DNA alkylating agents or UV irradiation (Hoege et al., 2002). In budding yeast and mammals, Rad6p/Rad18p orthologs catalyze monoubiquitination of PCNA on K164 and RNF8 is also important for monoubiquitination of PCNA upon exposure to UV irradiation in mammalian cells (Zhang et al., 2008). In addition, $CLR4^{Cdt2}$ can directly monoubiquitinate PCNA to promote translesion DNA synthesis. Both stalling of the replication fork and monoubiquitination of PCNA are perquisites for the exchange of replicative polymerases. Polη specifically interacts with the monoubiquitinated form of PCNA through an ubiquitin-binding domain (UBD) plus a PIP box (Bienko et al., 2005; Watanabe et al., 2004). Once synthesis through the lesion has been completed, Polη may be removed from the replication fork through a degradation mechanism involving $CRL4^{Cdt2}$-dependent ubiquitination requiring chromatin-associated PCNA. The $CRL4^{Cdt2}$ E3 ubiquitin ligase complex is a member of the cullin-RING family that promotes the polyubiquitination and degradation of the replication licensing factor Cdt1 (Arias and Walter, 2006; Higa et al., 2006; Jin et al., 2006; Senga et al., 2006). $CRL4^{Cdt2}$ consists of Cul4A or Cul4B, damage-specific DNA-binding protein 1 (DDB1), the RING-finger protein ROC1 and the DDB1 and Cul4-associated factor (DCAF), and substrate recognition factor/WD40 protein Cdt2. Additional substrates for $CRL4^{Cdt2}$ have been recently described, including the CDK inhibitor p21 (Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). Notably, most identified $CRL4^{Cdt2}$ substrates require their interaction with PCNA for their polyubiquitination. It is demonstrated that in normally cycling mammalian cells, $CRL4^{Cdt2}$ monoubiquitinates PCNA and that this activity is constitutively antagonized by the deubiquitinating enzyme USP1 (Huang et al., 2006). In response to DNA damage, however, Rad6/Rad18 is most critical for PCNA monoubiquitination, because Cdt2 depletion affects this monoubiquitination in some cell lines only partially. It is demonstrated that PCNA monoubiquitination via $CRL4^{Cdt2}$ synergizes with Rad6/18 in promoting TLS in undamaged cells (Terai et al., 2010).

Deubiquitination of K164 is also thought to promote the reassociation of Polδ with PCNA at the replication fork. In mammals, deubiquitination of PCNA is mediated by the deubiquitinase USP1 in conjunction with its interacting partner UAF1 in the absence of DNA damage (Cohn et al., 2007; Huang et al., 2006; Niimi et al., 2008). Monoubiquitinated PCNA accumulates upon DNA damage, indeed UV irradiation leads to the downregulation of USP1 protein and transcripts, but how monoubiquitinated PCNA is stabilized in response to other damaging agents is not yet known.

“Error free” repair is triggered by polyubiquitination of PCNA and results in the use of homologous recombination repair proteins to repair DNA damage (Zhang and Lawrence, 2005). During error free repair, which may involve template switching, chromatin-associated PCNA is first monoubiquitinated at K164 in a Rad6/Rad18 dependent manner. This prerequisite modification is then targeted for polyubiquitination through K63 linkages by HLTF and SHPRH in human cells (Unk et al., 2008). USP1 also negatively regulates polyubiquitination in mammals and therefore may participate in deubiquitination of PCNA during recovery after DNA repair by this pathway (Brun et al., 2010).

1.3.4 USP1 deubiquitinates PCNA

As explained in detail in the previous paragraph, PCNA ubiquitination is essential for the DNA repair process. Indeed, when a DNA replication fork stalls at damaged DNA lesions, the ubiquitin conjugation enzyme, RAD6, and the ubiquitin ligase, RAD18, monoubiquitinate lysine 164 of PCNA. Monoubiquitinated PCNA recruits translesion synthesis (TLS) polymerases, which can insert a base opposite the damaged lesion to bypass the damaged DNA. Alternatively, PCNA can be polyubiquitinated to promote DNA damage bypass by a homologous recombination mechanism.

After DNA damage bypass PCNA is deubiquitinated. USP1 (Ubiquitin-Specific Peptidase 1) was identified as a deubiquitinating enzyme for PCNA after DNA damage bypass (Huang et al., 2006; Motegi et al., 2008). USP1 reduces the accumulation of monoubiquitinated PCNA during normal cell division, which prevents mutagenesis by unwanted recruitment of error-prone TLS polymerases. In response to damage that blocks DNA replication USP1 is degraded, and consequently monoubiquitinated PCNA accumulates (Huang et al., 2006). However, the observation that levels of monoubiquitinated PCNA remain high, even in the presence of persistent levels of USP1 (Niimi et al., 2008) suggests that there might be additional mechanisms to regulate USP1 activity. The identification of UAF1 (USP1-associated factor 1) indicates that interacting proteins may regulate the activity of USP1 *in vivo* (Cohn et al., 2007). The loading and unloading of PCNA on DNA is executed by the replication factor C (RFC) complex in an ATP-dependent manner. The RFC complex is composed of five subunits, RFC1, -2, -3, -4, and -5 (Johnson and O’Donnell, 2005). RFC-dependent PCNA loading plays essential roles in PCNA-related processes during DNA replication and repair (Majka and Burgers, 2004). Recently, alternative RFC complexes containing the RFC2 to -5 core complex and a substitute ATPase have been identified in eukaryotes (Merkle et al., 2003). In human, three alternative RFC complexes exist: CTF18-RFC, RAD17-RFC, and ELG1-RFC. Although the canonical or alternative RFC complexes may function as clamp loaders, there is no evidence relating them to the processing of PCNA monoubiquitination.

A recent report demonstrated that human ELG1 protein is required to suppress genomic instability (Sikdar et al., 2009), similar to the yeast homologue Elg1p. Subsequently the same group identified human ELG1 as an interacting protein of the USP1-UAF1 complex (Lee et al., 2010). Monoubiquitinated PCNA accumulated in the chromatin following knockdown of either *ELG1* or *USP1*. The down-regulation of PCNA monoubiquitination by ELG1 was mediated through an N-terminal 17-amino acid sequence of ELG1, which directly interacts with UAF1. Taken together, these data indicate that ELG1 plays an important role in PCNA deubiquitination in cooperation with the USP1-UAF1 complex (Lee et al., 2010).

1.3.5 USP1 deubiquitinates ID proteins

Basic-helix-loop-helix (bHLH) transcription factors comprise the third largest family of transcription factors in the human genome (Tupler et al., 2001) and are essential regulators of development and differentiation through binding DNA elements termed E boxes (Massari and Murre, 2000). Class I bHLH homodimers are expressed broadly and promote expression of antiproliferative genes such as CDKN1A, CDKN2A, and CDKN2B (Yokota and Mori, 2002). Class II bHLH proteins show more restricted expression and form heterodimers with class I proteins to drive tissue-specific genes (Lassar et al., 1991; Weintraub et al., 1994). Through the combined induction of tissue-specific and antiproliferative genes, bHLH transcription factors serve as integrators of lineage commitment. DNA binding of bHLH proteins is limited by heterodimerization with inhibitor of DNA-binding proteins, or IDs. The ID family consists of four members, ID1, ID2, ID3, and ID4 (Lasorella et al., 2001), with overlapping spatial and temporal expression profiles. All four IDs bind the various bHLH proteins with similar affinities to regulate gene expression (Prabhu et al., 1997). IDs are essential for mammalian development, indeed disruption of two or more ID genes results in embryonic lethality (Lyden et al., 1999), while overexpression of ID proteins in transgenic mice produces malignancies (Kim et al., 1999). Similarly, elevated ID protein levels are observed in a broad range of dedifferentiated primary human malignancies, such as pancreatic carcinoma or neuroblastoma (Perk et al., 2005). Although ID proteins are scarce in normal adult differentiated tissues, they are abundant in proliferating tissues, including embryonic and adult stem cell populations, which suggests that IDs might maintain “stemness” (Yokota and Mori, 2002). The stem cell expression and tumour-promoting qualities of ID proteins suggest that *ID* genes may be pivotal in cancer stem cell biology. An engineered ID-suppressing HLH protein was reported to differentiate neuroblastoma tumors (Ciarapica et al., 2009). IDs are induced transcriptionally by several growth factors including bone morphogenic proteins, platelet-derived growth factor, epidermal growth factor, as well as by T cell

receptor ligation (Yokota and Mori, 2002). ID1, ID2, and ID3, but not ID4, are subjected to K48-linked polyubiquitination and subsequent degradation by the 26S proteasome. Consequently, IDs are short lived in most tissues (Bounpheng et al., 1999). The ubiquitously expressed APC/C-Cdh1 complex is an E3 ubiquitin ligase that governs ID stability and abundance (Lasorella et al., 2006), but ID proteins are stable in some contexts. In a recent study Dixit and colleagues demonstrated that USP1 is the deubiquitinating enzyme for ID1, ID2 and ID3, and as a consequence USP1 stabilizes ID proteins preserving them from proteasomal degradation (Figure 1.13).

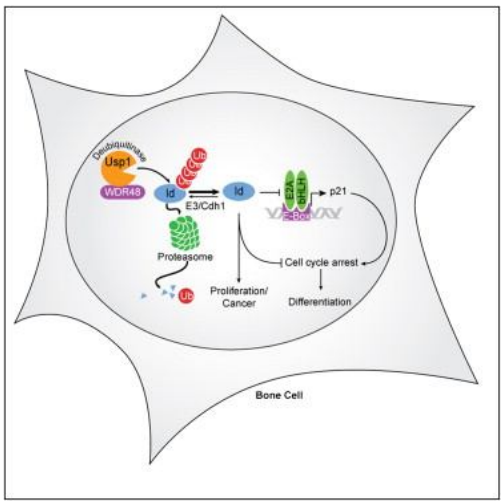

Figure 1.13 USP1 deubiquitinates ID proteins (ID1, ID2, ID3) preserving them from proteasomal degradation. IDs are abundant in embryonic and adult stem cell populations and are essential for "stemness" mainteinance. USP1 knockdown in osteosarcoma cells caused ID protein destabilization, induction of p21-dependent cell cycle arrest, reduction of mesenchymal stem cell markers, promoting osteogenic differentiation (Williams et al., 2011).

Moreover, they showed that ID proteins and USP1 are coordinately overexpressed in a significant fraction of human osteosarcomas. USP1 is overexpressed frequently in osteosarcoma cell lines and, by deubiquitinating the ID proteins, it inhibits bHLH-dependent expression of CDKI p21 resulting in uncontrolled cell proliferation. USP1 overexpression not only is necessary for the proliferation of several osteosarcoma cell lines, but it is also sufficient to prevent normal mesenchymal cell differentiation, maintaining the cells in a stem-like state. Osteosarcomas are heterogeneous tumors that comprises disorganized masses of different cellular types, such as osteoblasts, chondrocytes, and adipocytes. Indeed, these tumors are thought to develop from a mesenchymal stem cell population that can give rise to all three lineages (Tang et al., 2008). Osteosarcoma cell lines indeed fail to express typical osteoblast markers such as RUNX2, OSTERIX, SPARC/OSTEONECTIN and alkaline phosphatase (ALP) (Luo et al., 2008), whereas these tumour cells express surface markers characteristic of mesenchymal stem cells, including CD90, CD105, and CD106 (Di Fiore et al., 2009). In view of the role that ID proteins play in stem cell maintenance and regulation of

differentiation, the authors investigated whether either USP1 or ID knockdown in osteosarcoma would trigger osteoblastic differentiation. Accordingly, USP1 knockdown in osteosarcoma cell lines reduces the expression of mesenchymal stem cell markers and initiates an osteogenic development program, that leads to a decrease in proliferative capacity and potential reversal of transformed phenotype. These results render USP1 an attractive target for differentiation therapy in osteosarcoma. Moreover, ID2 or USP1 overexpression in mesenchymal stem cells inhibits osteogenic differentiation and preserves mesenchymal stem cell features, suggesting that USP1 might promote cell transformation. This is supported by the finding that in NIH 3T3 cells overexpression of USP1 triggers anchorage-independent cell proliferation in soft agar, which is a classic hallmark of oncogenic transformation (Hanahan and Weinberg, 2000). Moreover, when NIH 3T3 cells overexpressing USP1 are implanted subcutaneously into nude mice, they produce measurable tumors.

The observation that USP1 overexpression impairs osteoblastic differentiation of mesenchymal precursors, whereas USP1 loss causes osteoblastic differentiation of osteosarcoma cells, recapitulates *in vivo* since USP1 regulates normal bone development. Indeed *Usp1* -/- mice are osteopenic with defects in ossification of the cranial and long bones (Williams et al., 2011).

1.4 USP1 REGULATION

1.4.1 USP1 associates with UAF1/WDR48

Recent studies has demonstrated that DUBs associated to proteasome, interact with other non-catalytic proteins, or are subunits of a complex. This is the case for USP1, indeed, as recently demonstrated, it interacts with UAF1 (USP1-associated factor 1). UAF1 contains 677 aminoacids and harbors seven or eight potential WD40 repeats in the N-terminal region and a predicted coiled-coil domain in the C-terminal portion. UAF1 was demonstrated to be essential for USP1 stability, since cells depleted of UAF1 showed a reduction in USP1 levels, linked to an increase in the monoubiquitinated form of FANCD2 and PCNA. *In vitro* studies indicate that UAF1 not only interacts with and stabilizes USP1, but also stimulates its catalytic activity, indeed the activity of the USP1/UAF1 heterodimer was about 35-fold higher than the activity of USP1 alone. In conclusion, USP1 protein exists in a catalytically active protein complex with the UAF1 protein, which in addition to activating USP1 also serves to maintain its stability *in vivo*. The active USP1/UAF1 complex ensures that the Fanconi anemia pathway is kept at an adequate state under normal cell growth. When cells are subjected to genotoxic stress, transcription of the USP1 gene is rapidly shut off, leading to a decrease of USP1/UAF1 complex, thereby activating the Fanconi anemia pathway

through increased protein levels of Ub-FANCD2, ensuring maintenance of genomic stability (Cohn et al., 2007).

Recently, a study examined the role of the DUB complex, USP1/UAF1, in DNA repair. Disruption of USP1 and/or UAF1 in DT40 cells resulted in elevated FANCD2-Ub and PCNA-Ub levels. Interestingly, USP1 and UAF1 were epistatic in an HR pathway, and knockout of each gene, alone or in combination, resulted in a comparable level of substrate monoubiquitination and camptothecin sensitivity. Moreover, there are evidences that the USP1/UAF1 complex regulates DNA repair by promoting HR repair. Disruption of USP1 and/or UAF1 results in reduced HR; however, how UAF1 expression promotes HR activity is not known. One possibility is that the USP1/UAF1 complex can release the monoubiquitinated FANCD2/FANCI complex from chromatin, deubiquitinate FANCD2 and FANCI, and recycle these proteins for additional DNA repair events. Disruption of UAF1 or USP1 may result in depleted pools of free FANCD2 and FANCI or in the accumulation of monoubiquitinated FANCD2/FANCI complex, which may interfere with normal replication fork progression or DNA repair. Other recent studies suggest a role of protein ubiquitination in the regulation of HR repair. For instance, the ubiquitin conjugating enzyme, UBC13, initiates HR activity, and proteasome inhibition can disrupt HR function. The relative role of ubiquitin E3 ligases and DUBs in regulating HR is an important emerging field in DNA repair research. Other recent studies suggested the presence of regulatory mechanisms in the cell which suppress non-homologous end joining (NHEJ) and thereby promote HR repair. For instance, the FA pathway suppresses NHEJ and promotes HR. Disruption of the NHEJ protein Ku70 rescues HR in FANCC-deficient cells, and disruption of the NHEJ protein DNA-PK promotes HR in FANCD2-deficient cells. Still other studies have indicated that the DNA repair regulatory protein BRCA1 can also suppress NHEJ. BRCA1 appears to displace the NHEJ pathway-related protein 53BP1 from the site of double strand breaks. Loss of BRCA1 leads to increased 53BP1-mediated NHEJ activity and to increased toxicity due to chromosome breakage and translocations. A secondary loss in 53BP1 results in a rescue of these cells, a decrease in NHEJ, and a compensatory increase in HR. The mechanism of NHEJ suppression is largely unknown. According to one model, USP1/UAF1 may suppress NHEJ and promote HR by enhancing the processing of DSBs into forms with free (exposed) 3’ ends which are more suitable for strand invasion of the homologous template. It was proposed a model in which the FA pathway can function to suppress Ku70-driven NHEJ and to promote HR repair. For cells in which USP1 or UAF1 is lost, there is no longer suppression of NHEJ-driven DSB repair. In this case, elevated Ku70-driven NHEJ leads to toxicity and camptothecin-driven cell death. However, if there is an additional loss of Ku70, as in *UAF1 -/-/- Ku70* -/- double knockout DT40 cells, there is a loss of toxic NHEJ and a rescue of HR repair.

Hence, the double-knockout cells have improved survival in the presence of camptothecin and PARP inhibitor. Consistent with this model, the double-knockout cells exhibit persistently elevated FANCD2-Ub levels but have restored camptothecin-induced Rad51 foci assembly and disassembly. In summary, these results demonstrate that the USP1/UAF1 complex plays a critical role as a positive regulator for HR repair. Accordingly, disruption of USP1/UAF1 may provide a potent method for blocking HR repair and sensitizing cells to DNA interstrand cross-linking agents, or to the new class of PARP inhibitors which have entered clinical trials (Murai et al., 2011).

1.4.2 USP1 regulation in DNA damage response

The cellular levels of monoubiquitinated PCNA are regulated by two counteracting processes: monoubiquitination by RAD6 or RAD18 and deubiquitination by USP1. A recent study indicates that cellular exposure to UV results in USP1 degradation which leads to the accumulation of ubiquitinated PCNA. According to this model, in the absence of DNA damage, monoubiquitinated PCNA is subjected to constitutive deubiquitination by USP1, resulting in low steady-state levels of modified PCNA. However, in the presence of UV damage, USP1 switches its substrate target from ubiquitinated PCNA to its own C-terminal Gly-Gly motif, resulting in an increase in monoubiquitinated PCNA levels and TLS. The mechanism for this switch is not known. The data obtained from D'Andrea's group suggest that many known DNA damage response pathways (such as those regulated by BRCA1, ATR, ATM and NER proteins or by Fanconi anemia proteins) are not involved in USP1 autocleavage. Also, USP1 may be part of a larger DNA-damage sensor complex, where the recognition of UV lesions can trigger a direct conformational change in USP1 that allows autocleavage. The increase in both spontaneous and damage-induced mutagenesis in cells depleted of USP1 can be explained by the following models. First, increased levels of monoubiquitinated PCNA may result in the dysregulated function of Polη. Although Polη can accurately replicate DNA past cyclobutane-pyrimidine dimers (CPDs), its overuse, particularly in regions of undamaged DNA, may result in an overall increase in the frequency of mutations in a USP1-depleted cell. This has been observed *in vitro* where human Polη copies undamaged DNA template with much lower fidelity than replicative polymerases and indicates that the function of Polη must be tightly controlled to prevent potentially mutagenic DNA synthesis. Second, the high levels of monoubiquitinated PCNA (due to the loss of USP1) may result in the recruitment and competition of other error-prone TLS polymerases (such as Rev3-Rev7, Rev1, Polκ and Polι) with Polη for undamaged and damaged DNA templates. A recent study has identified two ubiquitin-binding domains (UBM and UBZ) that are evolutionarily conserved in all Y-family TLS polymerases (Rev1, Polκ, Polι and Polη). These domains are required by Polη and Polι (and

possibly other Y-family TLS polymerases) for interaction with monoubiquitinated PCNA. Also, *in vitro* studies have shown that monoubiquitinated PCNA, but not unmodified PCNA, is required for the activation of Rev1 to promote mutagenic DNA replication. Thus, a condition that upregulates PCNA monoubiquitination (such as USP1 knockdown) is likely to increase replication-coupled mutagenesis through the recruitment and activation of multiple TLS polymerases. One physiological role of USP1 could be to limit or suppress mutagenesis by restraining the TLS polymerase activity both in the absence and presence of UV-induced DNA damage. Whether or not other DUB enzymes regulate the activity of TLS polymerases has yet to be explored (Huang et al., 2006).

Recent studies argue for an alternative view for the function of the USP1 protein in DNA damage response, in which USP1 acts as a positive regulator of DNA repair. Its physiological predominant known substrate is the FANCD2 protein, the deubiquitination of which functions to promote the shuttling of this protein onto chromatin. Although USP1 is capable of self cleavage, this process does not appear to be crucial for the regulation of its activity.

The previously published studies on USP1 indicated a role for deubiquitination in the downregulation of DNA repair, since the observation that siRNA against USP1 in human cells makes them slightly more resistant to DNA damage (Huang et al., 2006), explained by the fact that persistently ubiquitinated FANCD2 or PCNA promote constitutive DNA repair. However, the alternative study, carried out in chicken DT40 cells, showed the opposite effect on DNA repair in cells where USP1 is depleted. Indeed, USP1 depletion increases FANCD2 and PCNA monoubiquitination but unexpectedly results in DNA crosslinker sensitivity, probably due to the fact that monoubiquitinated FANCD2 constitutively bound to chromatin, is not rapidly recycled from sites of repair. In contrast, persistent PCNA monoubiquitination hasn't significant impact on DNA repair or mutagenesis (Oestergaard et al., 2007). USP1 was previously shown to autocleave after DNA damage (Huang et al., 2006), while in DT40 cells USP1 autocleavage is not stimulated by DNA damage, and expressing an uncleavable mutant in the USP1 knockout cells partially rescues crosslinker sensitivity. It is clear that mutations in either the active site or at the Gly-Gly motif (autocleavage site) strongly stabilize USP1. Nevertheless, both of these mutants are cleaved after DNA damage-induced apoptosis, whereas the self cleavage of the wild-type USP1 is not stimulated by UV-induced DNA damage, therefore the apoptosis-induced degradation of USP1 does not involve self cleavage. It is also interesting that, although being present at levels 10- to 20-fold higher than the wild-type protein, the effect of mutating the Gly-Gly motif in USP1 functionality is minimal. Indeed, the kinetics of FANCD2 ubiquitination are marginally affected, since this mutated form of USP1 largely complements the knockout cell line with respect to DNA

crosslinker sensitivity. In conclusion, efficient DNA crosslink repair requires FANCD2 deubiquitination, whereas FANCD2 monoubiquitination is not dependent on USP1 autocleavage (Oestergaard et al., 2007).

1.4.3 APC/C-Cdh1 complex regulates USP1

Two ubiquitin E3-ligase complexes, SCF (Skp1/CUL1/F-box protein) and APC/C (Anaphase Promoting Complex/Cyclosome), control the timely transitions of cell cycle phases by promoting the degradation of many key cell cycle regulators. SCF complex mainly functions in G1, S and early M phases, whereas APC/C regulates mitosis including metaphase-anaphase transition and mitotic exit and maintains G1 phase. APC/C is a large (1,5 MDa complex) composed of at least 11 core subunits. It relies on two WD-40 repeat-containing adaptor proteins, Cdc20/fizzy(fzy)/p55-CDC and Hct1/srw1/fizzy-related(fzr)/Cdh1, to engage with its substrates. Destruction box (RXXLXXXXN/D/E) and KEN box are motifs frequently found in APC's substrates, but other motifs are also possible for recognition by APC/C-Cdc20 or APC/C-Cdh1. The consensus sequence of destruction box can be found in many proteins. However, not all of these proteins are APC's substrates. Thus, there must be other sequence constrains that are not understood yet, that define true APC's substrates. Moreover, some substrates only have an RxxL motif and yet are recognized by APC, indicating the last aminoacid in the consensus is not stringently conserved. APC/C-Cdc20 initiates the metaphase-anaphase transition through mediating the ubiquitination and degradation of cyclin B1 and securin. To prevent premature separation of sister chromatids and mitotic exit, APC/C-Cdc20 is inhibited by Mad2 and BubR1 through the spindle assembly checkpoint mechanism. Only when the sister chromatids are aligned at the metaphase plate and have established bivalent spindle attachment, the inhibition of APC/C-Cdc20 can be released. In contrast to APC/C-Cdc20, APC/C-Cdh1 is inactive in early mitosis when it is inhibited by phosphorylation and binding of Nup90/Rae1 complex. APC/C-Cdh1 only becomes active from late mitosis to G1. The difference in the timing of activation between APC/C-Cdc20 and APC/C-Cdh1 suggests a functional division between the two E3 ubiquitin ligases in mitosis. Recent analyses of mice deficient in Cdc20 or Cdh1 strongly support that notion. It appears that Cdc20 is required for metaphase to anaphase transition, whereas Cdh1 plays a nonessential role in mitotic exit but an essential role in G1/S regulation.

A large number of mitotic regulators are degraded at the end of mitosis, such as Cdc20, Aurora B, Plk1, etc., and are most likely the substrates of APC/C-Cdh1 (Table 1.2). Although many of these mitotic regulators do accumulate in the absence of Cdh1, they are eventually degraded, probably because of the stabilization of Cdc20 that compensates for the loss of Cdh1. As a result, Cdh1-

deficient cells can still proliferate. However, these cells accumulate mitotic errors and display difficulties in completing cytokinesis, resulting in the formation of binucleated cells at a high frequency. The lethality associated with the loss of Cdh1 is largely a result of failed development of placenta, an essential organ for embryonic life in mammals.

In HeLa cells, knocking down the expression of Cdh1 causes stabilization of Skp2, a F-box containing protein responsible for bringing p27Kip1 to the SCF complex for ubiquitination. As a result, p27 is destabilized in the cells with impaired Cdh1 function and the G1 phase is shortened in these cells. A recent work identified Ets2 as a new substrate of APC/C-Cdh1. Ets2 is a member of the Ets family of transcription factors which share a unique DNA binding domain, the ETS domain. It is well known that Ets2 is activated by Ras-Raf-MAPK signaling and mediates some effects of this important signaling pathway. The most prominent effect of Ras signaling is the stimulation of proliferation which relies in part on the induction of cyclin D1 expression by Est2. Increased expression of Ets2 has been associated with initiation and progression of various cancer types and its expression was altered in cervical cancer cell lines due to chromosomal changes in 21q22.1-22.2 where human ETS2 resides.

To enter S-phase, APC/C-Cdh1 must be inactivated. Several different mechanisms are in place to contain APC/C-Cdh1. First, the ubiquitination of APC-specific ubiquitin-conjugating enzyme (E2) UbcH10 by APC/C-Cdh1 itself provides a negative feedback mechanism that would eventually destroy APC/C-Cdh1 activity. Second, as Cdk activity accumulates, Cdh1 is phosphorylated. The phosphorylation promotes Cdh1 dissociation from APC. Third, phosphorylated Cdh1 is targeted by SCF ligase, further limiting the activity of APC/C-Cdh1. Finally, in late G1 phase, E2F activates the transcription of early mitotic inhibitor-1 (Emi1)/Rca1, which inhibits the activity of APC/C-Cdh1 as a pseudo-substrate.

Substrates	Function
Cdc20	Anaphase onset
Securin	Anaphase onset
Sgo1	Anaphase onset
Rcs1	Anaphase onset
XKid	Spindle assembly
Tpx2	Spindle assembly
Ase1	Spindle assembly
Aurora A	Mitotic exit
Aurora B	Mitotic exit
Plk1	Mitotic exit

Anillin	Mitotic exit
CKAP2	Mitotic exit
Cyclin B1	Mitotic exit
Cyclin A	Mitotic exit
Cdc6	DNA synthesis
Geminin	DNA synthesis
FoxM1	G1/G0 maintenance
SnoN	TGF-β signalling
Ets2	Ras signaling
Id2	Transcription

Table 1.2 Substrates of APC/C-Cdh1 (Li and Zhang, 2009).

In a very recent study, Huang's group explored whether the control of USP1 levels during cell cycle progression modulates the cellular response to UV DNA damage. They showed that during G1 phase USP1 is targeted for degradation by APC/C-Cdh1 to ensure that USP1 levels are kept in check before S phase entry. Low levels of USP1 enable robust UV-induced PCNA monoubiquitination during G1 phase, which is likely to allow the recruitment of TLS polymerases to UV lesions. These findings suggest that APC/C-Cdh1 plays a direct role in modulating the DNA repair choice in G1 and further solidify the link between cell cycle regulation and DNA repair. First they showed that in synchronized U2OS and HeLa cells USP1 protein levels are low during G1 phase and high during S phase of the cell cycle. Otherwise, the levels of USP1 co-factor UAF1/WDR48 didn't change during cell cycle. So they asked whether a ubiquitin ligase may be responsible for the degradation of USP1. As the APC/C-Cdh1 complex is critical for promoting the degradation of cell cycle regulators in G1, they tested whether this ubiquitin ligase also regulates the G1 levels of USP1. To this end, they silenced Cdh1 expression in both synchronized T98G and synchronized U2OS cells. They found that USP1 accumulated in both asynchronous and G1 cells depleted of Cdh1. This accumulation appears to be caused by USP1 stabilization. As expected (APC/C-Cdh1 is inactive during prometaphase), the accumulation of USP1 after Cdh1 silencing was not as prominent in M phase cells. Moreover, they found that G1-stabilized USP1 was still catalytically active, suggesting that USP1 proteolysis is likely a critical mechanism to inhibit USP1 activity during the G1 phase. They found that both exogenously and endogenously expressed USP1 can interact with members of the APC/C-Cdh1 complex. To test whether UV-induced DNA damage during G1 induces PCNA monoubiquitination, U2OS cells were synchronized in G1 and subsequently UV-irradiated. After UV damage, USP1 levels decreased below the G1 levels, whereas in untreated cells, USP1 reaccumulated as they exited G1 and started DNA replication.

Accordingly, after UV damage, Cdh1 levels remained elevated, correlating with the loss of USP1 and a delay in S phase entry. Interestingly, robust monoubiquitination of PCNA already occurred after UV damage (Figure 1.14). Thus, this study suggests a model in which degradation of USP1 by the APC/C-Cdh1 during G1 is critical for cells to properly recruit TLS polymerases for UV-mediated DNA gap repair (Cotto-Rios et al., 2011a).

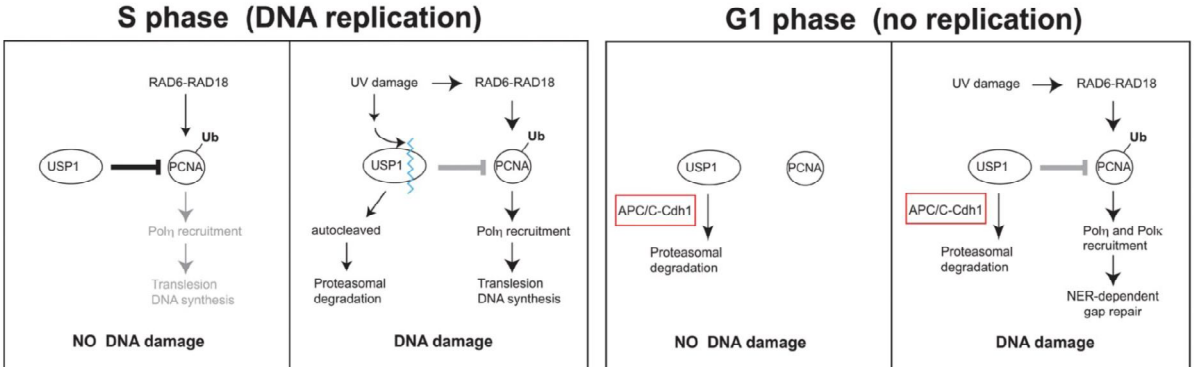

Figure 1.14 A schematic representation of how USP1 is regulated by proteolysis in G1 versus S phase cells in the presence or absence of UV DNA damage. (Cotto-Rios et al., 2011a).

Very recently, it was shown that USP1 undergoes phosphorylation, and more in detail, USP1 is phosphorylated in M phase by Cdks on S313 (Cotto-Rios et al., 2011b). This phosphorylation event may regulate its protein stability. It has been shown that phosphorylation of APC/C substrates can lead to their stabilization, as in the case of Cdc6 (Mailand and Diffley, 2005). Cdc6 is a key regulator for the initiation of DNA replication. Prior to S phase, Cdc6 is kept at low levels by APC/C-Cdh1-dependent proteolysis. Cdk-dependent phosphorylation of Cdc6 allows for its stabilization by preventing its association to Cdh1. Probably, phosphorylation of USP1 might also serve as a protective mechanism against APC/C-Cdh1-mediated degradation, since the S313 phosphorylation site is located within the region previously characterized as the Cdh1 recognition site. Additionally, phosphorylation of USP1 may have other regulatory functions, such as to facilitate the interaction with its co-factor UAF1. It could be hypothesized that when cells exit mitosis USP1 dephosphorylation might lead to disruption of the USP1-UAF1 complex and subsequent degradation of USP1 by Cdh1 during the G1 phase of the cell cycle. During G1 phase, USP1 is degraded to enable efficient PCNA monoubiquitination and TLS polymerase-dependent repair of persistent DNA lesions via gap repair synthesis. On the basis of these data, the authors propose the following model: during the G1 phase of the cell cycle, APC/C-Cdh1 targets USP1 for degradation, so it can no longer deubiquitinate the ID proteins, making them susceptible for

degradation by APC/C-Cdh1. This leads to upregulation of p21 and proper G1 timing to prevent unwanted DNA synthesis (Cotto-Rios et al., 2011b).

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

MG-132, lactacystin, pepstatin A, E64d and Z-leu-leu-CHO were purchased from Sigma Aldrich. siRNA for CAPN2 silencing (AAG UAC CUC AAC CAG GAC UAC) was purchased from DHARMACON, Inc. Dallas, TX (USA). siRNA targeting CAPN1 and LAMP2A were obtained from Santa Cruz Biotechnology. siRNA targeting other proteins were purchased from Eurofins MWG Operon (Germany): control siRNA targeting LACZ (CUG ACC AGC GAA UAC CUG UTT), the pool of four siRNAs targeting CAPNS1 (1-GAG CAU CUC UAU AAC AUG AUU TT, 2-CCA CAG AAC UCA UGA ACA UUU TT, 3-UCA GGG ACC AUU UGC AGU AUU TT, 4-GAA GAU GGA UUU UGA CAA CUU TT), USP1 (AAC CCU AUG UAU GAA GGA UAU TT), ATG5 (GCA ACU CUG GAU GGG AUU GTT), Cdh1 (AAU GAG AAG UCU CCC AGU CAG TT).

2.2 Cell culture

Wild-type and CAPNS1-/- mouse embryonic fibroblasts (Arthur et al., 2000) were a kind gift of Dr. Peter A. Greer (Ontario, Canada); U2OS, 293T, HT1080 cells and the mouse fibroblasts mentioned above were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). WI38 fibroblasts were grown in MEM supplemented with 10% fetal calf serum (FCS). U2OS HA-CAPNS1, a U2OS-derived cell line stably overexpressing calpain small subunit (CAPNS1), was created at LNCIB through transfection of pcDNA3-HA vector, in which *CAPNS1* gene was previously cloned. This cell line was grown in presence of G418 (0,2 mg/ml). U2OS pRS-control and pRS-shCAPNS1 were stably silenced for luciferase and calpain small gene, respectively. U2OS cells were infected through the retroviral vector pRetro-SUPER expressing short hairpin RNA directed against either luciferase or calpain small gene, and grown in presence of puromycin (2,5 µg/ml).

Cells were irradiated with 30J/m^2 254 nm UV light with Stratalinker UV Crosslinker, model 1800, from Stratagene.

2.3 Transfections and plasmids

U2OS and 293T cells at 60-80% confluency were transiently transfected using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's instructions. Plasmids encoding for tagged USP1 (Flag-USP1, GFP-USP1 and MYC-USP1) and encoding for mutated form of USP1 (MYC-USP1 1-746, MYC-USP1 1-523, GFP-USP1 GG670/671AA, GFP-USP1 C90A), together

with Flag-USP33, GFP-Polη and Flag-UAF1/WR48 were kindly provided by Dr. T. Huang. HA-CAST was obtained by cloning the gene encoding for calpastatin in a pcDNA3-HA vector.
Cells at 60-80% confluence were transiently oligofected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

2.4 Western Blot analysis and antibodies

Western Blot analysis was performed according to the standard procedures using the following primary antibodies: anti-actin, anti-tubulin, anti-Flag, anti-HA, anti-CAPNS1, anti-CAPN2, anti-ATG5, anti-WDR48 antibodies (Sigma Aldrich), anti-LAMP2 antibody (BD Biosciences Pharmingen), anti-Myc-tag antibody (Cell Signaling Technology), anti-PCNA, anti-CAPN1, anti-Cdc6 antibodies (Santa Cruz Biotechnology), anti-Cdh1 antibody (Calbiochem). Anti-USP1 antibodies (targeting N- and C-terminal regions) were kindly provided by Dr. T. Huang. Secondary antibodies coupled to horseradish peroxidase were obtained from Sigma Aldrich.

2.5 Immunofluorescence analysis

Cells were plated on glass coverslips in 3 cm culture dishes. After washing with PBS, cells were fixed in 3% paraformaldehyde in PBS, treated with 1% glycine in PBS, and permeabilized in 0,01% Triton-X100 in PBS. The staining was performed using specific antibodies incubated in 5% bovine serum albumin in PBS/0,01% Triton-X100 at 37°C followed by fluorescein isothiocyanate-conjugated secondary antibodies (Sigma Aldrich). Nuclei were decorated with Hoechst stain.
To visualize PCNA foci, a protocol to detect chromatin-bound proteins was used. Cells were washed once in 1X PBS and incubated in 0,5% Triton-X100/PBS 10' at 4°C. Cells were fixed in methanol/acetone (1:1) 10' at -20°C, then fixation mix was removed and cells were dried at room temperature. After re-hydration in 1X PBS, standard protocol for immunofluorescence was followed. Anti-PCNA antibody was purchased from Santa Cruz Biotechnology. FITC-conjugate secondary anti-mouse antibody (Sigma Aldrich) was used. Slides were analyzed on a Leica DM 4000B fluorescence microscope.

2.6 qRT-PCR

Total RNA from indicated cell lines was extracted using TRIzol Reagent (Invitrogen). Subsequently it was incubated with RQ1 RNase free DNase (Promega) for 30' at 37°C in a thermocycler. RQ1 DNase Stop Solution was added to terminate the reaction. First strand cDNA synthesis was performed with 1,5 μg of RNA using oligo dT and the SuperScript II Reverse Transcriptase (Invitrogen), according to manufacturer's instructions. Quantitative PCR was performed using the

SYBR Green Master Mix (Applied Biosystem) and analyzed on a StepOnePlusTM real time PCR machine (Applied Biosystem). mRNA levels were normalized with actin.

2.7 Immunoprecipitations

A protocol compatible with mass spectrometry was followed. True-blot beads (Mouse TrueBlot set cat. 88-7788-31, eBioscience) were washed 3 times with ice cold lysis buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0,2% TX-100; proteases inhibitors: 0,5 mM NaF, 1mM DTT, 1 mM Na_3VO_4, 1000X Protease Inhibitor Cocktail, Sigma) and then resuspended in 0,5 ml of lysis buffer supplemented with 0,1 mg/ml Dextran (cat. D1037, Sigma). Antibodies were added to the beads at a ratio of 1:100, and incubated on the rotator in the cold room for 45'. Beads were then washed twice with lysis buffer. Cells were washed with ice cold PBS and lysed by adding lysis buffer and dextran directly to the plate (on ice). Cells were scraped and the lysates were spinned for 10' on high speed. At this point 100 µl of lysates were removed into a fresh tube (input). Lysates were added to the washed antibody beads, and incubated in the cold room for 2,5 hours. Beads were washed 3 times and analysed by mass spectrometry or run in SDS-PAGE. Mass spectrometric analysis was performed in collaboration with Dr. Michael Myers (ICGEB, Trieste) using an Applied Biosystem 4800MALDI TOF/TOF instrument.

2.8 Single-cell gel electrophoresis (COMET assay)

Alkaline comet assay was performed according to manufacturer's protocol (Trevigen kit, 4250-050-K) with some modifications. To observe endogenous or induced DNA damage MEF cells wild-type or knockout for CAPNS1 gene were treated with UV irradiation ($30J/m^2$) or left untreated. After 48h or 72h cells medium was changed to remove apoptotic cells. Cells were washed in PBS, then gently scraped. 4×10^5/ml cells were combined with molten LMAgarose at a ratio of 1:10 and immediately pipetted onto CometSlide. Slides were treated with lysis and unwinding solution. After electrophoresis (21 Volts, 45'), slides were fixed in 70% ethanol, dried and stained with SYBR Green. Slides were observed by epifluorescence microscope and 200 random comets per sample were analysed with CometScore software (TriTek).

2.9 Statistical analysis

Results are expressed as means ± standard deviation of at least three independent experiments performed in triplicate or quadruplicate, unless indicated otherwise. Statistical analysis was performed using Student's t-test with level of significance set at $p < 0,05$. Values of $p < 0,05$ were considered to be significant.

3. RESULTS AND DISCUSSION

3.1 Identification of novel calpain interacting proteins through mass spectrometry analysis

Calpain plays an important role in the regulation of all stages of the stress response, leading to divergent biological responses depending on the specific timing, the compartment where the enzyme is activated and the endogenous substrates with respect to the specialized functions of the responding cells (Demarchi and Schneider, 2007). The main subject of interest in our laboratory is the study of the role of calpains, and in particular of CAPNS1 (calpain small subunit 1), in cellular processes that function as barriers against tumorigenesis, like apoptosis, autophagy and senescence. Recent findings indicate that CAPNS1 depletion alters the DNA damage response (DDR). Cells are able to detect and propagate the initial DNA damage signal to achieve cellular responses that include cell cycle arrest, DNA repair, senescence and apoptosis, which collectively have been termed the DNA damage response (DDR). Dysregulation of components involved in these processes contributes to genomic instability, which can lead to tumorigenesis.

CAPNS1-depleted mammalian cells are sensitive to a number of stimuli including DNA damaging agents such as etoposide and doxorubicin (Demarchi et al., 2005). Calpain is rapidly activated following radiation exposure and genotoxic stresses. In the absence of calpain activity p53 levels arise (Benetti et al., 2001), FoxO transcription factors appear more active (Bertoli et al., 2009), and there is a perturbation of H2AX phosphorylation dynamics (Demarchi et al., 2010), suggesting that there might be an increase in DNA damage, or a defect in DNA repair. Interestingly, also the loss of autophagic activity, as it occurs for instance in BECLIN1 deficient fibroblasts, is associated with an increase in genomic instability (Mathew et al., 2007). Moreover, in murine *Capns1* knockout cells we have observed an increased rate of ROS production, which could damage DNA, suggesting a possible role of calpain in mantaining genomic stability. It is therefore possible that in the absence of CAPNS1 DNA damage is increased.

In order to further characterize the role of calpain in the regulation of the DDR, we searched for unknown interactors of calpain by means of a proteomic approach. We performed a preparative co-immunoprecipitation experiment in HT1080 cells lysates, using an antibody recognizing the endogenous human calpain small subunit 1. We decided to analyze the endogenous proteins to avoid the interference of artifacts linked to the forced accumulation of a protein in the cell. In collaboration with Dr. Michael Myers (ICGEB, Trieste) we performed a mass spectrometric analysis of total CAPNS1 interacting proteins, using an Applied Biosystem 4800MALDI TOF/TOF instrument. Among other well known interactors of CAPNS1 (for instance calpain large subunits), a number of interesting and promising proteins emerged from this study, including GRP78/BIP,

recently shown to be required for autophagy (Li et al., 2008), and USP1 (Ubiquitin-Specific Peptidase 1), a deubiquitinating enzyme involved in DNA damage repair. As explained in the introduction, USP1 negatively regulates PCNA and FANCD2/FANCI monoubiquitination, and is involved in the repair of damaged DNA. Since cellular responses to DNA damage, besides constituting one of the most important fields in cancer biology, is probably connected to autophagy, we focused our attention on USP1/CAPNS1 interaction.

3.2 Verifying the interaction between CAPNS1 and USP1

We wanted to confirm *in vivo* the interaction between USP1 and CAPNS1. We performed a co-immunoprecipitation experiment of 293T cells lysates transfected with Flag-tagged USP1, using an antibody directed against endogenous human calpain small subunit 1. As negative control we transfected an unrelated deubiquitinating enzyme, namely USP33. We analyzed the results by Western Blot, and we observed that only Flag-tagged USP1 interacts with CAPNS1, as shown by the appearance of the specific band, while Flag-tagged USP33 fails to interact with CAPNS1, as we cannot detect any band (Figure 3.1). This result suggest that USP1 and CAPNS1 interacts in a specific way.

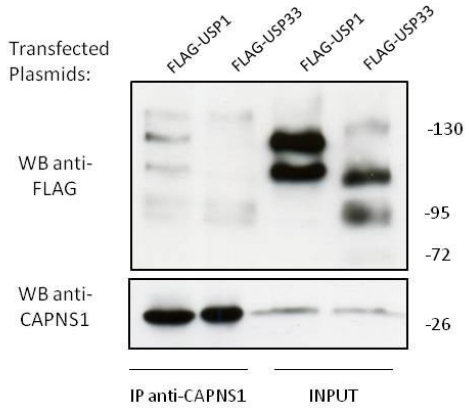

Figure 3.1 Flag-USP1 and Flag-USP33 expressing plasmids were transfected in 293T cells and 24 hours later the cells were lysed, and the lysates were used for immunoprecipitation against human endogenous CAPNS1. Immunoprecipitation products and respective inputs (0,2 %) were analyzed by Western Blot to detect the ectopic proteins and endogenous CAPNS1.

On the other hand we performed the opposite co-immunoprecipitation experiment: we used an anti-Flag antibody in USP1 overexpressing cell lysates, and we are able to detect a micro-calpain interacting band (data not shown). Further experiments are required to understand if the interaction is direct or mediated by the large catalytic subunits of micro- and milli-calpain.

Next, in order to analyze if CAPNS1 and USP1 were present in the same cellular compartments, we performed immunofluorescence assays with the ectopic proteins in cells. HA-tagged CAPNS1 stably expressing U2OS cells were transiently transfected with GFP-USP1, and after 24 hours the cells were fixed and analyzed by immunofluorescence. USP1 has a predominant nuclear localization, and we observed that also calpain may localize into the nucleus. Interestingly, we observed that USP1 and CAPNS1 may colocalize in a certain percentage of cells (Figure 3.2).

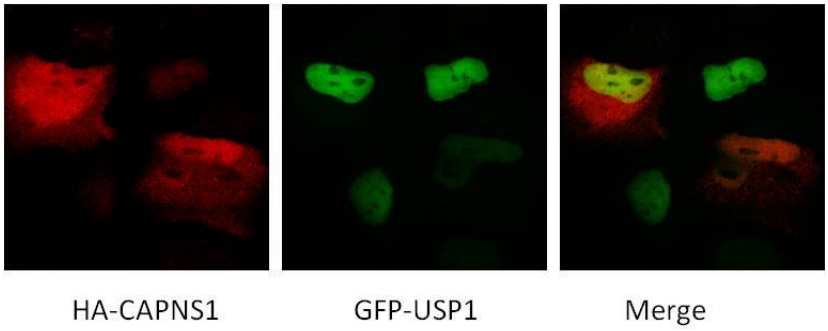

Figure 3.2 HA-CAPNS1 stably expressing U2OS cells were transiently transfected with GFP-USP1. 24 hours after transfection, cells were fixed, immunofluorescence assays were carried on with anti-HA antibody and analyzed by fluorescence microscopy.

3.3 Dissecting USP1/CAPNS1 interaction

In order to dissect more in detail USP1/CAPNS1 interaction, we transfected 293T cells with some constructs expressing Myc-tagged USP1 carrying deletions at the C-terminus: one comprising the first 746 aminoacids and one comprising the first 523 aminoacids (Figure 3.3).

Figure 3.3 Representative scheme of Myc-USP1 C-terminal deletion mutants

We repeated the co-immunoprecipitation experiment in 293T cell lysates overexpressing USP1 deletion mutants, and we observed that besides the wild-type protein, CAPNS1 also interacts with the truncated proteins, and this result suggests that USP1 first 523 aminoacids are sufficient for the interaction with CAPNS1 (Figure 3.4).

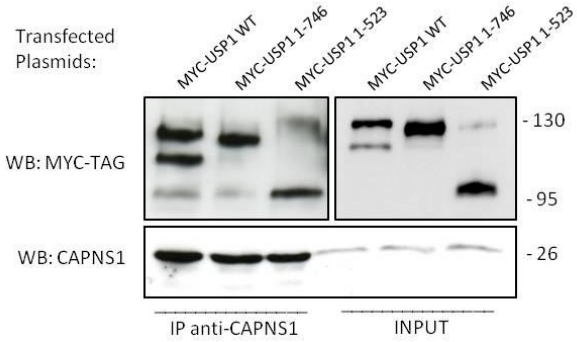

Figure 3.4 Myc-USP1 wild-type or mutants were transfected in 293T and 24 hours later the cells were lysed, and the lysates were used for immunoprecipitation against CAPNS1. Immunoprecipitation products and respective inputs (0,2%) were analyzed by Western Blot to detect the ectopic proteins and endogenous CAPNS1.

Further characterization of the interacting sites of the two proteins is needed. We are performing in collaboration with Dr. Paola Storici (Elettra, Trieste) a dissection of the regions involved in the interaction, by means of GST-pulldown experiments of CAPNS1 and USP1 deletion mutants.

3.4 Study of the effect of calpain upon USP1 stability

To investigate whether calpain could exert any biological effect upon USP1, we depleted calpain activity in a series of cell lines, and we followed the fate of endogenous USP1. To switch off calpain activity we transiently silenced the expression of calpain small subunit 1 (CAPNS1) with a specific siRNA. Indeed, it was demonstrated that CAPNS1 depletion is linked to a decrease in calpain large subunits protein levels, and thus to a decrease in calpain activity (Arthur et al., 2000). Moreover, we performed a time-course experiment of UV irradiation, in order to investigate whether calpain can modulate USP1 upon DNA damage conditions. First of all, we transfected human osteosarcoma U2OS cells with a CAPNS1-targeting siRNA, or a LACZ-targeting siRNA and a USP1-targeting siRNA as negative controls. Next, silenced cells were left untreated or exposed to 30J/m^2 UV irradiation, and we collected lysates at subsequent time points after UV irradiation release. We followed USP1 protein levels in Western Blot by means of two different antibodies targeting USP1: one directed against USP1 N-terminal region, and another directed against USP1 C-terminal region. N-terminal antibody recognize both the full length protein and the cleaved form of USP1, so we observed the presence of two USP1 specific bands, one at a higher apparent molecular weight (about 130 KDa) and one at a lower molecular weight (about 105 KDa). We observed that, as expected, USP1 levels decrease upon UV treatment, since USP1 is autocleaved and degraded to allow accumulation of monoubiquitinated PCNA (Huang et al., 2006). The more interesting observation is that in CAPNS1-depleted cells we found a strong decrease in

USP1 protein levels, compared to control-silenced cells, both in basal conditions and upon UV-induced DNA damage (Figure 3.5).

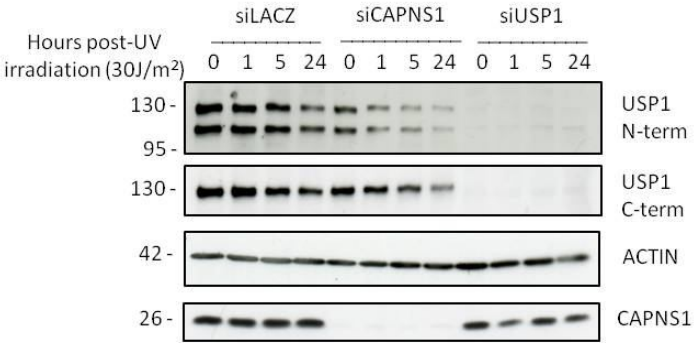

Figure 3.5 CAPNS1, LACZ or USP1 specific siRNAs were transfected in U2OS cells. Three days later cells were exposed to 30J/m^2 UV irradiation or left untreated (0). Samples were collected 1, 5 and 24 hours post-UV irradiation and utilized to monitor endogenous USP1 protein levels by Western Blot, by means of two different antibodies targeting either USP1 N-terminal or C-terminal regions.

This result was reproducible in various cell lines, such as normal human fibroblasts (WI38) and other human tumour cells such as non-small-cell lung carcinoma H1299 cells and MG-63 osteosarcoma cells, both lacking p53 expression (Figure 3.6 and data not shown).

Figure 3.6 CAPNS1, LACZ or USP1 specific siRNAs were transfected in WI38 fibroblasts. Three days later cells were exposed to 30J/m^2 UV irradiation or left untreated (0). Samples were collected 1, 5 and 24 hours post-UV irradiation and utilized to monitor endogenous USP1 protein levels by Western Blot (using both N-terminal and C-terminal targeting antibodies).

Next we took advantage of murine embryonic fibroblast (MEFs) cell lines, wild-type (WT) or in which *Capns1* gene was knocked out (KO) (Arthur et al., 2000). We observed that also in this cellular model there is a notable decrease in USP1 protein levels in cells in which *calpain small 1* gene is depleted, with respect to cells in which *calpain small 1* gene is normally expressed. Moreover, in a *Capns1* knockout cell line in which *Capns1* gene is reintroduced (R), we observed a rescue of USP1 to basal levels (Figure 3.7).

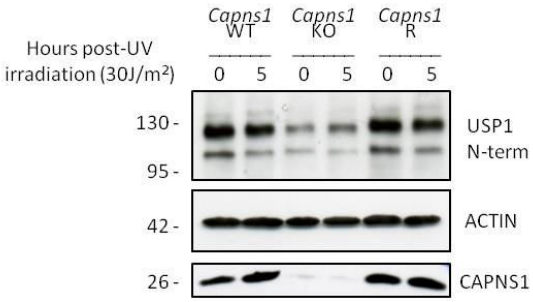

Figure 3.7 *Capns1* wild-type (WT), knockout (KO) or rescued (R) MEFs were left untreated (0) or irradiated with UV light. Samples were collected 5 hours post UV- irradiation and utilized to monitor endogenous USP1 protein levels by Western Blot.

To transiently knockdown CAPNS1 we used a pool of four different siRNAs targeting calpain small subunit 1. In order to investigate whether the effect that we observed in cells transfected with the pool of siRNAs is specific for calpain depletion or could be due to the regulation of an off-target, we transfected U2OS cells with the four siRNAs individually. We confirmed that USP1 levels are reduced also when calpain is depleted by single siRNAs, with different levels of efficiency, that reflect CAPNS1 knockdown efficiency (Figure 3.8).

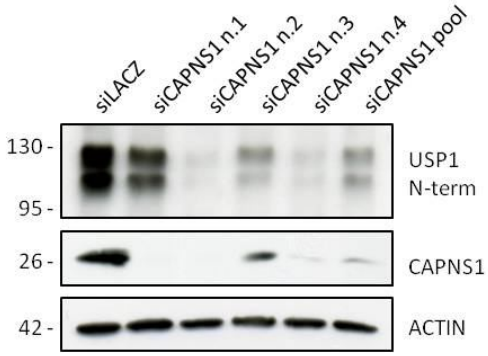

Figure 3.8 4 different CAPNS1 specific siRNAs (named n.1, n.2, n.3 and n.4) were transfected one by one or together in U2OS cells. Three days later samples were collected and utilized to monitor endogenous USP1 protein levels by Western Blot.

3.5 Effect of calpain upon USP1 substrate PCNA

PCNA is ubiquitinated by Rad6/Rad18 upon DNA damage, leading to the recruitment of TLS Polη (Watanabe et al., 2004), and subsequently is deubiquitinated by USP1 to reestablish the interaction with high fidelity DNA polymerases (Huang et al., 2006). USP1 negatively regulates PCNA monoubiquitination, so we expected that the lower levels of USP1 found in absence of calpain activity, lead to an increase in PCNA monoubiquitination. The effect of CAPNS1 depletion on

PCNA ubiquitination/deubiquitination kinetics induced upon DNA damage was analyzed employing Western Blot assays using lysates extracted from cells upon calpain depletion. We analyzed the levels of monoubiquitinated PCNA in U2OS cells stably expressing a control shRNA, a shRNA targeting CAPNS1 and overexpressing HA-tagged CAPNS1. We performed a time-course experiment of UV irradiation to induce PCNA monoubiquitination. Total extracts were prepared in basal conditions and upon DNA damage (UV irradiation, 30J/m^2). The levels of monoubiquitinated PCNA can be followed by the appearance of a band at a higher molecular weight. In control cells (pRS-control) we observed the increase of monoubiquitinad PCNA band 2 hours after UV treatment, followed by a decrease 5 hours after UV treatment, that indicate that PCNA begins to be deubiquitinated by USP1. On the contrary, in cells lacking calpain activity (pRS-shCAPNS1) we observed that monoubiquitinated PCNA levels are higher compared to control cells, and remained high also 5 hours after UV treatment, that indicate that in these cells USP1 activity is reduced. On the contrary, the cells overexpressing HA-CAPNS1 showed the opposite result. Indeed, we observed a decrease in PCNA monoubiquitination both in basal and in DNA damage conditions, with respect to control cells (Figure 3.9). This result can be due to the increased activity of USP1 in presence of overexpressed CAPNS1, that leads to an increased deubiquitination of USP1 substrates.

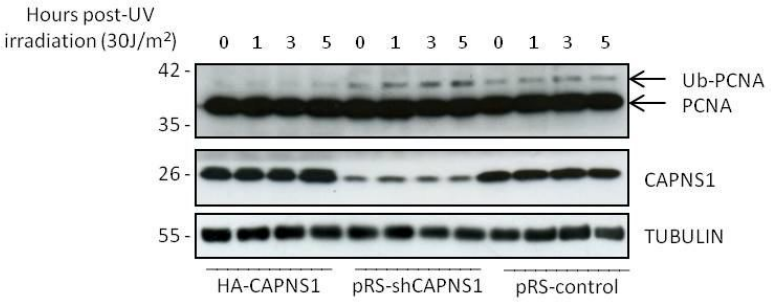

Figure 3.9 HA-CAPNS1 expressing U2OS cells, U2OS cells infected with pRetrosuper vector encoding for shCAPNS1 (pRS-shCAPNS1) or empty pRetrosuper vector (pRS-control) were irradiated with 30J/m^2 UV or left untreated (0), and respective lysates were collected at 1, 3 and 5 hours post-UV irradiation to monitor PCNA ubiquitination levels by Western Blot.

We evaluated monoubiquitinated PCNA levels also in mouse embryonic fibroblasts (MEFs). As expected, USP1 downregulation observed in MEFs knockout for calpain small subunit 1 corresponded to an increase in PCNA monoubiquitination, while the phenotype was rescued in cells were *Capns1* gene was reintroduced (Figure 3.10).

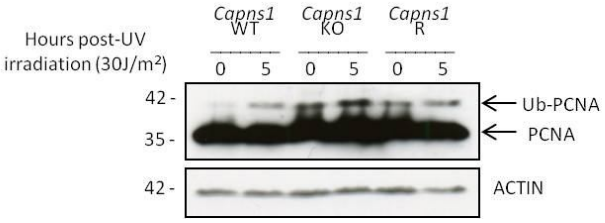

Figure 3.10. Wild-type (WT), *Capns1* knockout (KO) and *Capns1* rescued (R) MEFs were irradiated or not (0) with 30J/m^2 UV light and lysates were collected 5 hours later to monitor PCNA ubiquitination levels by Western Blot.

In parallel, we evaluated the role of calpain in the recruitment of PCNA on chromatin upon DNA damage by immunofluorescence studies employing standard protocols for the detection of chromatin bound proteins. In U2OS cells transfected with a control siRNA, or a CAPNS1-specific siRNA, we induced DNA damage by UV treatment, and we analyzed the percentage of cells containing PCNA foci at different time points (Figure 3.11, panel A). We observed that in control cells there is a transient increase in the number of cells presenting PCNA-induced foci, followed by a slight decrease at 24 hours post-UV irradiation, suggesting that after an initial PCNA recruitment on chromatin DNA damage repair occurred. In CAPNS1-depleted cells we observed a perturbation of PCNA recruitment upon chromatin: in particular there is an increase in the percentage of nuclei containing PCNA-induced foci after 24 hours upon UV induction, with respect to control cells (Figure 3.11, panel B).

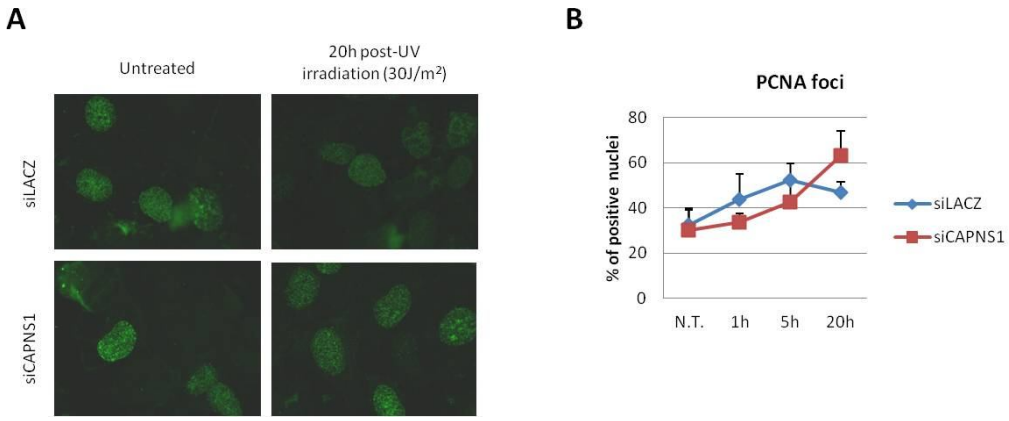

Figure 3.11 CAPNS1 or LACZ were depleted from U2OS cells using specific siRNAs and 72 hours later the cells were either left untreated or irradiated with 30J/m^2 UV. The cells were fixed 1, 5 and 20 hours later, and Triton insoluble PCNA was detected by immunofluorescence. Panel A: examples of PCNA foci. Panel B: average number of PCNA foci-containing cells at different time points after irradiation. 200 nuclei were analyzed for each sample; the error bars represent standard deviations of three independent experiments.

3.6 Determining if calpain effect upon PCNA is USP1-dependent

To demonstrate that the effect observed upon PCNA ubiquitination dynamics is mediated by USP1 dysregulation, we created U2OS cell lines stably overexpressing wild-type GFP-tagged USP1, or overexpressing two different GFP-tagged USP1 mutants: one carrying a mutation in the autocleavage site (GG670/671AA), and one carrying a mutation in the catalytic site (C90A) (Figure 3.12).

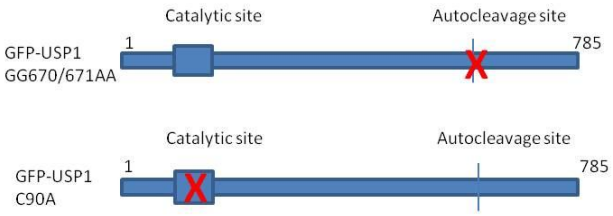

Figure 3.12 Representative scheme of GFP-USP1 mutated constructs: the first is an autocleavage site hyperstable mutant, the second a catalytically inactive mutant.

We transfected a control siRNA or a CAPNS1-targeting siRNA and induced DNA damage by UV treatment. We followed the fate of PCNA monoubiquitination by Western Blot analysis. The first observation is that USP1 mutated in the autocleavage site is more stable, and did not show degradation upon UV treatment (Figure 3.13, panel A). Moreover we observed that the overexpression of this hyper-stable mutant, and thus resistant to degradation, rescued the increase in PCNA monoubiquitinated form observed in CAPNS1-depleted cells (Figure 3.13, panel A). The rescue is not observed in cells overexpressing USP1 mutated in the catalytic site (Figure 3.13, panel B). This result led us to conclude that the perturbation of PCNA monoubiquitination coupled to CAPNS1 depletion is USP1-dependent.

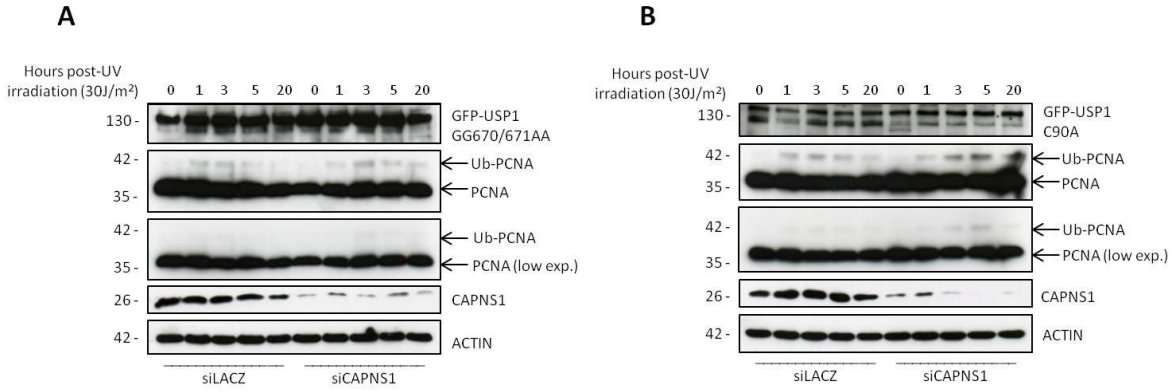

Figure 3.13 U2OS cells stably expressing hyper-stable GFP-USP1 mutated in its autocleavage site (panel A) or GFP-USP1 mutated in the catalytic site (panel B) were transfected with LACZ or CAPNS1 specific siRNAs and 72 hours

later were irradiated with 30J/m^2 UV or left untreated (0). Samples were collected at 1, 3, 5 and 20 hours post-UV irradiation to monitor PCNA ubiquitination levels by Western Blot.

3.7 Calpain role in translesion synthesis

PCNA is important for the switch between high fidelity DNA replication and translesion synthesis (TLS). When lesions in the DNA (such as ICLs) trigger a block of the replication forks, PCNA becomes monoubiquitinated and recruits TLS polymerases such as Polη to bypass the gap (Watanabe et al., 2004). We wanted to analyze the role of calpain in this PCNA-mediated process. Indeed, we asked whether the higher levels of PCNA ubiquitination observed in CAPNS1-depleted cells were linked to an increase in Polη loading on chromatin. We transiently overexpressed GFP-tagged Polη in U2OS cells and analyzed cellular localization through immunofluorescence experiments, at basal condition or upon UV-induced DNA damage. In normal condition Polη presented a diffuse nuclear staining, while five hours after UV irradiation, as expected we observed the appearance of nuclear foci in control cells (Figure 3.14, panel A). In cells lacking calpain activity (transfected with CAPNS1-targeting siRNA) we observed an increase in the number of cells presenting Polη foci even in absence of DNA damage, while in UV-treated cells there is an increased percentage of Polη-positive cells both in control cells and in CAPNS1-depleted cells. The increase observed in CAPNS1-depleted cells in basal conditions is reduced by the overexpression of wild-type USP1, and not by the overexpression of USP1 catalytic mutant (Figure 3.14, panel B). This result indicate that the catalytic activity of USP1 is required to regulate PCNA-mediated accumulation of Polη upon DNA damage sites.

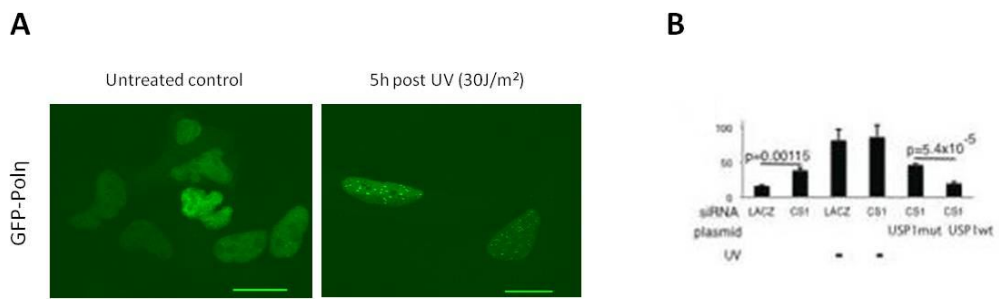

Figure 3.14 CAPNS1-depleted or LACZ-depleted U2OS cells were transfected with GFP-Polη and with a control vector or USP1 wild-type or catalytically inactive. 20 hours later cells were irradiated with 30J/m^2 UV or left untreated. Five hours later cells were fixed and analyzed by immunofluorescence. Panel A shows typical GFP-Polη nuclear staining or foci; the graph in panel B shows the percentage of GFP-Polη containing cells obtained in three independent experiments.

3.8 Role of calpain in UV damage response

Since we showed that calpain can modulate an important regulator of DNA damage response such as USP1, we investigated whether calpain could have a role in genome integrity maintenance. We took advantage of an assay called single-cell gel electrophoresis or COMET assay, that allows to detect double- and single-strand breaks in the DNA at single-cell level. The DNA of a single cell is subjected to an electric field and stained with SyBR Green. The intact DNA is visualized as a compact mass, that could be compared to a comet head, while fragmented DNA migrate faster in the electric field, and could be compared to a comet tail (Figure 3.15, panel A). The extension of the tail (measured by different parameters) gives an indication of the amount of total DNA damage of a cell population. COMET assays were carried on to detect DNA damage induction in basal conditions and upon UV light exposure (30J/m^2). Cells were collected before and 48 or 65 hours after irradiation and utilized for COMET assay. Pictures of 200 cells for each experiments were analyzed using a comet-scoring software. CAPNS1-knockout MEFs were compared to wild-type MEFs to evaluate any role of calpain in DNA damage induction or protection. In CAPNS1 wild-type cells we observed induction of DNA damage, and at longer time points a decrease in DNA damage, indicative of an efficient repair. On the other hand, CAPNS1 knockout cells showed a defect in DNA repair, suggesting that increasing levels in PCNA monoubiquitination could lead to an increase amount of errore-prone translesion synthesis (Figure 3.15, panel B).

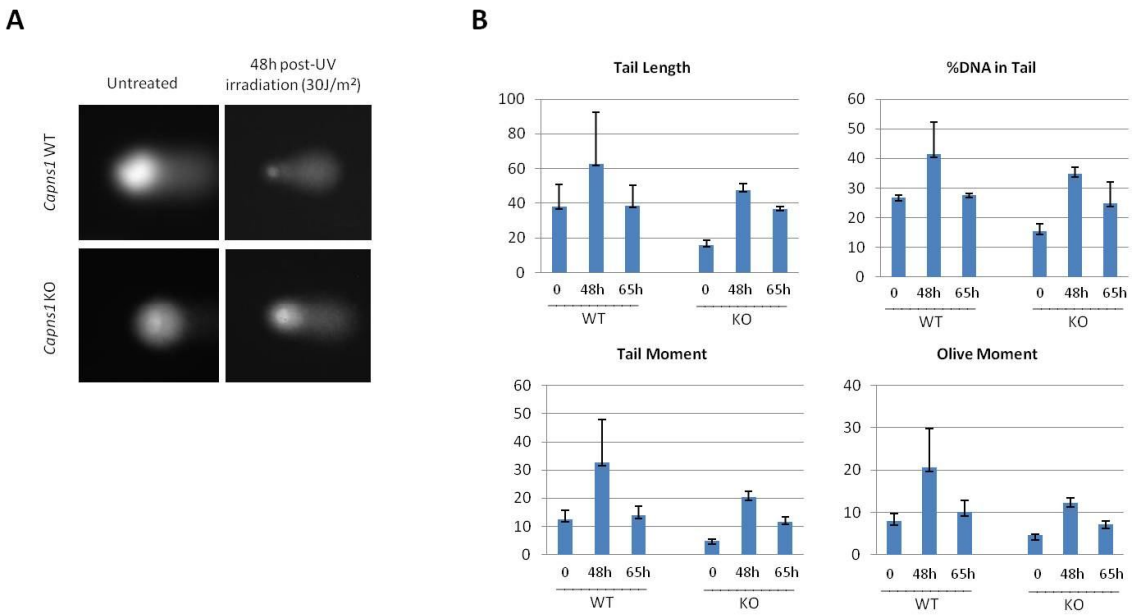

Figure 3.15 *Capns1* WT and *Capns1* KO MEFs were irradiated or not with 30J/m^2 UV light and collected to perform COMET assays following manufacturer's instructions 48 and 65 hours later. Panel A shows typical images of DNA of single cells after performing the assay; graphs in panel B reports the quantifications of some parameters indicative of DNA damage and recovery, such as tail length, percentage of DNA in the tail, tail moment and olive moment.

Indeed, calpain depletion is linked to a decrease of USP1 levels, and is coupled to an USP1-dependent stabilization of mono-ubiquitinated PCNA. Hyper-ubiquitination of PCNA is linked to a ehnanced Polη loading on chromatin, as described in the previous paragraph, and an increase in translesion synthesis, with possible increase in mutagenesis. Overall these data indicate that activation of efficient DNA repair mechanisms is dumped in *Capns1* knockout cells, upon UV-induced DNA damage.

3.9 Studying whether the catalytic activity of calpain is required for USP1 modulation

To further investigate the effect observed of CAPNS1-mediated regulation of USP1 stability, we asked whether USP1 mRNA levels were modulated by CAPNS1 depletion. We performed qRT-PCR experiments in cells transfected with control siRNA, CAPNS1-targeting siRNA or USP1-targeting siRNA as a negative control. We observed that while there is a strong decrease in USP1 protein levels (Figure 3.16, panel A), there isn't any significant change in USP1 mRNA levels in cells lacking CAPNS1 activity compared to control cells (Figure 3.16, panel B). USP1 mRNA levels didn't correlate with USP1 protein levels in CAPNS1-depleted cells, so we concluded that CAPNS1-mediated USP1 regulation occurs at a post-transcriptional level.

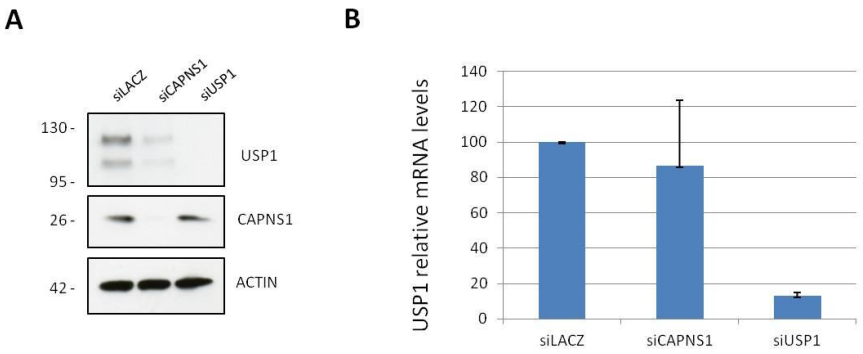

Figure 3.16 U2OS cells were transfected with LACZ, CAPNS1 or USP1 siRNA. 72 hours later cells were lysates or processed for qRT-PCR analysis. Panel A shows USP1 protein levels upon indicated siRNAs. The graph in panel B shows the quantification of USP1 mRNA, with the indication of standard deviation of three independent experiments. For each mRNA the control RNA extracted from LACZ silenced cells is set as 100.

Next we asked whether calpain catalytic activity was required for USP1 regulation. We used cellular models in which the function of calpain is impaired, such as cells overexpressing calpastatin, the endogenous inhibitor specific for calpain, or cells treated with inhibitors of proteases in general, or calpain-specific inhibitors. Lysates of U2OS cells overexpressing increasing concentrations of HA-tagged calpastatin were analyzed by Western Blot, and we observed a

calpastatin dose-dependent decrease in endogenous USP1 levels compared to untransfected cells (Figure 3.17).

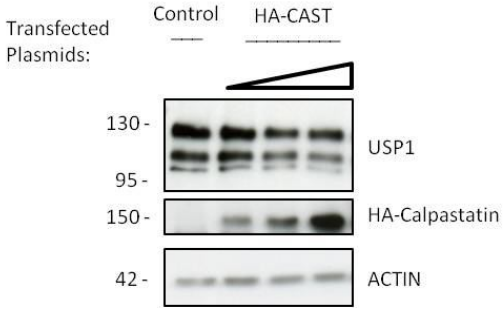

Figure 3.17 U2OS cells were transfected with increasing concentrations of HA-calpastatin expression plasmid (500 ng, 750 ng or 1µg) or an empty vector (control). 24 hours later cells were collected to monitor USP1 levels by Western Blot.

Moreover, we treated U2OS cells with different inhibitors, such as MG132 that inhibits both proteasome and calpains, lactacystin that specifically inhibits proteasome, pepstatin or a combination of pepstatin and E64d, drugs tipically used to inhibit the cathepsins (lysosomal enzymes), and Z-leu-leu-CHO, an inhibitor 100-fold more selective for calpain over proteasome compared to other calpain inhibitors (Tsubuki et al., 1996). We analyzed endogenous USP1 protein levels and, as expected, there was a reduction upon treatment with calpain inhibitors such as MG132 and Z-leu-leu-CHO, suggesting that inhibition of the catalytiv activity of calpain is responsible for decrease in USP1 protein levels. Moreover, we observed a stabilization in USP1 levels upon treatment with cathepsins inhibitors, and this suggest that USP1 can be degraded in the lysosome (Figure 3.18).

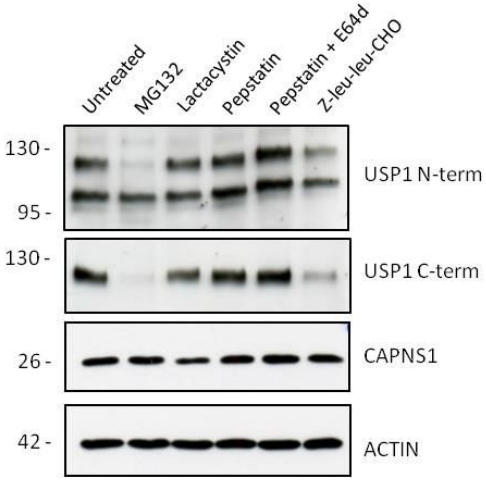

Figure 3.18 U2OS cells were incubated with the indicated drugs, and 20 hours later respective lysates were analyzed by Western Blot to detect endogenous USP1 levels, both through anti-N-terminal and C-terminal region antibodies.

Then, to determine which catalytic subunit was responsible for USP1 regulation, we transfected a siRNA specific for micro-calpain catalytic subunit and for milli-calpain catalytic subunit. USP1 protein levels showed a decrease upon micro-calpain silencing, compared to the decrease observed upon CAPNS1 knockdown, and didn't change upon milli-calpain silencing, thus suggesting that micro-calpain isoform could be responsible for USP1 modulation (Figure 3.19).

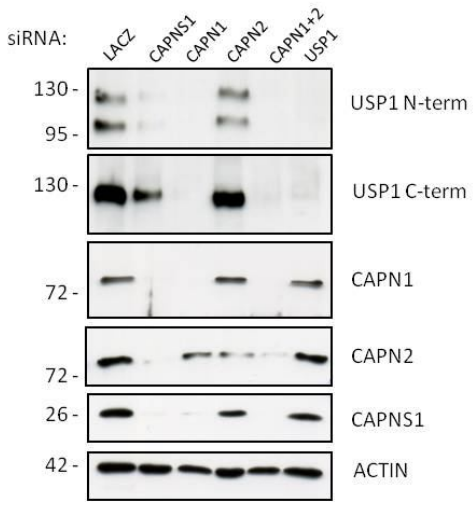

Figure 3.19 U2OS cells were transfected with the following siRNAs: control siRNA (LACZ), calpain small subunit 1 (CAPNS1), micro-calpain large subunit (CAPN1), milli-calpain large subunit (CAPN2), a combination of CAPN1 and CAPN2, or USP1. 72 hours later cells were lysated and analysed by Western Blot to monitor USP1 levels.

3.10 Study of the involvement of autophagic pathways in USP1 degradation

Calpain plays an important role for proper activation of macroautophagy (Demarchi et al., 2006), so we asked whether USP1 degradation in absence of calpain activity could involve autophagic pathways. Autophagy mediates the bulk degradation of intracellular components such as macromolecule complexes and subcellular organelles through lysosomal-dependent mechanisms and is essential for the maintenance of cellular homeostasis (Klionsky, 2005). Three modes of autophagy have been identified in mammals. Macroautophagy cargo is sequestered into a specialized double-membrane structure called an autophagosome. Autophagosomes then fuse with lysosomes, and their content is subsequently degraded by hydrolytic enzymes. Microautophagy cargo is generated by direct budding of the lysosomal membrane. In chaperone-mediated autophagy (CMA), substrates are recognized by a chaperone complex containing Hsc70 (heat shock cognate of 70 kDa) and delivered into lysosomes through interactions with LAMP-2A (lysosome-associated membrane protein 2A). CMA is activated during prolonged nutrient deprivation, which results in the degradation of cytosolic proteins in a molecule by molecule fashion (Massey et al., 2005).

We first blocked macroautophagy pathway by silencing *ATG5*, an essential gene for the induction of this pathway. We observed that USP1 is downregulated when macroautophagy is impaired (Figure 3.20), suggesting that the inactivation of ATG5 could lead to an increased activity of other degradation pathways.

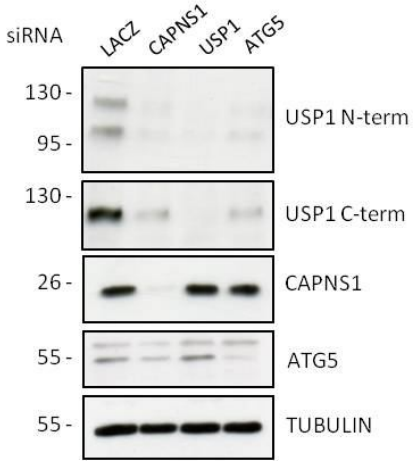

Figure 3.20 LACZ, CAPNS1, USP1 or ATG5 were depleted by siRNA transfections in U2OS cells. 72 hours later cells were lysated and analysed by Western Blot to monitor USP1 levels.

Indeed, it is well establish that chaperone-mediated autophagy (CMA) is hyper-activated when macroautophagy is impaired (Kaushik et al., 2008), so we hypothesized that USP1 degradation can occur via CMA, activated by CAPNS1 depletion. To test this hypothesis we impaired CMA by silencing a protein important for autophagosome formation, LAMP2A. Also in these conditions, however, we observed a decrease in USP1 protein levels, thus suggesting that USP1 could be alternatively degraded by different pathways (Figure 3.21).

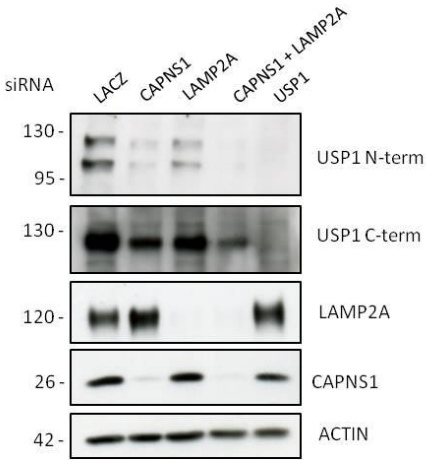

Figure 3.21 LACZ, CAPNS1, LAMP2A, a combination of CAPNS1 and LAMP2A, or USP1 were depleted by siRNA transfections in U2OS cells. 72 hours later cells were lysated and analysed by Western Blot to monitor USP1 levels.

3.11 CAPNS1 effect upon UAF1/WDR48 protein levels

USP1 needs association with UAF1/WDR48 to be active and stabilized (Cohn et al., 2007). To better understand the mechanism by which calpain affect USP1 stability we searched for an effect of calpain upon USP1 co-factor and activator UAF1/WDR48. We observed that in calpain-depleted cells there is a downregulation of UAF1/WDR48 protein compared to control cells (Figure 3.22, panel A), while UAF1/WDR48 mRNA levels didn't change (Figure 3.22, panel B), suggesting a post-transcriptional regulation.

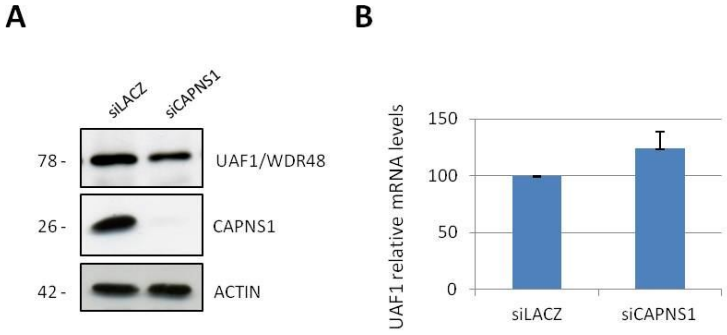

Figure 3.22. U2OS cells were transfected with LACZ, or CAPNS1 siRNA. 72 hours later cells were lysates or processed for qRT-PCR analysis. Panel A shows UAF1/WDR48 protein levels upon indicated siRNAs. The graph in panel B shows the quantification of UAF1/WDR48 mRNA, with the indication of standard deviation of three independent experiments. For each mRNA the control RNA extracted from LACZ silenced cells is set as 100.

Next we asked whether UAF1/WDR48 downregulation is responsible for USP1 downregulation in CAPNS1-depleted cells. We treated U2OS cells with calpain specific inhibitor Z-leu-leu-CHO, and we overexpressed UAF1/WDR48 to investigate whether USP1 co-factor overexpression could rescue USP1 levels in calpain-inhibited cells. What we observed was a partial rescue of USP1 levels (Figure 3.23), so we concluded that CAPNS1 regulates USP1 only partially by modulating its cofactor UAF1/WDR48.

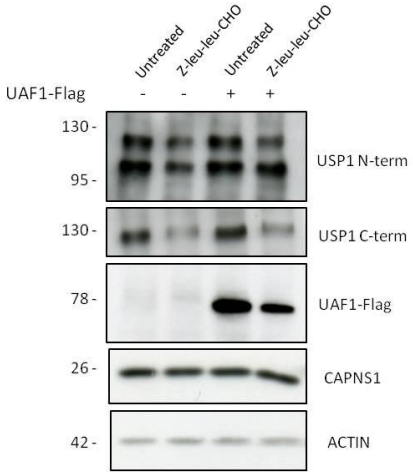

Figure 3.23 U2OS cells were transfected with Flag-UAF1/WDR48 expression plasmid or an empty vector. 24 hours later cells were incubated for 6 hours with Z-leu-leu-CHO or left untreated and then used to monitor USP1 levels by Western Blot.

3.12 Study of the involvement of Cdh1 in CAPNS1-mediated regulation of USP1

A recent study suggests a model in which USP1 is proteasome degraded by the APC/C-Cdh1-mediated ubiquitination during G1, and this event is required to properly recruit TLS polymerases for UV-mediated DNA gap repair (Cotto-Rios et al., 2011a).

Since it is known that Cdk5 activator p35 is a calpain substrate (Sato et al., 2011) and that Cdk5 phosphorylates Cdh1 inactivating it (Maestre et al., 2008), we asked whether calpain effect upon USP1 stabilization could be mediated by APC/C-Cdh1 complex. We transfected U2OS cells with a control siRNA, a siRNA targeting CAPNS1, a siRNA targeting Cdh1 and co-transfected a CAPNS1- and Cdh1-siRNA together. We followed USP1 endogenous levels and observed that, as expected, there was a decrease upon CAPNS1 knockdown, while any significant change upon Cdh1 knockdown. The interesting finding was that knocking down simultaneously both CAPNS1 and Cdh1 we observed a rescue of USP1 levels (Figure 3.24), and this result suggests that the inactivation of APC/C-Cdh1 complex in calpain-depleted cells lead to USP1 accumulation.

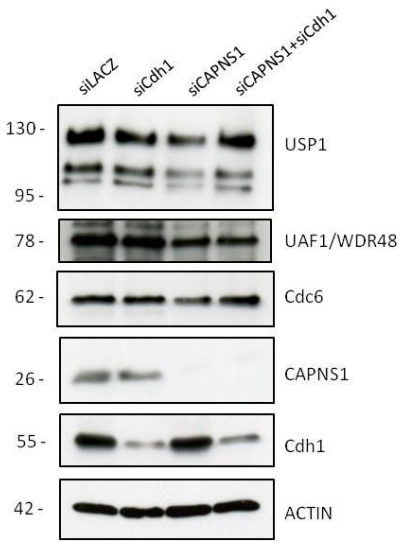

Figure 3.24 LACZ, Cdh1, CAPNS1, and a combination of CAPNS1 and Cdh1 siRNA were transfected in U2OS cells. 72 hours later cells were lysated and analysed by Western Blot to monitor USP1 levels.

We propose a model in which calpain depletion leads to the inactivation of Cdk5 that fails to phosphorylate Cdh1, leading to the activation of APC/C-Cdh1 complex. This could lead to USP1 downregulation in calpain-depleted cells (Figure 3.25). Accordingly, upon Cdh1 knockdown we observed USP1 accumulation, and this is observed also for another APC/C-Cdh1 complex, such as Cdc6 (Li et al., 2009), while the levels of USP1 cofactor, UAF1/WDR48, didn't change, suggesting that this effect is specifically directed against USP1. This working model provides one possible explanation for the phenotype that we observed upon CAPNS1 depletion and deserves a further in depth-examination, since USP1 downregulation leads to dramatic effect in the cells, such as dysregulation of DNA damage response.

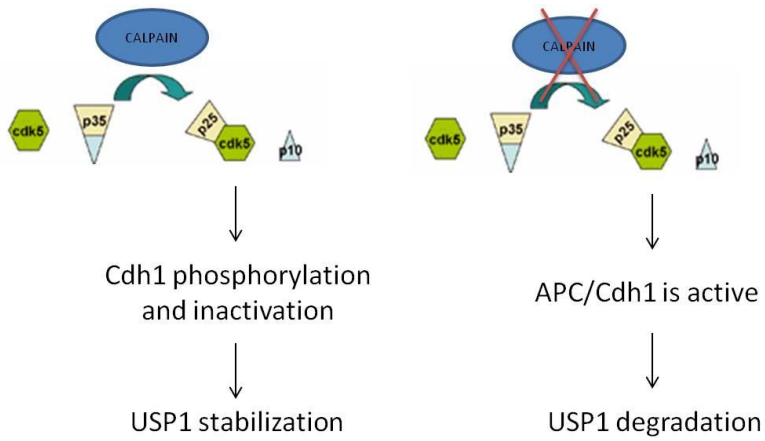

Figure 3.25 Schematic representation of a working model in which calpain regulation of p35/p25 activator of Cdk5 could affect Cdh1 phosphorylation and thus APC/C-Cdh1 activity, that leads to a regulation of USP1 stabilization.

4. CONCLUSIONS

Ubiquitous micro- and milli-calpain require the CAPNS1 regulative subunit for function, and therefore are inactive in its absence (Goll et al., 2003). The work carried out for this thesis led us to identify a novel CAPNS1 interactor: the deubiquitinating enzyme USP1. Increasing evidences place USP1 in a crucial place for the regulation of the DNA damage response and genome integrity maintenance. Cells are able to detect and propagate the initial DNA damage signal to achieve cellular responses including cell cycle arrest, DNA repair, senescence and apoptosis, which collectively have been termed the DNA damage response (DDR). Dysregulation of components involved in these processes contributes to genomic instability, which can lead to tumorigenesis. USP1 negatively regulates the monoubiquitination of proteins involved in important cellular pathways, such as the Fanconi anemia (FA) pathway, and the translesion synthesis (TLS). USP1 deubiquitinates the DNA replication processivity factor PCNA and FANCD2/FANCI heterodimer, a key component of the FA pathway involved in DNA repair and protection from chromosome instability. The Fanconi core complex, activated upon DNA damage detected during DNA synthesis, triggers the monoubiquitination of FANCD2/FANCI heterodimer and its subsequent relocalization on chromatin-associated foci, where it recruits DNA replication and repair factors required for homologous recombination repair. USP1 deubiquitinates FANCD2 when cells exit S phase or recommence cycling after a DNA damage insult (Nijman et al., 2005), and it is required for correct FA pathway functioning, FANCD2 nuclear foci assembly and release from chromatin (Kim et al., 2009). Indeed, inactivation of murine USP1 results in genomic instability and a Fanconi Anemia phenotype. UV damage induces the degradation of USP1, allowing PCNA monoubiquitination and activation of translesion DNA synthesis (TLS) that bypasses DNA lesions with low stringency basepairing requirements. USP1 degradation involves an initial C-terminal autocleavage event producing a 75 KDa product, followed by proteasomal degradation (Huang et al., 2006). An alternative study demonstrated that both catalytic inactive- and autocleavage site-mutants undergo proteolytic processing after UV exposure. Moreover, expressing a noncleavable mutant of USP1 in USP1 knockout cells partially rescues crosslinker sensitivity (Oestergaard et al., 2007). These apparently controversial results indicate that there could be another mechanism for USP1 regulation. Indeed, recently it was demonstrated that during G1 phase USP1 is targeted for proteasomal degradation by APC/C-Cdh1 ubiquitin ligase complex to ensure that USP1 levels are kept low before S phase entry. Low levels of USP1 enable UV-induced PCNA monoubiquitination during G1, which is likely to allow the recruitment of TLS polymerases to UV lesions (Cotto-Rios et al., 2011a).

The finding that USP1 interacts with calpain small subunit 1 (CAPNS1) came out from an immunoprecipitation experiment in which the proteins interacting with endogenous CAPNS1 where analyzed by means of mass spectrometry. The choice to analyze the interactions between endogenous proteins was given by the will to avoid eventual forced interactions mediated by the accumulation of a protein in the cells. Among the interacting protein we found, as expected, well known partners of calpain, for instance calpain large catalytic subunits micro- and milli-calpain. This assured us that the immunoprecipitation experiment was correctly carried out. Among the unknown CAPNS1 partners there were, besides USP1, other interesting proteins worthy of further studies, such as GRP78/BIP, a protein involved in autophagy (Li et al., 2008). We decided to focus on USP1 deubiquitinating enzyme for its importance in genome integrity maintenance and response to cellular stress. In order to further characterize CAPNS1/USP1 interaction, we confirmed in 293T cells extracts the interaction between endogenous CAPNS1 and ectopic USP1. To exclude the possibility that the interaction was not specific, we used as a negative control an unrelated deubiquitinating enzyme, USP33, and we found that it didn't interact with CAPNS1, indicating that USP1/CAPNS1 interaction is specific. Further experiments are needed to investigate whether the interaction is direct or mediated by the catalytic subunits, micro- and/or milli calpain. To this end GST-USP1 recombinant proteins will be produced and utilized in GST pull-down assays. Moreover we utilized deletion mutants of USP1 to map the region responsible for the interaction, by means of co-immunoprecipitation experiments between endogenous CAPNS1 and ectopically expressed USP1 deletion mutants. We found that the first region of USP1, comprising aminoacids from 1 to 523, is sufficient to assure the interaction. Further experiments on deleted proteins are ongoing to dissect more in detail which regions of calpain and USP1 are required for interaction. Moreover we analyzed cellular localization of USP1 and CAPNS1 by means of immunofluorescence analysis: our results indicate that the ectopic proteins are localized in the same cellular regions, predominantly into the nucleus.

Once established that the two proteins interact with each other and are present in the same cellular compartment, we asked whether calpain is required for USP1 stability or deubiquitinating activity. To answer this question we knockdown CAPNS1 in a series of cell lines and we analyzed by Western Blot experiments eventual changes in endogenous USP1 protein levels. The striking result that we observed is a decrease in USP1 levels in absence of CAPNS1, that could indicate a requirement of CAPNS1 in the maintenance of USP1 stability. We obtained reproducible results in different human cellular systems: osteosarcoma U2OS cells, osteosarcoma MG-63 cells, non-small cell lung carcinoma H1299 cells, normal fibroblasts WI38 cells. The evidence that also in cell lines lacking p53 activity, such as MG-63 and H1299, USP1 levels decrease demonstrates that this effect

is p53-independent. We asked whether also in murine cells the same regulation occurred, so we took advantage of a cellular model of mouse embryonic fibroblasts (MEFs) wild-type or knockout for *Capns1* gene (Arthur et al., 2000). Also in these cells there is a decrease in USP1 levels in cells lacking CAPNS1, and more importantly, in a *Capns1* -/- cell line in which the gene was reintroduced, USP1 levels return back to control cells levels. This demonstrated that the changes in USP1 levels are specifically due to CAPNS1 and not to other proteins or processes eventually regulated by CAPNS1 knockdown.

Since USP1 is downregulated during DNA damage to allow the accumulation of PCNA monoubiquitination, we asked whether CAPNS1 could affect USP1 stability also upon DNA damage conditions. We subjected cells to UV-induced DNA damage, and also in these conditions we observed USP1 downregulation upon CAPNS1 knockdown.

Next, we asked whether CAPNS1 can modulate USP1 activity. To answer this question we monitored the monoubiquitination levels of a substrate of USP1, PCNA. We expected that the decrease in USP1 levels coupled to CAPNS1 depletion leads to an increase in PCNA monoubiquitination, since USP1 is less stable. Indeed, we observed an increase in PCNA monoubiquitinated form in cells lacking CAPNS1 with respect to control cells, indicating that CAPNS1 can modulate USP1 activity. In particular we observed a reverse correlation between CAPNS1 and monoubiquitinate PCNA levels: indeed cells lacking CAPNS1 show higher levels of PCNA modified form, while cells overexpressing CAPNS1 show low levels of PCNA monoubiquitination. To confirm that the effect upon PCNA monoubiquitination is due to USP1 modulation by CAPNS1, we overexpressed in CAPNS1 knockdown cells USP1 mutated in the autocleavage site, and therefore more stable. The overexpression of this mutant reduced the levels of monoubiquitinated PCNA in CAPNS1 depleted cells, indicating that the effect upon PCNA is USP1-dependent.

Besides this result, we monitored the efficiency of PCNA recruitment upon chromatin in DNA damage conditions by immunofluorescence experiments. In CAPNS1-depleted cells increased monoubiquitination of PCNA is linked to a perturbation in the dynamics of PCNA loading onto chromatin: in particular, while in control cells the increase in PCNA foci formation induced upon DNA damage is followed by a decrease after a certain time interval, in CAPNS1-depleted cells this kinetic is perturbed, since PCNA foci persist after long time intervals. These results suggest that in CAPNS1-depleted cells the response to DNA damage is altered, since the decrease of PCNA foci on chromatin indicates an efficient repair of DNA lesions, condition that is reduced when CAPNS1 is knockdown. Next, we wanted to study the role of calpain in the PCNA-modulated switch between high fidelity replication and translesion synthesis. PCNA monoubiquitination is required

for the recruitment at sites of damaged DNA of low-fidelity translesion synthesis polymerases that allow the bypass of DNA damage. We analyzed the localization of ectopic Polymerase η, that is one of Y-family of translesion synthesis polymerases, to monitor its recruitment upon chromatin. In control cells, upon DNA damage, Polη moves from a diffuse nuclear localization to distinct chromatin-associated foci. The percentage of cells containing Polη foci is higher in CAPNS1 depleted cells than in control cells, before DNA damage induction. The number of cells positive for Polη foci in CAPNS1-depleted cells is reduced upon overexpression of USP1 wild-type, but not by an inactive USP1. This result indicates that the regulation of USP1 activity is important for the CAPNS1-mediated modulation of translesion synthesis switch. Next, we investigated the role played by calpain in genome integrity maintenance by means of COMET assays carried on to detect DNA damage induction in basal conditions and upon UV light exposure. CAPNS1-depleted cells presented an overall defect in the activation of DNA repair compared to control cells. This phenotype is probably linked to the decrease in USP1 levels that leads to a defective activation of repair pathways such as FA pathway and homologous recombination, and accumulation of monoubiquitinated PCNA in this cells, that leads to increased error-prone translesion synthesis.

Subsequently we tried to understand how calpain regulates USP1 stability. First of all, to determine at what level the regulation occurs, we compared USP1 mRNA levels in CAPNS1-depleted cells to control cells, and we observed no significant changes, suggesting that the regulation occurs at post-transcriptional levels. Then we asked whether calpain catalytic activity is required to the regulation of USP1 stability, and to deplete calpain activity we overexpressed calpain specific inhibitor calpastatin, or treated the cells with a specific inhibiting peptide. We observed that calpain activity is essential for USP1 regulation because upon calpain inhibition USP1 protein levels still decreased. Moreover, by depletion of calpain large catalytic subunits, micro- and milli-calpain, we found that micro-calpain depletion, but not milli-calpain depletion, leads to a decrease in USP1 protein levels, suggesting that the calpain isoform responsible for USP1 regulation is micro-calpain.

Since calpain is essential for correct autophagy activation, we asked whether USP1 degradation in absence of calpain activity could be mediated by autophagy. Moreover, we observed that by treating cells with a combination of inhibitors of cathepsins, enzymes that degrade the lysosomal cargo, USP1 protein levels are stabilized. These observations led us to hypothesize that USP1 could be degraded by a pathway that converge into the lysosome, besides that by the proteasome. We impaired macroautophagy pathway by knocking down the essential protein ATG5, and we observed that USP1 is still downregulated when macroautophagy is blocked, suggesting that macroautophagy inactivation could be compensated by increased activation of other degradation pathways, for instance chaperone-mediated autophagy (CMA). In CMA protein targeted to degradation are

recognized by a chaperone complex containing Hsc70 (heat shock cognate of 70 KDa) and delivered into lysosomes through interactions with LAMP-2A (lysosome-associated membrane protein 2A). CMA is activated during prolonged nutrient deprivation and triggers the degradation of cytosolic proteins in a molecule by molecule fashion (Massey et al., 2005). To impair CMA we depleted LAMP2A, important for autophagosome formation. Also in this case we observed a decrease of USP1 protein levels, suggesting that USP1 could be degraded by alternatively activated different pathways.

USP1 stability and activity is increased by the interaction with a cofactor, UAF1/WDR48. Since we showed that CAPNS1 depletion is linked to a reduction of UAF1/WDR48 protein levels, without affecting mRNA levels, we hypothesized that reduction of USP1 cofactor could bring to a downregulation of USP1. To verify this hypothesis we overexpressed UAF1/WDR48 in cells were calpain was inhibited: USP1 levels didn't return to basal levels, indicating that also other mechanisms should be involved in CAPNS1-mediated USP1 regulation. The regulation of UAF1/WDR48 by calpain is very interesting and needs further characterization since UAF1/WDR48 can stabilize and activate also other deubiquitinating enzymes, such as USP12, involved in histone H2A and H2B deubiquitination (Joo et al., 2011), and USP46, a modulator of GABA signaling (Todi and Paulson, 2011).

The recent finding that USP1 is targeted to proteasomal degradation by APC/C-Cdh1 ubiquitin ligase complex (Cotto-Rios et al., 2011a) led us to ask whether calpain could regulate APC/C-Cdh1-mediated downregulation of USP1. One well established substrate of calpain is p35 activator of Cdk5 (Sato et al., 2011), a kinase that is responsible for an inactivating phosphorylation of Cdh1, a member of APC/C-Cdh1 complex (Maestre et al., 2008). Our hypothesis was that calpain depletion leads to a decrease in Cdk5 activity that affect Cdh1 phosphorylation, leading to an increased APC/C-Cdh1 activity and a subsequent destabilization of USP1. This hypothesis was supported by our observation that Cdh1 knocking down in CAPNS1 depleted cells triggers USP1 stabilization.

This study led us to understand how calpain regulates the deubiquitinating enzyme USP1. Recently it was demonstrated that USP1 is able to deubiquitinate and keep stable ID proteins (Inhibitor of DNA binding), essential to inhibit differentiation and maintain stemness. USP1 knockdown in osteosarcoma cells leads to ID protein degradation, cell cycle arrest, and osteogenic differentiation. These observations make USP1 a regulator of stemness maintenance in osteosarcoma, and thus suitable as a target for differentiative therapy (Williams et al., 2011). The importance of calpain regulation of USP1 in this cell model could lead to a therapeutical inhibition of calpain. It is known that calpain can be targeted for anti-cancer therapy, since it is involved in survival of tumour cells,

neoplastic invasion and resistance to chemotherapeutic agents. The new function of calpain described in this thesis work, that is the regulation of deubiquititnating enzyme USP1, acquires more importance since USP1 is involved in the regulation of different pathways implicated in cancer biology, and in particular the recent finding that USP1 inactivation can lead to osteosarcoma differentiation. The detailed understanding of the mechanism by which calpain regulates USP1 function could lead to the development of novel drugs targeting calpain and USP1.

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