Identifying Novel Interaction Between SENP1 and the Deubiquitinating Enzyme Ubiquitin-Specific Protease 7 (USP7)

KANWARPARTAP DHINDSA

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Abstract

Ubiquitin Specific Protease 7 (USP7) is a well-characterized deubiquitinating enzyme involved in numerous cellular pathways. Despite extensive studies, many USP7-regulated proteins and pathways remain unidentified. One emerging area of interest is the SUMOylation and deSUMOylation pathways, dynamic post-translational modifications increasingly recognized for their importance in protein regulation and disease progression. Through bioinformatics analysis, we identified SENP1, a deSUMOylating enzyme that contains a P/A/ExxS consensus motif, characteristic of known USP7 substrates. We hypothesized that USP7 interacts with and regulates SENP1, thereby modulating its stability and activity. Using various methods, including co-immunoprecipitation, silencing and overexpression in HCT116 cells, our findings demonstrate that USP7 interacts with and deubiquitinates SENP1, thus regulating its stability. Given SENP1's involvement in various diseases, including cancer progression, and hypoxia signalling, our findings provide a novel insight into USP7-mediated regulation of deSUMOylation and highlights its potential as a therapeutic target.

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List of Abbreviations

AR	Androgen receptor
CAT	Catalytic core domain
CHFR	Checkpoint with Forkhead and RING finger domain
CHX	Cycloheximide
Co-IP	Co-Immunoprecipitation
CtBP	carboxyl-terminus binding protein
CTD	Carboxy-Terminal Domain
CP	Core Particle
DESI1	deSUMOylating isopeptidase 1
DESI2	deSUMOylating isopeptidase 2
DNMT1	DNA Methyl Transferase 1
DSB	Double-stranded breaks
DUB	Deubiquitinating Enzyme
DUSP	Domain in USP
EBNA1	Epstein-Barr Nuclear Antigen 1
EBV	Epstein-Barr virus
ELOB	Elongin B
ELOC	Elongin C
EV	Empty Vector
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin ligase
FOXO	Fork head box O
FOXO4	Fork head box O4
HAUSP	Herpesvirus Associated Ubiquitin Specific Protease
HCC	Hepatocellular carcinoma
HCT116	Human Colorectal Carcinoma Cell Line
HCT116 USP7$^{-/-}$ KO	USP7 Knockout Human Colorectal Carcinoma Cell Line
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HECT	Homologous to the E6AP carboxyl terminus
HEK293T	Human Embryonic Kidney Cell Line Stably Expressing the SV40 Large T Antigen
HIF-lα	Hypoxia-inducible transcription factor-1α
HSV-1	Herpes Simplex Virus type 1
H3K9me3	Histone H3 Lysine 9 trimethylation
ICP0	Infected Cell Protein 0
JAMM/MPN+	JAB1/MPN/MOV34 metalloenzyme domain
KSHV	Kaposi's Sarcoma herpesvirus
LANA	Latency-Associated Nuclear Antigen
MCM-BP	Mini-chromosome maintenance complex-binding protein
MDC1	Mediator of DNA damage checkpoint 1
MDM2	Murine double minute 2
MJD	Machado-Josephin Domain protease

MMP	Matrix metalloproteinase
NB	Nuclear bodies
NER	Nucleotide excision repair
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
NSCLC	Nonsmall cell lung cancer
NTD	N-Terminal Domain
OTU	Ovarian Tumor related proteases
PAGE	Polyacrylamide Gel
PBS	Phosphate-buffered saline
PCGF	Polycomb group RING finger
PCNA	Proliferating cell nuclear antigen
PHF8	PHD Finger Protein 8
PIAS	Protein inhibitor of activated signal transducer and activator of transcription
PLK1	Polo-like kinase 1
PPM1G	Protein phosphatase 1G
PRC-1	Polycomb Repressive Complex-1
PTEN	Phosphate and tensin homolog
RanGAP1	Ran GTPase-activating protein 1
RBR	RING-between-RING
RING	Really Interesting New Gene
RNF169	Ring Finger Protein 169
RP	Regulatory Particle
SAE1	SUMO-activating enzyme subunit 1
SAE2	SUMO-activating enzyme subunit 2
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SENPs	SUMO-specific proteases
siRNA	small interfering RNA
SIRT1	Sirtuin 1
SUMO	Small Ubiquitin-related Modifier
TBS	Tris-buffered saline
TDG	Thymine DNA glycosylase
TLS	Translesion DNA synthesis pathway
TRAF	Tumor necrosis factor Receptor Associated Factor
Ub	Ubiquitin
UBA52	Ubiquitin A-52 Residue Ribosomal Protein Fusion Gene
UBA80	Ubiquitin Carboxyl Extension Protein 80 Gene
UBB	Ubiquitin B Gene
UBC	Ubiquitin C Gene
UBC9	Ubiquitin-Conjugating Enzyme 9
Ubl	Ubiquitin-like
UCH	Ubiquitin C-terminal Hydrolase
UHRF1	Ubiquitin-like, containing PHD and RING finger domains 1
USP	Ubiquitin-Specific Protease

USPL1	Ubiquitin-specific protease-like 1
USP7	Ubiquitin-Specific Protease 7
vIRF1	viral Interferon Regulatory Factors 1
vIRF2	viral Interferon Regulatory Factors 2
vIRF4	viral Interferon Regulatory Factors 4
XPC	Xeroderma pigmentosum complementation group C
Znf-UBP	Zinc-finger ubiquitin-binding domain

Chapter 1: Introduction

1.1 Proteome Regulation Through Ubiquitin-Mediated Degradation

Maintaining the structural integrity of the proteome is vital for normal cellular functions and ultimately, organism viability.[1] One highly conserved pathway that plays a crucial role in modulating the proteome by providing the integral balance between protein synthesis and degradation is the ubiquitin-proteasome degradation pathway. Alterations in this pathway can disrupt many biological processes, including cell cycle control, DNA damage response, and apoptosis.[2,3] Central to this pathway is ubiquitin, a small yet highly conserved protein that serves as a molecular tag for protein modifications, regulating activity, localization and protein turnover.

1.2 Ubiquitin

Ubiquitin (Ub) is a 76-amino acid small protein that is highly conserved and ubiquitous among all eukaryotes. [2–4] In mammalian cells, Ub is encoded by four different genes, two of which, Ubiquitin B Gene (*Ubb*) and the Ubiquitin C Gene (*Ubc*) produce polymers of ubiquitin linked together while the other two, Ubiquitin A-52 Residue Ribosomal Protein Fusion Gene (*UBA52*) and Ubiquitin Carboxyl Extension Protein 80 Gene (*UBA80*) comprise a single ubiquitin that is fused to ribosomal proteins. [5–8] Although these genes may seem redundant, their importance is highlighted in *Ubb* and *Ubc* knockout mice, which are embryonic lethal or infertile, respectively.[7–9]

Proteins are subject to different ubiquitin modifications, divided into three different types, which dictate their cellular fate. The first type known as mono-ubiquitination, refers to the

covalent attachment of a single ubiquitin monomer to a lysine residue on a substrate protein (Figure 1). In contrast, multi-monoubiquitylation refers to the addition of individual ubiquitin monomers to different lysine residues of the same substrate (Figure 1).[4,10] Generally, these two types of modification are associated with non-proteolytic function, including protein interaction and localization, cellular activity, DNA repair and endocytosis.[11] The third type of modification is known as polyubiquitination, which refers to the addition of multiple ubiquitin monomers on the same lysine residue of the target protein, effectively creating ubiquitin chains (Figure 1). These chains are assembled on a single lysine residue through the formation of isopeptide bonds between the carboxyl terminus of one monomer and the ε-amino group of the lysine on the next monomer.[10]

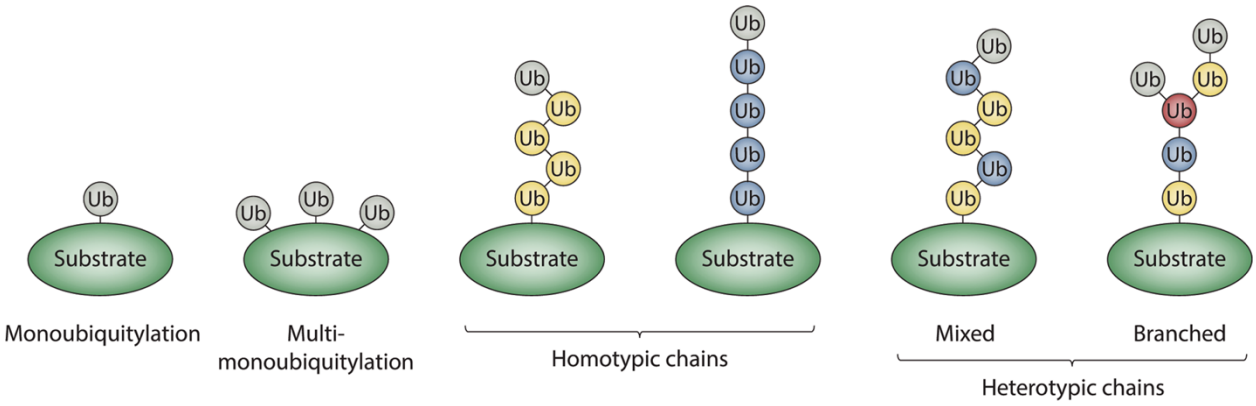


Figure 1. Different Types of Ubiquitin Linkages.
Ubiquitin can be conjugated to substrate proteins in different ways: as a single ubiquitin on a single lysine residue (monoubiquitylation), or multiple single ubiquitin molecules to different lysine residues (multi-monoubiquitylation), or as a chain of ubiquitin molecules on the same lysine residue, which can either be homotypic or heterotypic (polyubiquitylation). Terminal ubiquitin molecules are shown in grey, while branch point ubiquitin is depicted in red. Ubiquitin that is modified on one acceptor site is coloured in yellow or blue. Reproduced from French et al.[10]

The functional outcome of the target protein depends on which of the seven ubiquitin lysine residues (K6, K11, K27, K29, K33, K48 and K63) the isopeptide linkage is formed (Figure 2). Out of the seven lysine residues, K48 and K63-linked polyubiquitination are the most well-studied. K48-linkages are the most prevalent chains in the biological process.[4] This linkage

is associated with targeting substrates for proteasomal-mediated degradation by the 26S proteosome. On the other hand, K63-linkages mainly regulate vital intracellular signalling processes such as DNA damage repair, autophagy, and cytokine signalling. In summary, ubiquitin is a small, multifunctional protein that serves as a versatile signalling molecule, orchestrating a wide range of cellular processes essential for maintaining homeostasis.

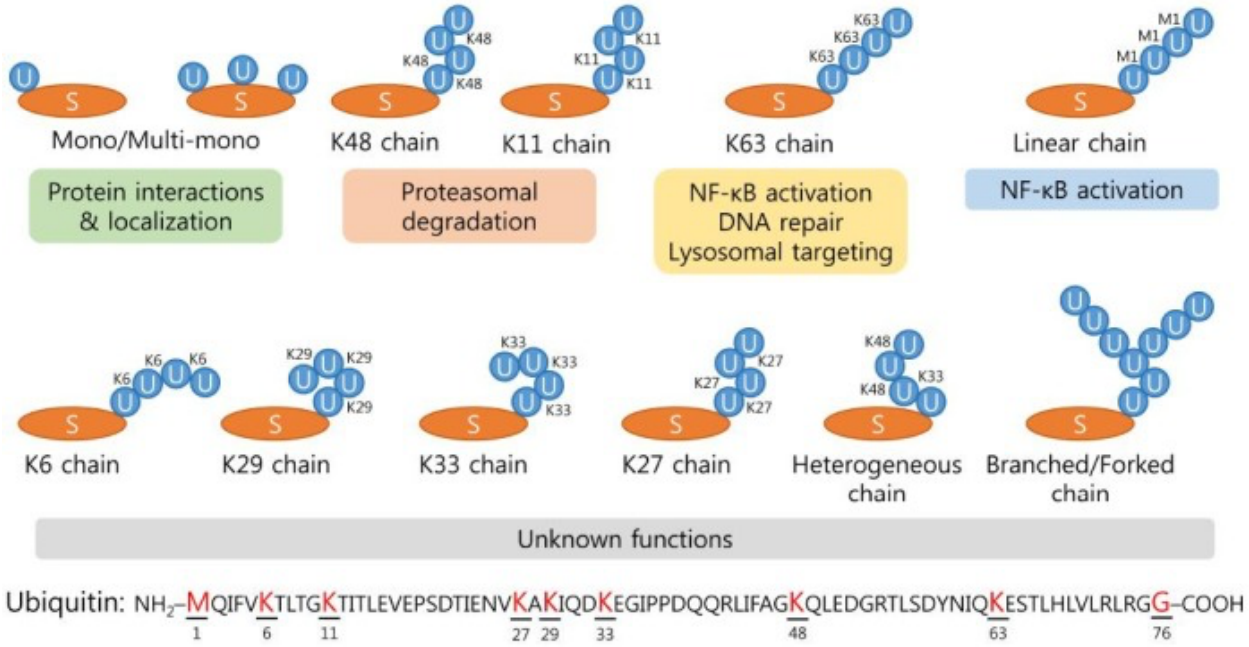


Figure 2. Different Types and Functions of Ubiquitin Chain Linkages.
Ubiquitin contains seven different lysine residues, each capable of forming distinct ubiquitin linkages. The position and type of linkage will dictate the fate of the substrate protein, influencing various cellular processes such as proteasomal degradation, DNA repair and immune response. Reproduced from Park & Ryu.[12]

1.3 Protein Ubiquitination

Protein ubiquitination is a multi-step enzymatic process orchestrated by three crucial enzymes: the ubiquitin-activating enzyme or E1 enzyme, the ubiquitin-conjugating enzyme or E2 enzyme, and the ubiquitin ligase or E3 ligase (Figure 3). Firstly, through an ATP-dependent reaction, the precursor ubiquitin is activated by the E1 enzyme, forming a thioester-linked ubiquitin between the cysteine residue in the active site of E1 and the C terminus of ubiquitin.[4,13]

The activated ubiquitin is then transferred from the E1 enzyme to the E2 enzyme, again through the active cysteine site in the E2 enzyme, forming a thioester bond. Finally, the E3 ligase facilitates the transfer of ubiquitin by simultaneously binding the E2 enzyme and the target protein, catalyzing the transfer either directly by direct transfer from the E2 enzyme or indirectly after creating a thioester bond with E3.[13] The human genome encodes for two E1 conjugating enzymes (UBA1 and UBA6), 38 E2s and approximately over 600 E3s.[14] The extensive diversity of E3 ligases plays a critical role in conferring substrate specificity of the ubiquitin system. Currently, E3 ligases can be classified into four distinct categories based on structural features and mechanism of action. These classes include HECT type, U-box type, RING-finger type, and RBR type.

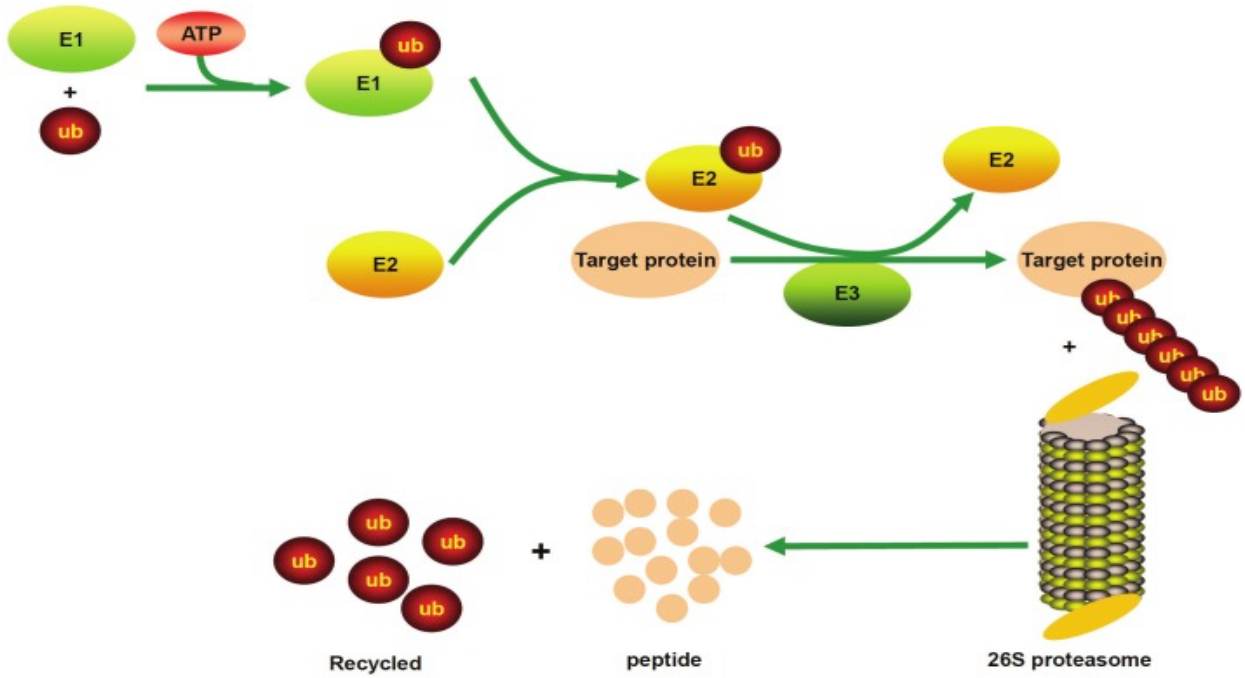


Figure 3. Overview of Ubiquitin-Proteasome Pathway.
Ubiquitin is attached to target proteins through a cascade involving three distinct enzymes: E1 (the ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (the ubiquitin ligase). In an ATP-dependent process, the E1 enzyme activates and transfers a ubiquitin to the E2 enzyme, which is transferred to the E3 enzyme, allowing for the direct or indirect transfer of the ubiquitin to the target protein. This process can be repeated multiple times, creating polyubiquitylated chains, marking the target protein for 26S proteasome degradation. Reproduced from Tu et al.[2]

HECT (homologous to the E6AP carboxyl terminus) E3 ligase family, one of the largest and earliest studied families, ubiquitinates substrates indirectly through a two-step process.[4] First, the ubiquitin is transferred from the E2 conjugating enzyme to the active cysteine site within the conserved homologous HECT domain of the E3 ligase.[4] Subsequently, the ubiquitin is then attached to the lysine residue of the target protein (Figure 4A).

The U-box E3 ubiquitin ligases comprise a relatively small but evolutionarily conserved family, characterized by a U-box domain located within the C-terminus. This class of E3 ligases are essential for protein quality control. Unlike the HECT E3 ligases that indirectly transfer ubiquitin to target proteins, members of the U-box E3 ligases act as scaffolds, simultaneously

interacting with both the E2 enzyme and the target protein and facilitating the direct transfer of ubiquitin (Figure 4C).

The RING (really interesting new gene) E3 ligases are the largest type of E3 ubiquitin ligases. There are over 600 RING-type ligases characterized by their RING domain. Similar to the U-box E3 ligases, the RING E3 ligases catalyze ubiquitination through a direct mechanism bypassing the E3-ub intermediate (Figure 4B).[4]

The RBR (RING-between-RING) E3 ligases are a newly discovered class of E3 ligases, and their mechanism of action is similar to that of the HECT E3 ligases, such that they function in a two-step reaction. The RBR E3 ligases contain a RING2 domain that allows for the transfer of ubiquitin from the E2 ligase onto the E3 ligase, forming a thioester intermediate with the E3. Subsequently, the ubiquitin is covalently attached to the lysine of the target protein. Although similar to HECT E3 ligases, RBR ligases tend to create linear ubiquitin chains on target proteins, suggesting a distinct mechanism (Figure 4D).[4]

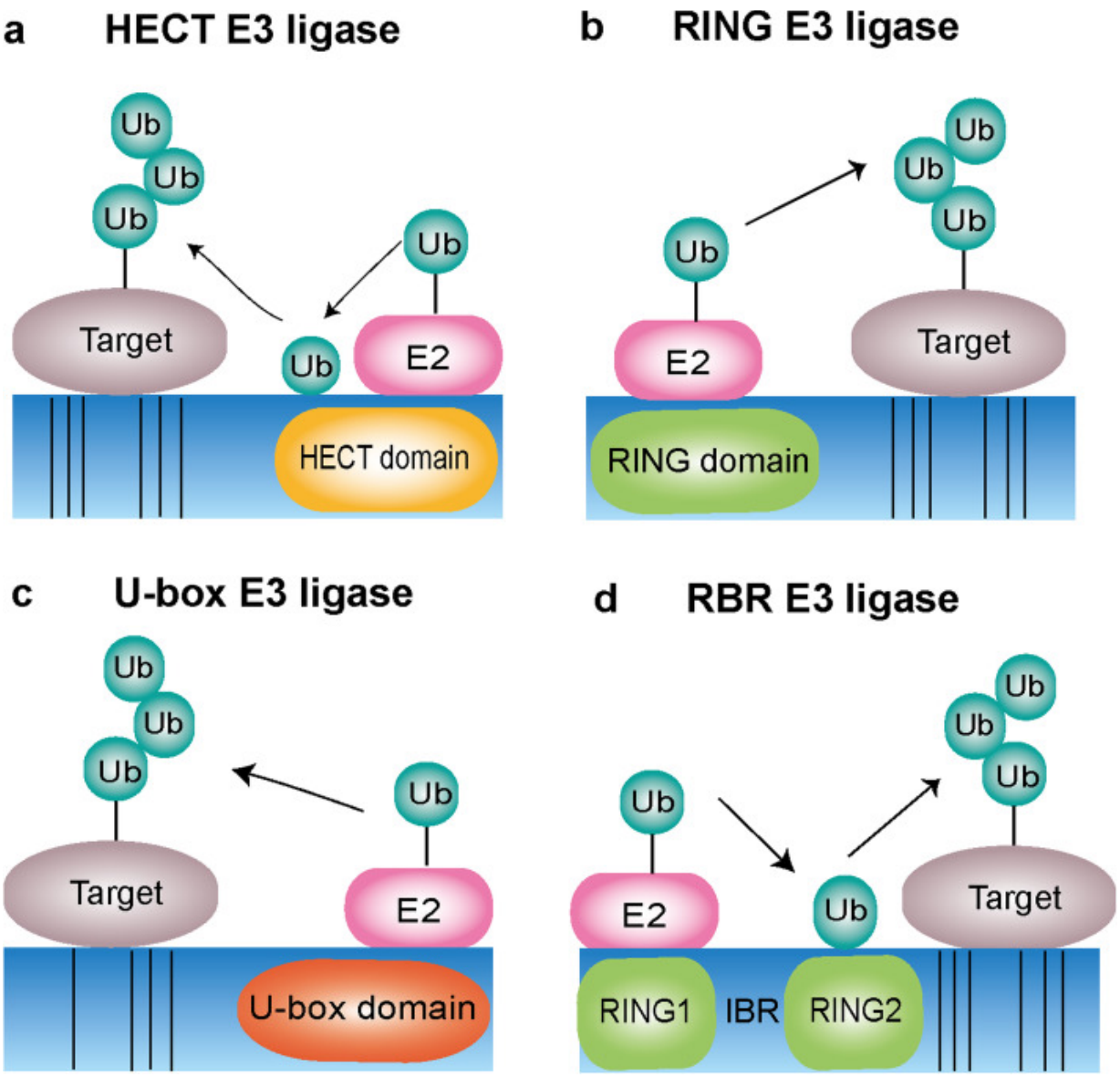


Figure 4. Different Types of Ubiquitin E3 Ligases and Their Method of Ubiquitin Transfer.
(A & D) HECT and RBR E3 ligases mediate transfer of ubiquitin via an indirect, two-step process where the ubiquitin molecule is first transferred from the E2 enzyme to the E3 ligase and then to the target protein.
(B & C) RING and U-box E3 ligases directly facilitate the transfer of ubiquitin from the E2 enzyme to the target protein, bypassing the E3-ubiquitin intermediate.
Reproduced from Yang et al.[4]

1.4 Protein Deubiquitination

In some instances, a previously ubiquitinated protein may no longer require ubiquitination for its activity, localization or stability. As a result, there is a special class of

enzymes known as deubiquitinating enzymes (DUBs) that are critical in controlling protein function by cleaving ubiquitin from substrate proteins. As a result, DUBs prevent substrate proteins that were previously ubiquitinated and designated to the 26S proteasome from being degraded, thus regulating their stability within the cell. Through their activity, DUBs play a critical role in regulating cellular homeostasis by controlling key processes such as protein function and turnover, intracellular trafficking and participation in diverse signalling pathways.[15] Dysregulation of DUBs can disrupt these processes and has been implicated in various diseases, including different cancers and neurological disorders.[16] In addition to regulating covalently attached ubiquitin from target proteins, DUBs are also essential in maintaining the cellular pool of free ubiquitin by cleaving and processing newly synthesized ubiquitin fusion proteins. The maintenance of a free pool of ubiquitin is critical in responding to rapid changes within the cellular environment, such as stress-induced damage.[15] Therefore, the coordinated interplay between both the ubiquitination and deubiquitination pathways is crucial for preserving cellular homeostasis.

The human genome encodes approximately 100 different DUBs, which can be divided into 2 major classes: metalloproteases and cysteine proteases.[15] The metalloproteases class of DUBs have a conserved JAB1/MPN/MOV34 (JAMM/MPN+) domain that requires a zinc cofactor for enzyme activity.[15] This class of DUBs catalyze isopeptide bond hydrolysis through a zinc-dependent mechanism involving a catalytic serine residue. Although the human genome encodes 14 proteins containing the JAMM/MPN+ domain, only seven can bind the zinc ion for catalytic activity, and just six have been experimentally verified as functional deubiquitinating enzymes capable of removing ubiquitin from substrate proteins.[15]

Unlike the metalloproteases that require a zinc ion for catalysis, the class of cysteine proteases utilize a catalytic triad composed of a cysteine, histidine and an asparagine or aspartate residue to mediate the isopeptide hydrolysis in ubiquitin conjugates. This class of DUBs can be broken down into six families of proteins based on sequence and structural conservation. These families include Ubiquitin C-terminal Hydrolase (UCH), Ubiquitin-Specific Protease (USP), Ovarian Tumor (OTU), Machado-Josephin Domain (MJD), K48 polyubiquitin-specific MINDY domain families, and the newly discovered zinc finger with UFM1-specific peptidase domain.[15] Out of these six families, the USP family is the largest, comprising 58 members each exhibiting diverse domain architecture, substrate specificity and biological functions, for example USP4 which is a paralog of USP15 and USP11, contain a DUSP (domain in USP) located within the N-terminus, two UBL (ubiquitin-like) and a bi-part catalytic domain which is also known as a USP domain.[17] On the other hand, USP7 does not contain a DUSP domain. Instead, it contains a MATH domain also known as a TRAF (Tumor necrosis factor Receptor Associated Factor) domain situated within the N-terminus which is not present in any other USP family member.[17] Other USP family members such as USP3, USP5, USP13, USP16, USP22, USP33, USP44, USP49 and USP51, contain a zinc-finger ubiquitin-binding domain (Znf-UBPs) situated within the N-terminal region, which is necessary to recognize free ubiquitin chains either derived from newly synthesized linear chains or cleaved chains from substrates.[18] Additionally, many USPs have insertions and terminal extensions allowing for diverse protein-interaction domains, contributing to substrate specificity.

The catalytic domains of members of this family exhibit a characteristic hand-like structure consisting of three subdomains: the thumb, palm and fingers (except for CYLD, which lacks the finger sub-domain).[16] The catalytic site, consisting of the catalytic triad, is located at

the interface between the thumb and palm, while the fingers contribute to the positioning and stabilization of the interaction.[15] The catalytic triad of all USP members, except for USP7 and USP15, is catalytically competent and is in an active conformation even when unbound to a substrate.[17] In contrast, USP7 and USP15 undergo structural changes upon substrate binding that activate their catalytic site.[17]

The molecular mechanism of cysteine proteases relies on a reactive cysteine residue within the catalytic site. Polarization of this site is mediated by a histidine residue, which is often enhanced by a nearby asparagine or aspartate. This causes deprotonation of the thiol group forming the thiolate ion, which mediates a nucleophilic attack on the isopeptide bond between ubiquitin and its substrate. This forms a thioester intermediate, which is broken by a hydrolysis reaction by the deubiquitinating enzyme, resulting in the release of ubiquitin from the substrate and resetting the deubiquitinating enzyme to its active thiolate form for another catalytic cycle (Figure 5).

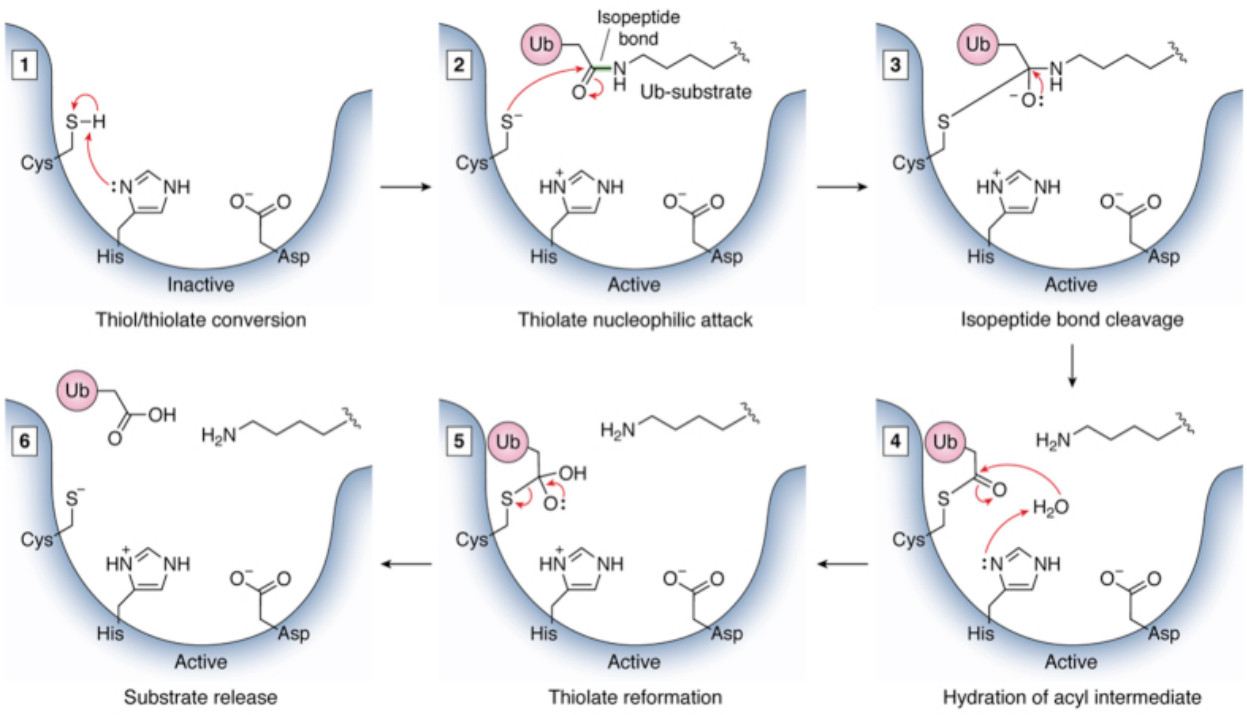


Figure 5. Mechanism of Cysteine Protease Deubiquitinating Enzymes (DUBs). Cysteine proteases DUBs remove ubiquitin from target proteins through a series of steps. First, the inactive cystine is converted to its reactive form, which allows for nucleophilic attack on the isopeptide bond between the ubiquitin and substrate. This releases the ubiquitin from the substrate but creates an intermediate between the ubiquitin and the DUB. Finally, a water molecule hydrolyzes the intermediate bond, releasing the free ubiquitin. Reproduced from Snyder and Silva.[15]

1.5 26S Proteasome

The 26S proteasome is a 2.5 MDa, multi-catalytic ATP-dependent protease complex that mediates the degradation of cytosolic, nuclear and membrane proteins in all eukaryotic cells.[19] The proteasome is made up of two distinct complexes, the 20S core particle (CP) and a 19S regulatory particle (RP). The 20S CP has an overall barrel-shaped structure composed of four stacked heteroheptameric rings, effectively creating the proteolytic chamber. The two outer α-rings contain seven distinct α-subunits, allowing for the formation of a pore that functions as a gate, regulating the proteins that are allowed to enter and exit the complex.[19] Likewise, the β-rings also contain seven distinct β-subunits, of which three possess protease activity.

The 19S RP is a multifunctional complex that is divided into two regions: the “base” and the “lid”.[19] The base consists of six ATPase subunits that form a hexameric ring along with four non-ATPase subunits, which serve as ubiquitin receptors, initially recognizing and binding ubiquitinated proteins targeted for degradation.[19] These receptors recognize substrates that have at least four ubiquitin molecules attached.[14] Once bound, proteasome-associated deubiquitinases catalyze the removal of the ubiquitin linkages. The lid consists of nine subunits that serve as deubiquitinases, removing ubiquitin chains from incoming substrates. Among the various ubiquitin chain types, the 26S proteasome predominantly targets and degrades K48 linkages, although there is evidence of mixed linkages of K11 and K29 with K48 that serve as proteasome degradation signals.[20] In addition, while K63 linkages do not normally serve as a proteasomal degradation signal, these chains can associate with the proteasome and lead to degradation of the conjugated protein.[20] Additionally, one or two 19S RPs can attach to a single 20S CP to form either the 26S proteasome or the 30S proteasome, though the term “26S proteasome” is often referred to both configurations.[20] The 19S RP also facilitates substrate unfolding and mechanical translocation into the 20S CP’s proteolytic chamber.

1.6 USP7

Identified in 1994, Ubiquitin-Specific Protease 7 (USP7), also known as Herpesvirus Associated Ubiquitin Specific Protease (HAUSP), is one of the most well-studied DUBs that is evolutionary conserved among eukaryotes. USP7 is a 1102 amino acid (135 kDa) cysteine protease that is primarily localized in the nucleus. It was initially discovered through its interaction with the viral protein Infected Cell Protein 0 (ICP0), also known as Vmw110 from Herpes Simplex Virus type 1 (HSV-1).[21] Since its discovery, USP7 has been reported to interact with many additional viral proteins such as Epstein-Barr Nuclear Antigen 1 (EBNA1) of Epstein-

Barr virus (EBV), viral Interferon Regulatory Factors 1, 2 and 4 (vIRF1, vIRF2, and vIRF4) and Latency-Associated Nuclear Antigen (LANA) of Kaposi's Sarcoma herpesvirus (KSHV).[22] Apart from viral proteins, many cellular proteins have been associated with USP7, with p53 being the first and most well-characterized.[23] USP7 knock-out mice exhibit embryonic lethality during development, likely due to USP7's role in the p53 and MDM2 axis, which leads to p53 stabilization and subsequent growth arrest.[22,24] Since then, USP7 has been unveiled to interact with a wide array of substrate proteins across different cellular pathways, underscoring the critical role of its regulation in maintaining cellular function (Figure 6).

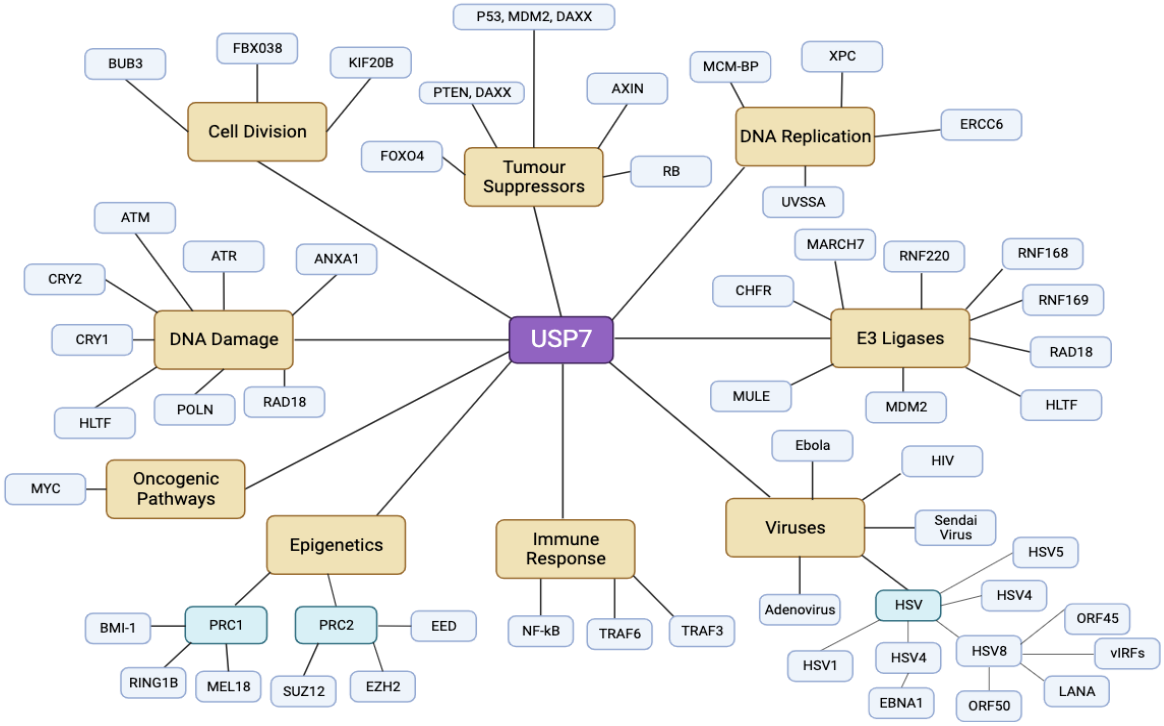


Figure 6. Cellular Roles and Substrates of USP7.
USP7 interacts with and deubiquitinates numerous proteins that are essential in various cellular processes. Schematic created with BioRender.com.

1.7 USP7 Structural Organization

USP7 is composed of several domains that are essential for substrate interaction, catalysis and regulation (Figure 8). Briefly, the N-terminus of USP7 contains the TRAF-like (Tumor

necrosis factor Receptor Associated Factor) domain, which spans residues 1-205. The C-terminus contains five ubiquitin-like (Ubl 1-5) sub-domains spanning residues 564-1102. Situated between these regions is the catalytic core (CAT) domain that spans residues 208-560.

The N-terminus of USP7 comprises of intrinsically disordered region of approximately 50 amino acids that includes a polyglutamine region, the function of which remains unclear.[25] Following this disordered region is the TRAF-like domain, which has been extensively studied and characterized as one of the two major substrate binding domains within USP7.

The TRAF domain adopts an eight-stranded anti-parallel β-sandwich structure, which contains a shallow surface groove in the middle allowing for substrate protein interaction, including p53, Mdm2 (murine double minute 2), MDMX, UbE2E1 and MCM-BP (mini-chromosome maintenance complex-binding protein).[25,26] Extensive studies have revealed that USP7 binds interacting protein substrates that contain a P/A/ExxS (where P=proline, A= alanine, E= glutamic acid, x=any amino acid, and S= serine) consensus motif through its ^{164}DWGF^{167} (where D =aspartic acid, W=trypophan, G= glycine and F= phenylalanine) sequence, which is positioned within the substrate-binding groove.[27] The D164 and W165 residues within the ^{164}DWGF^{167} sequence are critical in forming essential contacts with substrates. Mutations of D164 to alanine have been shown to reduce binding to EBNA1, p53 and MDM2, while mutation of W165 completely abolishes binding to all three substrates. [25,27]

The central catalytic (CAT) domain of USP7 contains a highly conserved Cysteine-Histidine box, also known as the catalytic triad, composed of Cysteine (Cys^{223}), Histidine (His^{464}) and Aspartate (Asp^{481}). This triad is critical in binding ubiquitin and catalyzing the hydrolysis of the isopeptide bond between ubiquitin and its substrate. Structurally, the CAT domain adopts a characteristic right-hand configuration consisting of three subdomains: the

thumb, palm and fingers.[15,28] The finger subdomain consists of a prominent binding surface which serves as the primary platform for ubiquitin binding.[28] The active catalytic triad is situated in a cleft between the palm and the thumb subdomains. Mechanistically, large structural changes occur upon ubiquitin binding, converting the inactive catalytic domain structure to its active form, which positions the three active site residues near each other (Figure 7).[25] Furthermore, USP7 catalytic activity is enhanced nearly 100-fold through the interaction of Ubl4-5 with a regulatory switching loop adjacent to the active site[25,29]. This interaction promotes a more favourable arrangement of the catalytic triad and increases the enzyme's affinity for ubiquitin[29]. Once activated, His^{464} deprotonates the thiol group of Cys^{223}, activating it for a nucleophilic attack on the isopeptide bond that links ubiquitin to the substrate. The Asp^{481} residue functions to stabilize and orient His^{464}, thereby enhancing its ability to polarize and effectively deprotonate cysteine[30].

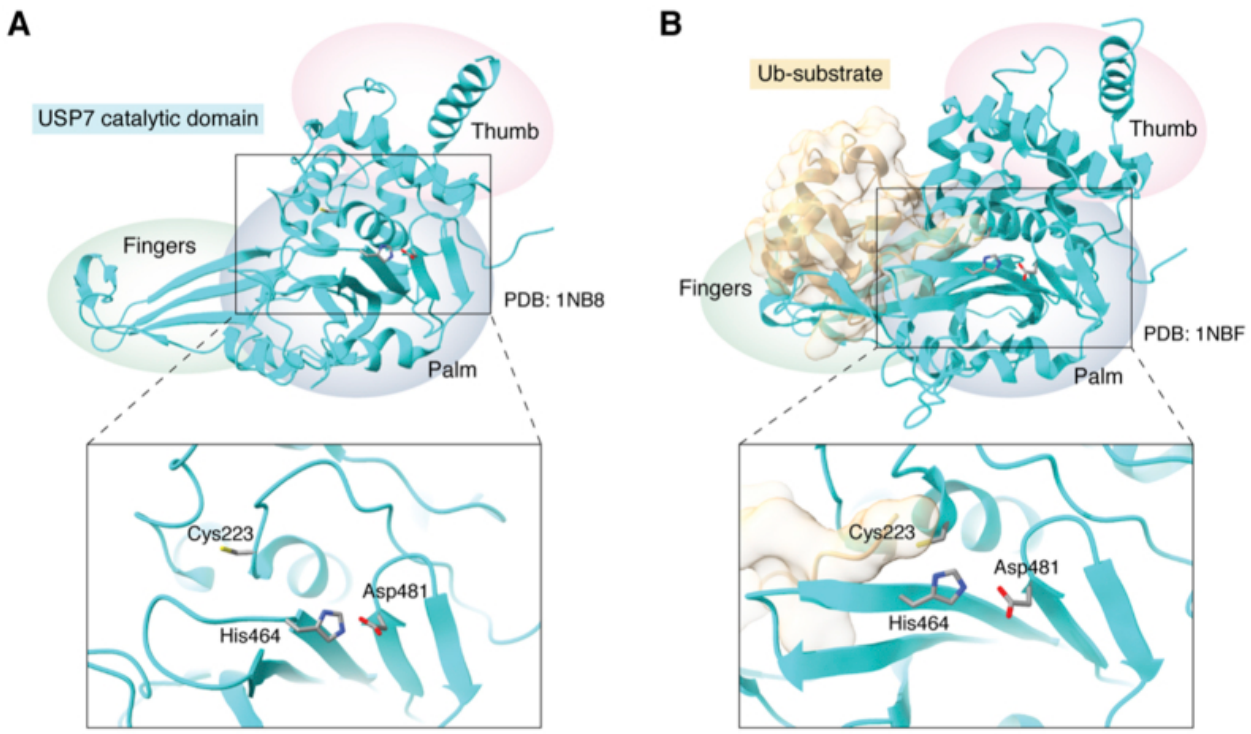


Figure 7. Catalytic Site of USP7 in its inactive and active conformation.
A) In the inactive state, the catalytic cysteine of USP7 (cyan) is positioned far away from the other residues of the catalytic triad. Upon ubiquitin binding (yellow), there is a conformational change that positions the catalytic cysteine closer to the catalytic triad, allowing for deprotonation and the conversion of cysteine to its reactive form. Reproduced from Snyder and Silva.[15]

Bridging the catalytic domain and the five C-terminal ubiquitin-like domains (Ubl 1-5) of USP7 is a flexible α-helix that spans 26 amino acids.[31] Despite low sequence homology among the UBL domains and with ubiquitin itself, each UBL domain adopts a common β-grasp ubiquitin fold contributing to USP7's regulatory and substrate recognition function.[25] The five UBL domains function in a 2:1:2 architecture where Ubl1/Ubl2 and Ubl4/Ubl5 form dimers, which are connected to Ubl3 via hinges. This architecture allows for flexibility and regulation of the enzymatic activity of USP7. The CTD of USP7 provides an additional platform for substrate binding and plays a critical role in enhancing its catalytic activity. The additional USP7 substrate binding site was determined to be situated within Ubl2 through its initial interaction with ICP0.

Structural analysis determined that ICP0 recognizes and binds to a negatively charged region within Ubl2 via its ^{618}PRKCARKT^{625} sequence.[32] The interaction interface within Ubl2 was determined to be an acidic region spanning residues ^{758}DELMDGD^{764} (where D=aspartic acid, E=glutamic acid, L=leucine, M=methionine, and G=glycine) which recognizes and binds to substrates with a consensus motif consisting of KxxxK (where K=lysine and x=any amino acid).[32] The two lysine residues within the substrate consensus motif are critical in forming salt bridges with two aspartate residues (Asp^{762} and Asp^{764}) within USP7. Mutations of these residues significantly reduce USP7's affinity for its substrates.[32] Since the initial discovery of ICP0 with the CTD of USP7, many other substrates have been identified to use the KxxxK consensus motif to recognize and bind USP7 such as Ubiquitin-like, containing PHD and RING finger domains 1 (UHRF1), DNA Methyl Transferase 1 (DNMT1) and Ring Finger Protein (RNF169).[32]

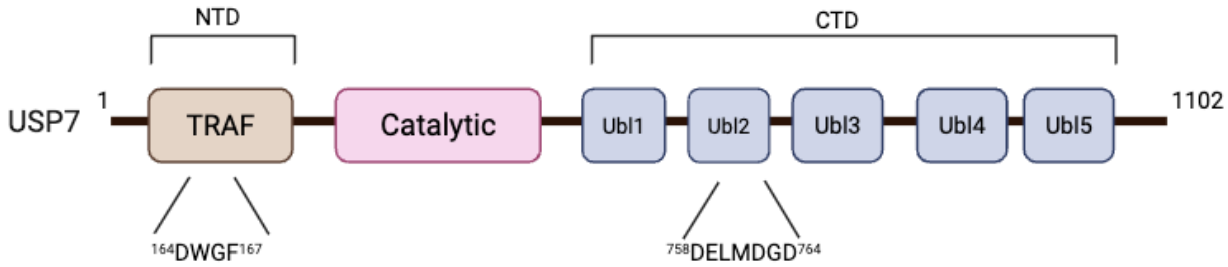


Figure 8. Schematic representation of USP7 domains.
USP7 is composed of several domains that are essential for substrate interaction and catalytic activity. The N-terminal TRAF contains a ^{164}DWGF^{167} motif that binds substrate proteins. USP7 also contains a second binding site located within the C-terminal UBL region consisting of ^{758}DELMDGD^{764} that is well-known to interact with substrate proteins. Situated between these regions is the catalytic domain that mediates deubiquitination of substrate proteins.

1.8 USP7 and DNA Damage Response

USP7 regulates many cellular pathways through its deubiquitinase activity, including DNA damage response, cell cycle control, and the modulation of many transcription factors, underscoring its critical role in maintaining cellular homeostasis (Figure 6).

Central to the DNA damage response pathway is the tumour suppressor protein p53, a protein that has been extensively characterized for its interaction with USP7. p53 is an essential transcription factor that regulates a vast number of genes in response to DNA damage. These genes typically encode proteins that function in cell cycle arrest, DNA damage repair and apoptosis.[25] USP7 interacts with and deubiquitinates p53 as well as its negative regulator MDM2, an E3 ligase that functions to polyubiquitinate p53, resulting in its proteasomal degradation by the 26S proteasome.[23] Under normal, undamaged conditions, p53 is targeted for degradation by MDM2, resulting in minimal p53 levels in the cell. USP7 plays a critical role during this step by deubiquitinating and stabilizing MDM2, counteracting its autoubiquitination, allowing MDM2 to ubiquitinate and degrade p53. This is a result of USP7s higher affinity to MDM2 during undamaged cellular conditions due to phosphorylation at Ser18 of USP7 by kinase CK2.[25,30] Additionally, USP7 also stabilizes MDM4 and SUV39H1 methyltransferase, which are MDM2 interacting partners and substrates, further contributing to p53 repression under undamaged conditions.[25,33] MDM4 interacts with and prevents autoubiquitination of MDM2, enhancing its ligase activity to p53. SUV39H1 co-localizes with p53 at target gene promoters and trimethylates lysine 9 on histone H3 (H3K9me3), establishing repressive chromatin and inhibiting p53-dependent transcription of those genes.[25,33] SUV39H1 function is regulated by SIRT1, a histone deacetylase that, interestingly, is also deubiquitinated and stabilized by USP7, highlighting the complex crosstalk among these proteins to regulate the levels of p53.[33] Upon DNA damage, ATM-dependent dephosphorylation of Ser18 by Protein phosphatase 1G (PPM1G) decreases the affinity of USP7 towards MDM2.[25,34] As a result, MDM2 self-ubiquitinates, resulting in its proteasomal degradation. This allows USP7 to interact

with and deubiquitinate p53, resulting in its stabilization and consequent activation of p53-dependent DNA damage response.[25]

In addition, USP7 is also directly involved in regulating the nucleotide excision repair (NER) pathway caused by DNA lesions during excessive UV radiation. During the NER process, Xeroderma pigmentosum complementation group C (XPC) proteins recognize DNA lesions and initiate the NER response. However, during UV-induced DNA damage, XPC gets polyubiquitinated.[35] Although XPC polyubiquitination is important for enhancing the binding capacity to DNA lesions, it also exposes them to proteolysis. Therefore, USP7 deubiquitinates and stabilizes XPC, preventing premature degradation.[25,30,36]

Finally, USP7 is involved in regulating double-stranded DNA breaks, a toxic form of DNA lesions that, if left uncorrected, can lead to chromosomal fragmentation and rearrangement.[25] USP7 deubiquitinates PHD Finger Protein 8 (PHF8), a histone demethyltransferase that recruits and facilitates the localization of Ku70 and BLM helicase to double-stranded breaks (DSBs).[37] Additionally, USP7 interacts and deubiquitinates mediator of DNA damage checkpoint 1 (MDC1) that functions to detect DSBs.[38] MDC1 binds to phosphorylated histone H2AX which facilitates the recruitment of RNF8, an E3 ligase.[25,39] The accumulation of RNF8 promotes the recruitment of RNF168, another E3 ligase which catalyzes K63-linked polyubiquitination of histone H2A and H2AX.[25,40] This ubiquitin signal serves as the platform for DNA damage response factors, including 53BP1, and Rap80, to sites of DSBs. Interestingly, USP7 also stabilizes both RNF168 and RNF169, further regulating the access of proteins to damaged chromatin.[30,41,42]

1.9 USP7 and Cell Cycle Control

Cell cycle control is essential in ensuring DNA integrity, proper replication and chromosomal segregation. USP7 is known to play a critical role in controlling and regulating substrates involved within these processes. Many reports have indicated that inhibition or knockdown of USP7 results in a reduction in cell cycle proliferation. Some key substrates that USP7 regulates include cyclin A2[37], Checkpoint with Forkhead and RING finger domain (CHFR)[43], proliferating cell nuclear antigen (PCNA) and Rad18[44].

Cyclin A2 is a critical regulator of S phase initiation and facilitates the transition to the G2 phase of the cell cycle. As mentioned previously, USP7 deubiquitinates and stabilizes the histone demethylase PHF8.[37] PHF8 activates transcription through demethylating histones, which in turn results in the activation of a subset of genes involved in cell cycle progression, one of which is cyclin A2.[37] Cyclin A2 functions to increase cell growth and proliferation. However, aberrant activation of this process has been associated with breast carcinogenesis.[25,45]

CHFR is an E3 ubiquitin protein ligase that functions as a mitotic checkpoint. CHFR plays an important role as a tumour suppressor, controlling cell cycle progression and mitotic protein expression for optimal chromosomal stability.[46] CHFR function to delay cell cycle progression during mitosis through ubiquitination of critical proteins such as Polo-like kinase 1 (Plk1).[47] USP7 deubiquitinates and stabilizes CHFR, preventing it from proteasomal degradation, thus preserving its checkpoint function and allowing it to control the progression of the cell cycle.[43]

Finally, USP7 is known to regulate both PCNA and Rad18, an E3 ubiquitin ligase. In response to DNA damage, PCNA is monoubiquitinated by Rad18 which is key in activating the translesion DNA synthesis (TLS) pathway.[46] During this process, specialized polymerases which

have PCNA and ubiquitin-interacting domains, replicate across damaged bases. However, these polymerases are error-prone and are only activated in response to mono-ubiquitinated PCNA. USP7 deubiquitinates PCNA, turning off the TLS pathway and it also deubiquitinates Rad18, stabilizing it, resulting in tight regulation of the TLS pathway, ensuring that it is not excessively active, which can result in mutagenesis or underactive, which can stall replication forks.[44,46]

1.10 USP7 and Transcription Factors

USP7 modulates a vast number of transcription factors, thus influencing the expression of their target genes. For example, USP7 is known to target and deubiquitinate FOXO4, a member of the Fork head box O (FOXO) transcription factors.[46,48] These transcription factors are critical in regulating various biological processes, including cellular metabolism, apoptosis and cell-cycle regulation. In response to oxidative stress, FOXO4 is monoubiquitinated, resulting in its nuclear localization and subsequent increase in the transcriptional activity of its target genes. USP7 regulates FOXO4 functionality by deubiquitinating it, resulting in its re-localization into the cytoplasm and subsequent inhibition of its transcriptional activity.[48]

Another well-known transcription factor that is regulated by USP7 is the oncogenic protein c-Myc. Myc is a master regulator, either directly or indirectly, of thousands of genes involved in different biological processes. USP7 deubiquitinates and stabilizes c-Myc, resulting in its upregulation at both the protein and mRNA levels.[46,49] Myc overexpression is implicated in many different forms of cancer due to its ability to increase cell proliferation as well as allowing cancer cells to re-enter the cell cycle and bypass cell-cycle checkpoints. Therefore, the coordinated interplay between ubiquitination and deubiquitination is key in maintaining proper c-Myc stability and function.

1.11 USP7 and its Role in Cancer

USP7 plays a critical role in diverse cellular pathways by deubiquitinating a wide range of substrates, particularly those involved in cell cycle control, DNA damage and apoptosis pathways. Dysregulation of USP7 and its substrates is involved in the initiation and progression of various diseases, such as cancer. Certain cancers, such as epithelial carcinoma, breast, lung, prostate, and nonsmall cell lung cancer (NSCLC) have been associated with high levels of USP7.[50] In breast cancer, USP7 has been shown to deubiquitinate and stabilize PHD finger protein 8 (PHF8), leading to an upregulation of cyclin A2, which promotes DNA synthesis and is critical for cell growth and proliferation.[37] In addition, USP7 is known to deubiquitinate the tumor suppressor, phosphatase and tensin homolog (PTEN). Monoubiquitination of PTEN promotes its nuclear localization, where it contributes to genomic stability by suppressing cell growth. However, deubiquitination of PTEN by USP7 promotes its shuttling to the cytoplasm, where its accumulation is associated with more aggressive, late-stage cancers, such as observed in prostate cancer as well as leukemia, due to the loss of its tumour-suppressor function.[51] USP7 is also known to interact with and deubiquitinate androgen receptor (AR), leading to its stabilization, which is observed in prostate cancer.[12] In addition, the interaction between USP7 and AR is essential for androgen-activated AR binding to chromatin, which facilitates the expression of a subset of genes that induce proliferation of prostate cancer cells.[52] Thus, the dynamic regulation of USP7 is vital in regulating protein substrate stability and cellular homeostasis.

1.12 SUMOylation

The addition of Small Ubiquitin-related Modifier (SUMO) onto target proteins through a process called SUMOylation is vital in regulating several crucial functions of cells, such as cell

cycle, apoptosis, metastasis, DNA damage repair, etc.[53] SUMO proteins are approximately 10 kDa in size and resemble the three-dimensional structure of ubiquitin, despite having only an 18% amino acid sequence similarity to it. [54,55] The SUMO protein family consists of five paralogs in mammals: SUMO1, SUMO2, SUMO3, SUMO4 and SUMO5. Each contains an unstructured stretch of 10-25 amino acids at the N-terminus that is not found in any other ubiquitin-related protein.[54,55] Of these, SUMO1-SUMO3 are ubiquitously expressed, whereas the expression of SUMO4 and SUMO5 is restricted to certain tissues. SUMO2 and SUMO3 share a 95% similar sequence identity but have only a 45% similarity to SUMO1. SUMO4, in turn, exhibits an 86% sequence similarity to SUMO2 and SUMO3.[54] SUMO5 is considered a more distinct member, characterized by its very strict tissue specificity, expressed highly in testes, peripheral blood tissues and in primates.[53]

Similar to the ubiquitin-proteasome degradation pathway, SUMOylation is a reversible multi-step pathway that is regulated by distinct enzymes (Figure 9). The first step of the SUMOylation pathway involves the maturation of the SUMO protein through SUMO-specific proteases (SENPs), which expose diglycine motifs at its C-terminus which are essential for ligation. The mature SUMO proteins then attach to an ATP-dependent E1-activating enzyme heterodimer (SAE1/SAE2). This ligation functions as an intermediate in the formation of a thioester bond between the mature SUMO protein and the catalytic cysteine residue of SAE2 and also serves as an activator of SUMO. Following the formation of the ligation intermediate, the SUMO protein is transferred from SAE2 to the E2 conjugating enzyme, UBC9, forming a thioester linkage between the catalytic cysteine residue of UBC9 and the C-terminal carboxyl group of SUMO. Finally, the mature and activated SUMO is transferred from UBC9 to the substrate protein via the formation of an isopeptide bond between the carboxyl glycine residue of

SUMO and the lysine residue on the substrate protein. This step is often facilitated by E3 SUMO ligases, which enhance substrate specificity and accelerate the conjugation process.

1.13 SUMO E3 Ligases

There are three families of SUMO E3 ligases, the largest family characterized by the presence of a SP-RING motif.[54] This motif resembles that of the classical RING domain such that the proteins that contain this domain bind both their targets and UBC9 directly and act as scaffolds that position the target substrate and thioester-charged UBC9 in a favourable orientation for SUMO transfer.[54] SP-RING proteins can be divided into different groups, the largest consisting of the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family proteins. In mammals, five PIAS proteins have been identified: PIAS1, PIAS3, the splice variants PIASxα, PIASxβ, and PIASy.[54] All members share a conserved 400-residue N-terminal domain, as well as a SAP domain which is necessary for binding nucleic acids and a SUMO-interaction/binding motif. Other SP-RING ligases include MMS21, which is part of an octameric SMC5-SMC6 complex essential for vegetative growth and DNA repair.[54]

The second family of SUMO E3 ligases is the vertebrate-specific nuclear pore protein RanBP2 (also known as Nup358). RanBP2 is an essential component of the nucleocytoplasmic transport machinery and plays a dual role in nuclear import and SUMO E3 ligase activity.[56] Unlike the SP-RING E3 ligases, RanBP2 has no known counterpart in the ubiquitylation cascade. It directly binds to SUMO1 and UBC9 and functions to significantly enhance the SUMO1 transfer from UBC9 to target proteins such as the nuclear body component, Sp100.[56] RanBP2 facilitates SUMOylation by positioning the SUMO-UBC9 thioester complex in an

optimal position for a nucleophilic attack by the lysine residue on the substrate protein, thereby increasing the efficiency and specificity of SUMO conjugation.[54]

The final class of SUMO E3 ligases is the human polycomb group protein, Pc2. Polycomb proteins form large multimeric complexes within the nucleus, known as polycomb bodies, that regulate gene silencing, often through depositing and maintaining epigenetic modifications on the chromatin.[54]Pc2, a transcriptional repressor, was discovered to interact with the transcriptional corepressor carboxyl-terminus binding protein (CtBP). This interaction mediates the recruitment of UBC9 to polycomb bodies, thereby significantly enhancing the transfer of SUMO to CtBP and promoting its SUMOylation.[57]

1.14 Biological Function of SUMOylation

Typically, in normal conditions, a small fraction (typically less than 5%) of a given protein is SUMOylated at a time.[54] Yet, many transcription factors and transcriptional co-regulators become significantly activated when the acceptor lysine site is mutated to an arginine[54]. This suggests that SUMO modification on the transcriptional machinery plays a negative role in the regulation of transcriptional activity. This is in part due to SUMO modifications altering interactions with other macromolecules. SUMOylation can promote protein-protein interaction such as the interaction between SUMO E3 ligase RanBP2 and Ran GTPase-activating protein 1 (RanGAP1).[54] Consequently, it can also hinder protein-protein interaction as seen with the transcriptional repressor ZNF76, whose SUMO-acceptor site overlaps with the binding site of TATA-binding protein.[54] Additionally, SUMOylation of transcription factors and transcriptional co-regulators recruits downstream effectors such as histone deacetylases (HDACs), which condense chromatin, thereby repressing transcription.[54]

Additionally, the role of SUMO regulation in modulating DNA repair enzymes has been extensively studied. During the mismatch base pair process, a key enzyme known as thymine DNA glycosylase (TDG) recognizes mismatched base pairs and excises the incorrect base pair.[54,58] However, during this process, TDG has a high affinity for the abasic site, preventing it from detaching from DNA, resulting in it getting stuck within the reaction cycle. [54] This is solved when TDG is SUMOylated, causing a conformational change which weakens TDG's interaction with the DNA. Subsequently, TDG enter the nucleoplasm where the SUMO modification is removed by isopeptidases allowing TDG to re-enter the cycle and bind the next DNA mismatch.[54]

Furthermore, SUMOylation is well known for its role in regulating nuclear and subnuclear localization of proteins. For instance, the first ever recorded instance of SUMO modification facilitating nuclear localization was associated with RanGAP1, which is involved in nucleocytoplasmic transport. RanGAP1, lacking the SUMO modification, is located within the cytoplasm. However, upon conjugation with SUMO facilitated by E3 ligases, RanBP2-RanGP1 is localized to the nuclear pore complex (NPC).[58,59] This was the first documented instance in which SUMO modification directly influenced the subcellular localization of target proteins. Additional proteins such as MDM2, and DAXX, have also been discovered to undergo SUMOylation, resulting in altered subcellular localization.[60,61]

1.15 DeSUMOylation

Proteins modified by SUMO are also subject to the removal of the modification through a process known as deSUMOylation, carried out by enzymes referred to generically as SUMO proteases. In humans, deSUMOylation is primarily mediated through a family of enzymes known as SUMO-specific proteases (SENPs) (Figure 9). These enzymes belong to the C48

family of cysteine proteases that possess a conserved catalytic domain located within the C-terminus.[62] This domain features a classical catalytic triad comprising histidine, aspartate, and cystine, along with a conserved glutamine residue, which forms the oxyanion hole, a structural feature that is required for stabilizing the active site during catalysis.[62] There are six SENPs in humans which can be divided into three families based on specificity and sequence similarity. The first family consists of SENP1 and SENP2 which have specificity for SUMO1-3. The second family consists of SENP3 and SENP5 which favour SUMO 2/3 while the third family consists of SENP6 and SENP7 which also prefer SUMO 2/3.[63] Apart from the SENPs, recent studies have identified three new SUMO proteases in humans: deSUMOylating isopeptidase 1 and 2 (DESI1 & DESI2), and ubiquitin-specific protease-like 1 (USPL1).[63]

SENP1 is primarily localized in the nucleoplasm and contains both a nuclear localization signal within the N-terminus and a nuclear export signal in its C-terminus. It is the most extensively studied member of the SENP family and will be discussed in detail in the following section. SENP2, another well-studied deSUMOylase, gives rise to three alternatively spliced mRNA variants, each encoding proteins with distinct subcellular localizations. One isoform is situated in the cytoplasm, while the others are found at the nuclear pore complex and within nuclear foci.[63] SENP2 is vital for embryogenesis as *SENP2*$^{-/-}$ embryo die during embryonic day 9.5. This could be in part due to higher SUMO levels on pc2/CBX4, leading to transcriptional repression of Gata4/6, which are essential for embryonic development.[63] In addition, SENP2 regulates numerous proteins such as the well-known tumor suppressor p53, by modulating SUMOylation levels on Mdm2.[63] SENP2 is also known to play an important role in the cellular response to genotoxic stress, during which its expression is upregulated. This increase in SENP2 promotes the removal of SUMO from NEMO, which is a key activator of NF-κB. As a result,

NF-κB activity is reduced, which ultimately reduces SENP2 production, a negative feedback mechanism that helps control cell survival under stress conditions.[62]

SENP3 and SENP5 are both localized primarily within the nucleolus and have their nucleolar localization signals situated within the N-terminus.[62] SENP3 has been shown to regulate ribosome biogenesis by associating with and deSUMOylating the nucleolar protein NPM1, thereby preventing its modification by ADP-ribosylation factor-mediated SUMOylation.[62] SENP5 has been suggested to play a role in mitosis as its knockdown inhibits cell proliferation and leads to abnormal nuclear morphology, including the formation of binucleate cells.[62] SENP6 and the least well-studied SENP7 do not possess SUMO precursor processing activity in comparison to the other SENPs. They are both localized within the nucleoplasm. SENP6 has been shown to regulate genomic integrity by maintaining centromere and kinetochore structure during mitosis.[63] Additionally, it is known to deSUMOylate RPA1, preventing RAD51, a repair protein, from being recruited to DNA damage sites.[63]

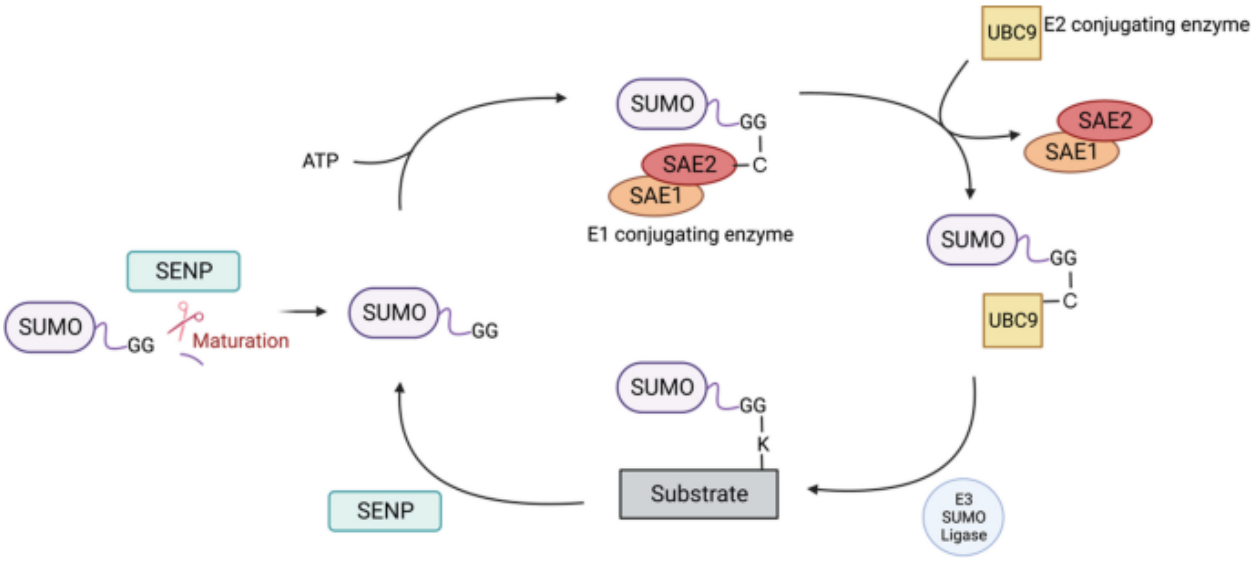


Figure 9. Overview of the SUMOylation Pathway.
SUMO is attached to its target protein through a cascade of enzymes: SUMO-specific proteases (SENPs), an E1 activating enzyme, an E2 conjugating enzyme and often an E3 SUMO ligase. First, SUMO is processed and converted to its mature form by SENPs, where it binds and becomes activated by the E1 enzyme. The SUMO is then transferred to the E2 enzyme, where it catalyzes the transfer of SUMO to the target protein, which can be facilitated by the recruitment of E3 ligases. SENPs also mediate the removal of SUMO-modified proteins releasing free SUMO for reuse. Reproduced from Huang et al.[55]

1.16 Sumo-Specific Protease 1 (SENP1)

SENP1 is the first mammalian SUMO-specific protease to be identified and is primarily localized within the nucleus.[64] Its gene is located on chromosome 12q13.11. While SENP1 can deconjugate all SUMO isoforms, studies in SENP1 knockout (*SENP1*$^{-/-}$) embryos show a failure to process SUMO1, suggesting that SENP1 functions as the principal C-terminal hydrolase for SUMO1 during embryogenesis. In addition, *SENP1*$^{-/-}$ mutations result in embryonic lethality, typically occurring between days 13 and 15 of gestation and are associated with severe anemia characterized by a reduced number of erythrocytes compared to wild-type embryos.[65]

Structurally, SENP1, a 644 amino acid protein, is composed of two main domains: a long N-terminal region that is poorly conserved among the SUMO proteases, and a highly conserved C-terminal domain that contains the catalytic core responsible for its enzymatic activity. N-terminal residues 171-177 of SENP1 are responsible for nuclear localization, as substitution of

this motif results in a loss of nuclear localization.[66] Although this sequence does not resemble a conventional nuclear localization sequence (NLS), it is proposed that SENP1 may enter the nucleus by piggybacking on another cellular protein, with this short motif mediating the necessary interaction.[66] Studies have demonstrated that the cysteine residue at position 603 (Cys^{603}) is essential for the catalytic activity of SENP1 in mediating deSUMOylation. Mutation of Cys^{603} into a serine renders SENP1 catalytically inactive and incapable of deconjugating SUMO-1. Interestingly, this mutation alters the subcellular localization of SENP1- while wild-type SENP1 is found in both the nucleus and cytoplasm, the catalytically inactive mutant localizes exclusively to the nucleus at high expression.[62,67] This observation was thought to reflect the involvement of SUMO-1 in the nuclear import processes.[66] Additionally, not only does the mutation prevent SENP1 from deconjugating SUMO-1, but it also exhibits a dominant negative phenotype, by promoting the accumulation of SUMO-1 conjugates and the loss of free SUMO-1.[66]

1.17 Cellular Roles of SENP1

Increasing evidence suggests that SENP1 targets a wide range of proteins for deSUMOylation, thus regulating their gene expression and stability. Notably, SENP1 enhances Androgen Receptor (AR)- dependent transcription through its ability to deconjugate histone deacetylase 1 (HDAC1). This deSUMOylation reduces HDAC1's deacetylase activity on AR, resulting in increased AR transcriptional activity.[62,67] In addition, SENP1 is also known to deSUMOylate SIRT1, another histone deacetylase involved in stress response. Upon exposure to DNA-damaging agents such as oxidative stress and UV radiation, the interaction between SENP1 and SIRT1 is enhanced.[68] SENP1-mediated deSUMOylation of SIRT1 leads to a reduction in its deacetylase activity, which results in the accumulation of pro-apoptotic SIRT1

substrates.[68] This promotes apoptosis, highlighting the critical role of SENP1 regulation on SIRT1 activity and cellular fate under genotoxic stress.

Moreover, SENP1 is well-known to be implicated in the progression of various cancers, including breast[69], lung[70], prostate[71], liver[72], pancreatic[73] cancer, and leukemia[74]. In these malignancies, SENP1 expression is consistently found to be significantly upregulated. In breast cancer, *SENP1* mRNA and protein are overexpressed, which stabilizes c-Myc, a key transcriptional regulator that is frequently upregulated in various cancers. c-Myc plays a central role in controlling diverse cellular processes, including cell proliferation, apoptosis, and DNA replication. SENP1 enhances c-Myc stability by deSUMOylating it which in turn reduces its polyubiquitination and prevents its degradation.[70]As a result, c-Myc levels and its transcriptional activity are elevated. Conversely, silencing of SENP1 reduces c-Myc levels, triggering cell cycle arrest and decreased cell proliferation in breast cancer cells.[69]

Similar to its role in breast cancer, SENP1 mRNA and protein levels are significantly upregulated in lung cancer tissues, resulting in increased cell proliferation. In A549 lung carcinoma epithelial cells, knockdown of SENP1 using small interfering RNA (siRNA) led to a marked reduction in both cell number and cell proliferation. In addition, SENP1-silencing enhanced ionizing radiation-induced cell cycle arrest, promoting cell-cycle arrest in the G1 stage, increased γ-H2AX- a marker associated with DNA damage and tumor radiosensitivity and enhancing apoptosis.[70]

In prostate cancer, elevated SENP1 levels are correlated with increased tumor aggressiveness and disease severity. Overexpression of SENP1 enhances the invasion and migration capabilities of prostate cancer cells, whereas SENP1 silencing significantly decreases these functions.[72] It is well-known that prostate cancer cells tend to form secondary tumours in

the bone, and increasing evidence suggests that SENP1 upregulation contributes to this process by upregulating matrix metalloproteinases (MMPs)- enzymes that contribute to bone remodelling and are overexpressed during the progression of prostate cancer. Specifically, in PC3 and DU145 prostate cancer cells, SENP1 is essential for the expression of MMP2 and MMP9, as silencing of SENP1 results in a significant reduction in both proteins.[71]

Similar to other cancers, SENP1 is upregulated in hepatocellular carcinoma (HCC), and its elevated expression correlates with a poorer survival rate.[72] Silencing SENP1 in HCC cells leads to reduced proliferation, migration and invasion, as well as an increase in the G0/G1 cell cycle arrest, similar to the effects observed in other cancer types. In HCC, SENP1 deSUMOylates UBE2T, a protein that is involved in DNA repair and cell cycle regulation and is also highly overexpressed in HCC tissues. UBE2T deSUMOylation results in its stabilization and activation of the Akt signalling pathway, thereby enhancing tumor growth, migration and invasiveness.[72] Knockdown of SENP1 decreases UBE2T levels, which inhibits the p-Akt pathway and suppresses the oncogenic features of HCC cells.[72] In addition, SENP1 also regulates key components of the Wnt/β-catenin signalling pathway, including Wnt1 and β-catenin, both of which are critical for promoting cancer stemness and malignant behaviour.[75] SENP1 deSUMOylates and stabilizes β-catenin, allowing for its upregulation where it can maintain populations of tumour-initiating and cancer stem cells along with promoting the expression of cell-cycle related proteins such as cyclin B1 and Cyclin C to drive carcinogenesis and contribute to the progression of HCC.[76]

1.18 Project Rationale and Objective

USP7 is a well-characterized deubiquitinating enzyme known to regulate numerous cellular pathways by stabilizing proteins. However, its role in modulating the deSUMOylation

machinery remains unidentified. To address this gap, our group was interested in investigating whether USP7 regulates the protein levels of SENP1. Understanding how SENP1 stability is maintained is particularly important, as SENP1 has been implicated in various pathological processes, including cancer progression and cellular response to hypoxia.

Using ScanProsite and Eukaryotic Linear Motif computational biology resources, we found that SENP1 contains the P/A/ExxS motif, which could potentially mediate an interaction with the ^{164}DWGF^{167} binding pocket in the N-terminus of USP7. Although P/A/ExxS motif was identified, in this study we focused on using cell biology approaches such as co-immunoprecipitation and overexpression to examine whether USP7 interacts with and regulates SENP1, rather than determining whether this specific motif mediates the interaction. Our first objective was to confirm whether USP7 interacts with SENP1. Once an interaction was established, our next objective was to determine if SENP1 is a substrate for USP7-mediated deubiquitination. Once a regulatory relationship was established, we wanted to see if SENP1 protein abundance is regulated by USP7.

Chapter 2: Materials and Methods

2.1 Mammalian Cell Culture and Transfection

Human colorectal carcinoma HCT116 wild-type for USP7 ($USP7^{WT}$) cells and HCT116 $USP7^{-/-}$ knockout ($USP7^{KO}$) cells were grown in McCoy's media (Wisent Inc.) supplemented with 10% Fetal Bovine Serum (Gibco) and 1mg/ml Penicillin-Streptomycin (Wisent Inc.) at 37°C and 5% CO_2. The human embryonic kidney cell line stably expressing the SV40 large T antigen (HEK293T) was grown in DMEM media (Wisent Inc.) supplemented with 10% Fetal Bovine Serum (Gibco) and 1mg/ml Penicillin-Streptomycin (Wisent Inc.) at 37°C and 5% CO2. HCT116 WT/ KO, and HEK293T cells were transfected with various plasmids using PolyjetTM In Vitro DNA transfection Reagent (SignaGen Laboratories) according to manufacturer instructions.

2.2 Plasmids

Plasmid constructs for mammalian transfection are listed below (Table 1).

Table 1. Plasmid Constructs used for Mammalian Transfection and Expression.

Protein	Vector	Tag	Status
FLAG-SENP1	pcDNA3.1	FLAG	Wild-type
Myc-USP7	pcDNA3	Myc	Wild-type
Myc-USP7 C223S	pcDNA3	Myc	C223S mutation
FLAG-EV	pcDNA3	FLAG	Empty vector
Myc-USP7 (N-terminus domain)	pcDNA3	Myc	1-208 residues
Myc-USP7 (N-terminus + Catalytic Domain)	pcDNA3	Myc	1-560 residues
Myc-USP7 (C-terminus domain)	pcDNA3	Myc	560-1102 residues
HA-Ubiquitin (Ub)	pcDNA3	HA	Wild-type

2.3 Cell Lysis and Preparation

Before cell lysis, HCT116 and HCT116 USP7$^{-/-}$ KO, and HEK293T cells were washed with cold phosphate-buffered saline (PBS) (Wisent Inc.). They were then collected in micro-centrifuge tubes and centrifuged at 1500 RPM for 5 minutes. The cell pellets were lysed using NP-40 lysis buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 1% (v/v) NP-40) or RIPA buffer (150mM NaCl, 50 mM Tris-HCl, 1% (v/v) NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitors (1mM benzamidine, 1 mM PMSF, 1x Complete protease inhibitor cocktail (Roche)). Cells were sonicated on ice at 10% amplitude 5 times, 1 second ON, 10 seconds OFF followed by centrifugation at 13,000 RPM for 15 minutes at 4°C to pellet cellular debris. The protein concentration of the clarified lysate was measured using BCA assay following the manufacturer's instructions (Thermo Scientific Pierce BCA Protein Assay Kit).

2.4 SDS-PAGE Gel and Western Transfer

20 µg of lysate samples were prepared using 5x SDS loading dye (5% β-mercaptoethanol, 10% sodium dodecyl sulfate (SDS), 30% glycerol, 250 mM Tris-HCl [pH 6.8], 0.02% bromophenol blue) at 100°C for 5 minutes. Lysates were resolved using SDS polyacrylamide gel electrophoresis (SDS-PAGE) for 90 minutes at 120 volts. The proteins were transferred to a PVDF membrane for 90 minutes at 104 volts at 4°C. The membranes were then blocked for 1 hour at room temperature with 5% skim milk in PBS-T (0.1% Tween20) with rocking.

2.5 Immunoblotting and Antibodies

The following primary antibodies were used for immunoblotting or for immunoprecipitation studies: polyclonal rabbit anti-USP7 (Bethyl Laboratories, A300-033A), monoclonal mouse anti-GAPDH (Santa Cruz Biotechnology Inc., 47724) or monoclonal rabbit anti-GAPDH (Cell Signalling, 14C10), mouse anti-FLAG (Sigma-Aldrich, F1804-1MG), monoclonal rabbit anti-SENP1 (Abcam, EPR3844) and mouse anti-Myc (Invitrogen, 2243074). The transferred membranes were incubated with 2 ml of diluted primary antibody overnight, shaking at 4°C. Post-primary incubation, the membranes were washed 3 times in 5-minute intervals, shaking with Phosphate-buffered saline with Tween 20 (PBS-T). The washed membranes were incubated with secondary antibodies for 1 hour at room temperature. The following secondary antibodies were used for immunoblotting: HRP-conjugated goat polyclonal mouse IgG (ThermoFisher, 31430) and anti-rabbit HRP-conjugated (Cell Signalling, 7074). Post-secondary incubation, the membranes were washed 3 times with PBS-T in 15-minute intervals with shaking. The membranes were then incubated with Amersham ECL Select Western Blotting Detection Reagent (Cytiva) for 1 minute before being imaged using an Azure 280 imager (Azure Biosystems).

2.6 Co-immunoprecipitation

HEK293T cells were seeded into 10 cm plates 18-24 hours before transfection to reach confluency of 70-80% at the time of transfection. They were transfected with the indicated plasmids. 24 hours post-transfection, cells were harvested and lysed as mentioned above. 20 ug of protein lysate was used for input lanes for immunoblotting. 1000 μg of protein lysate was incubated with anti-FLAG M2 magnetic beads or Dynabead magnetic beads conjugated to Myc by an anti-Myc antibody overnight, rotating at 4°C. The next day, beads were washed with Tris-buffered saline (1xTBS) (150 mM NaCl, and 20 mM Tris [pH 7.5]) three times for 5 minutes rotating at 4°C and eluted using 1x SDS. SDS-PAGE and Western blotting were conducted as mentioned above using the indicated antibodies.

2.7 Ubiquitination Assay

293T cells were co-transfected with HA-Ub or FLAG-SENP1 plasmids for 24 hrs. Cells were lysed as mentioned above. 20 μg of protein lysates was used for inputs, while 1000 μg of protein lysates was used for the immunoprecipitation by incubating lysates with Anti-FLAG M2 magnetic beads overnight, rotating at 4°C. The next day, beads were washed with TBS three times and eluted using 1x SDS. SDS-PAGE and Western blotting were conducted as mentioned above using the indicated antibodies.

2.8 Deubiquitination Assay

HEK293T and HCT116 WT cells were transfected with the indicated plasmids. Cells were treated with 40 μM MG132 (Millipore-Sigma) for four hours before harvesting. Cells were collected and lysed as mentioned above. 20 μg of protein lysates was used for inputs, while 1000 μg of protein lysates was used for the immunoprecipitation by incubating lysates with Anti-

FLAG M2 magnetic beads overnight, rotating at 4°C. The next day, beads were washed with TBS three times and eluted using 1x SDS. SDS-PAGE and Western blotting were conducted as mentioned above using the indicated antibodies.

2.9 Cycloheximide Chase

HCT 116 WT and HCT 116 USP7-/- KO cells were treated with 50 μg/ml cycloheximide (Millipore-Sigma) for the indicated timepoints. Cells were harvested and lysed as mentioned above. 20 ug of protein lysate was used to run the SDS-PAGE, as mentioned above. Blots were probed with indicated antibodies and imaged using the Azure 280 imager.

2.10 Blot Quantification and Statistical Analysis

All quantifications of the blots were done using ImageJ software. All proteins of interest were normalized to GAPDH. Data is shown as mean ± standard error of the mean (SEM). Differences between groups were analyzed using Student's t-test or one-way ANOVA followed by Tukey HSD Test, and $P<0.05$ was considered statistically significant displayed as * ($P<0.05$).

Chapter 3: Results

3.1 SENP1 is a USP7-interacting protein

The ^{164}DWGF^{167} motif located within the MATH domain of USP7 is known to bind interacting proteins that contain a P/A/ExxS consensus sequence, where x can be any amino acid. While surveying potential USP7-interacting partners using bioinformatic tools, ScanProsite and Eukaryotic Linear Motif (ELM.EU.ORG) computational biology resource, we found that SENP1 contains a PSSS motif, which could potentially mediate protein interaction with USP7. As shown in Figure 10A, the SENP1 consensus sequence matches closely with other well-characterized USP7 substrates. Thus, we hypothesized that USP7 interacts and regulates SENP1 activity, thus impacting its protein stability.

Prior to elucidating the functional role of USP7 on SENP1 in mammalian cells, it was first important to validate the physical interaction between USP7 and SENP1. This was done by performing co-immunoprecipitation assays. HEK293T cells were transiently transfected with expression plasmids for either FLAG-SENP1 or Myc-USP7. Cell extracts were then immunoprecipitated for anti-FLAG using FLAG M2 magnetic beads. As shown in Figure 10B (last lane), SENP1 co-immunoprecipitates with USP7, confirming that both proteins interact in these cells. Similarly, immunoprecipitation of cell extracts with Anti-Myc conjugated Dynabead magnetic beads pulled down exogenously expressed FLAG-SENP1, providing additional evidence for an interaction between USP7 and SENP1 (Figure 10C, last lane).

To assess this interaction under a more physiologically relevant condition, HEK293T cells were transfected with either FLAG-SENP1 or an empty vector (EV) control. Following immunoprecipitation with an Anti-FLAG antibody, we observed that endogenous USP7 co-

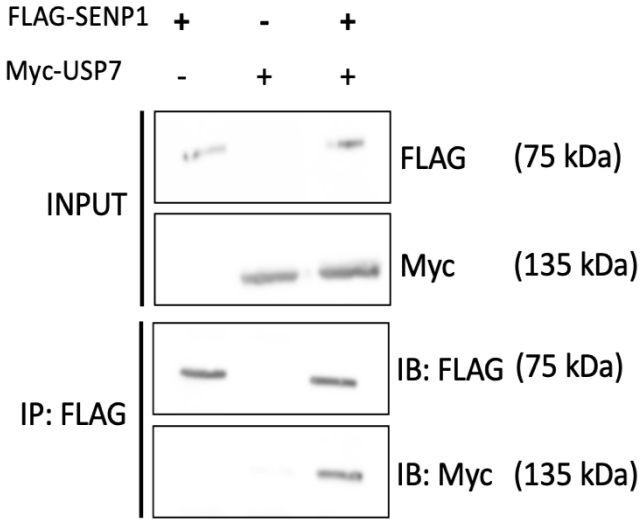
immunoprecipitates with SENP1 (Figure 10D, last lane), providing further evidence of the interaction between SENP1 and USP7. Likewise, immunoprecipitation of HEK293T cells transfected with Myc-USP7 or EV with Anti-Myc conjugated Dynabead magnetic beads demonstrated that endogenous SENP1 was pulled down, confirming the reciprocal interaction between SENP1 and USP7 (Figure 10E, last lane). Overall, our data together suggest that SENP1 is a novel protein that interacts with USP7.

A)

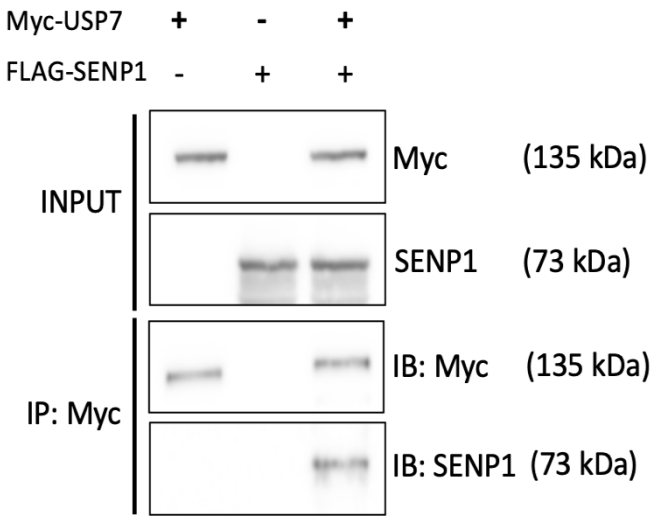
Consensus Sequence P/A/E x x S

p53	**P**	G	G	**S**	R
CHFR	**P**	S	T	**S**	T
MDM2	**P**	S	T	**S**	T
CDK8	**P**	S	T	**S**	Q
BMI1	**P**	S	T	**S**	S
MDM2	**P**	S	S	**S**	H
SENP1	**P**	S	S	**S**	S

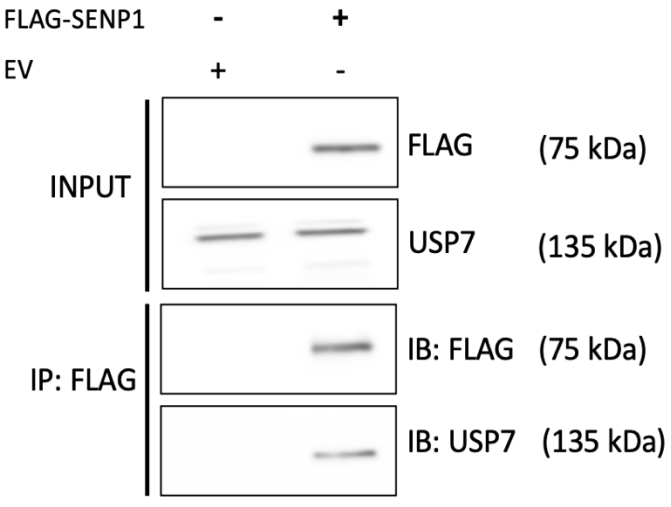
B)



C)



D)



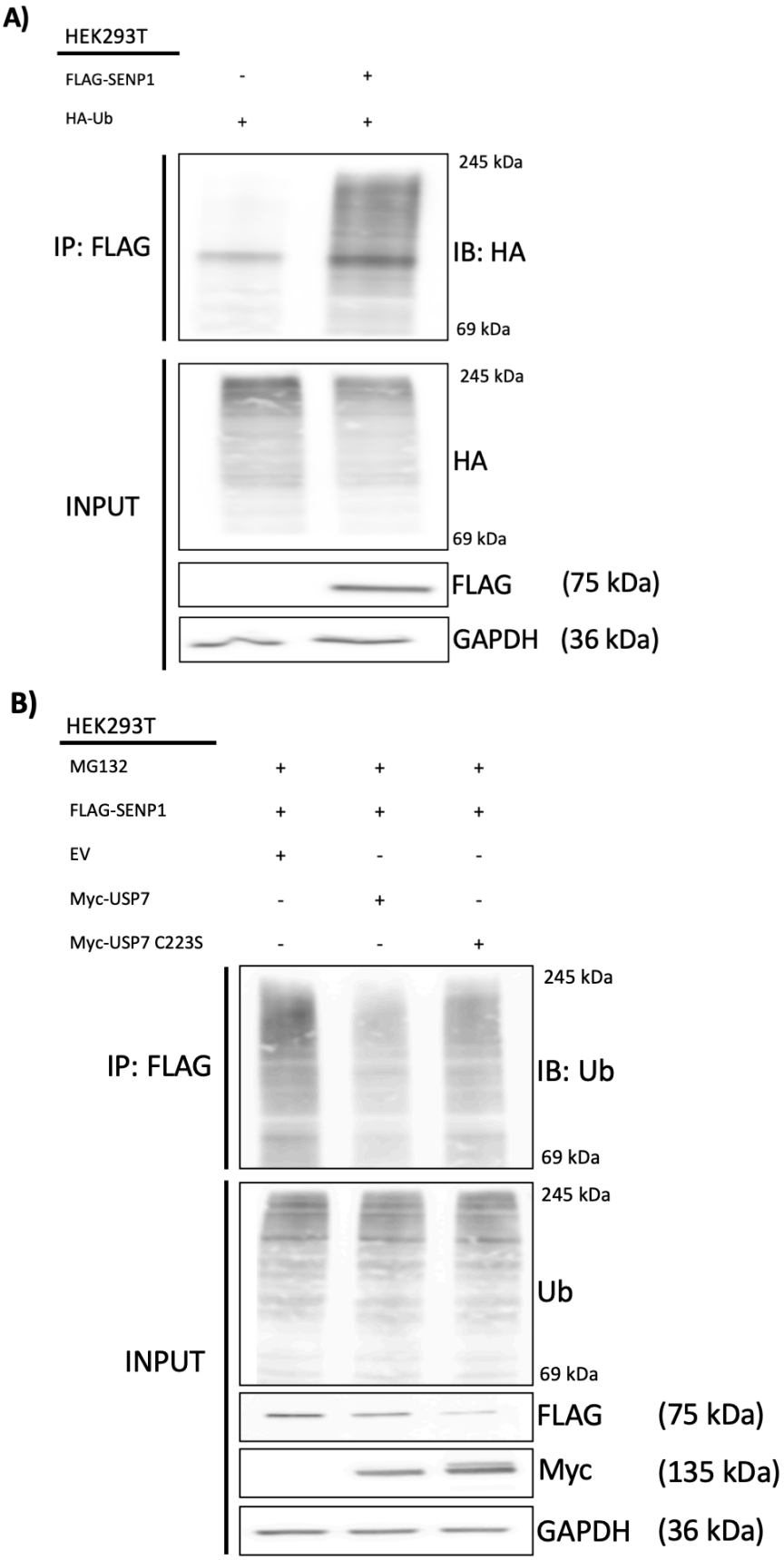
E)

Figure 10. SENP1 is a USP7-interacting protein.
A, Bioinformatics search using ScanProsite and Eukaryotic Linear Motif (ELM.EU.ORG) computational biology resource revealed a PSSS motif within SENP1 that could potentially regulate USP7-SENP1 interaction.
B, 3 ug of FLAG-SENP1 or Myc-USP7 were transfected in HEK293T cells; 1000 ug of proteins were extracted for co-IP using FLAG Magnetic M2 beads. Western blotting was performed with anti-FLAG or anti-Myc antibody (n=3).
C, 3 ug of Myc-USP7 or FLAG-SENP1 were transfected in HEK293T cells; 1000 ug of proteins were extracted for co-IP using Myc-conjugated Dynabeads magnetic beads. Western blotting was performed with SENP1 or anti-Myc antibody (n=1).
D, 3 ug of FLAG-SENP1 or empty vector were transfected in HEK293T cells; 1000 ug of proteins were extracted for co-IP using FLAG Magnetic M2 beads. Western blotting was performed with anti-FLAG or endogenous USP7 antibody (n=1).
E, 3 ug of Myc-USP7 or empty vector were transfected in HEK293T cells; 1000 ug of proteins were extracted for co-IP using Myc-conjugated Dynabeads magnetic beads. Western blotting was performed with anti-Myc or endogenous SENP1 antibody (n=1).

3.2 USP7 deubiquitinates SENP1 *in vivo*.

To determine if USP7 regulates SENP1 protein stability through its deubiquitinase activity, we first examined whether SENP1 undergoes ubiquitination. A cell-based ubiquitination assay was performed in HEK293T cells co-expressing FLAG-SENP1 and HA-ubiquitin (HA-Ub). If SENP1 is ubiquitinated, a characteristic high-molecular weight smear would be detected, corresponding to polyubiquitinated SENP1. Indeed, as shown in Figure 11A, SENP1 exhibited an increase in ubiquitination (last lane), indicating that it undergoes polyubiquitination in mammalian cells.

To determine if SENP1 is a substrate and is deubiquitinated by USP7, a deubiquitination assay was conducted. Wild-type USP7 or its catalytically inactive mutant USP7 (C223S) was overexpressed in HEK293T cells. The catalytically inactive USP7 mutant (C223S) contains a missense substitution at amino acid 233, where the cysteine residue is substituted for a serine residue, preventing USP7 from initiating catalysis.[23] As a result, mutant USP7 lacks deubiquitylation activities, rendering it catalytically inactive. Overexpression of wild-type USP7 led to a reduction in the endogenous ubiquitin levels of SENP1 (Figure 11B, lane 2) compared to control (lane 1). In contrast, the C223S mutant resulted in increased endogenous ubiquitination (Figure 11B, lane 3) compared to the wild-type Myc-USP7 transfected lane (lane 2). Similarly, wild-type USP7 or its catalytically inactive mutant USP7 (C223S) was overexpressed in HCT116 cells. Overexpression of wild-type USP7 led to reduced HA-Ub conjugated SENP1 levels (Figure 11C, lane 3) compared to the control (lane 2), whereas the C223S mutant led to increased HA-Ub modified SENP1 levels (Figure 11C, lane 4). Taken together, these results demonstrate that SENP1 is a direct substrate that undergoes deubiquitination by USP7.


A)
HEK293T
FLAG-SENP1 - +
HA-Ub + +
IP: FLAG
245 kDa
IB: HA
69 kDa
INPUT
245 kDa
HA
69 kDa
FLAG (75 kDa)
GAPDH (36 kDa)
B)
HEK293T
MG132 + + +
FLAG-SENP1 + + +
EV + - -
Myc-USP7 - + -
Myc-USP7 C223S - - +
IP: FLAG
245 kDa
IB: Ub
69 kDa
INPUT
245 kDa
Ub
69 kDa
FLAG (75 kDa)
Myc (135 kDa)
GAPDH (36 kDa)

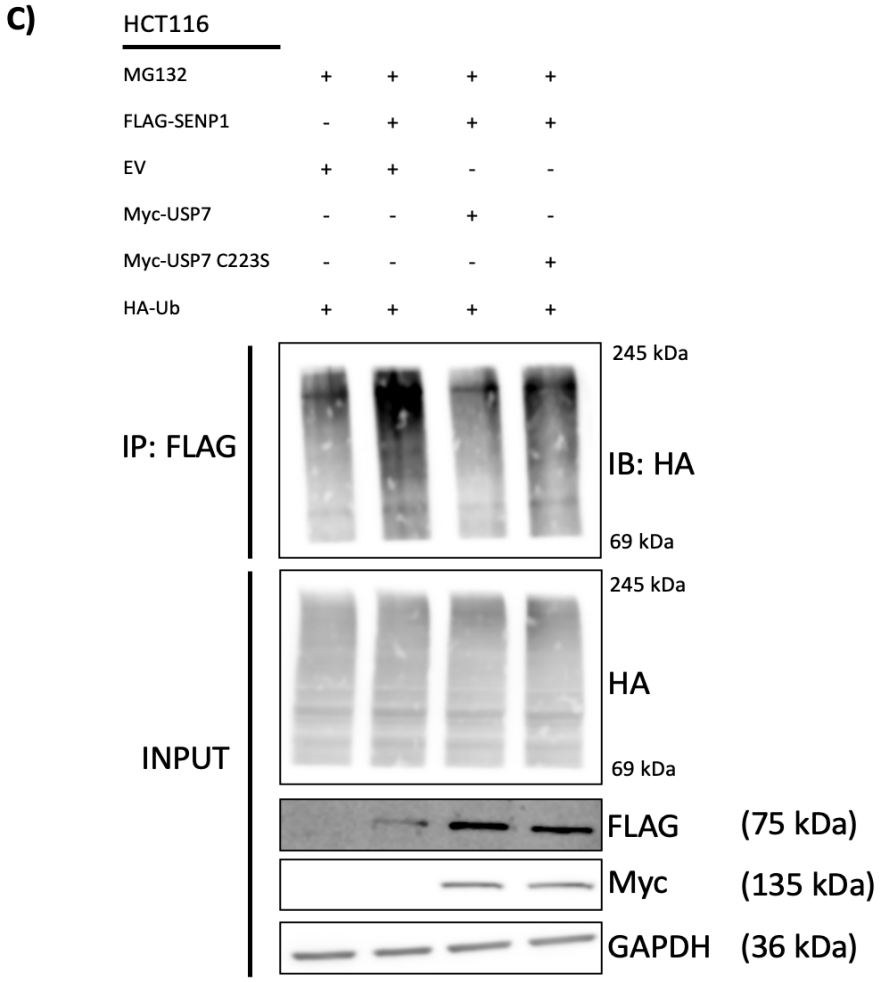
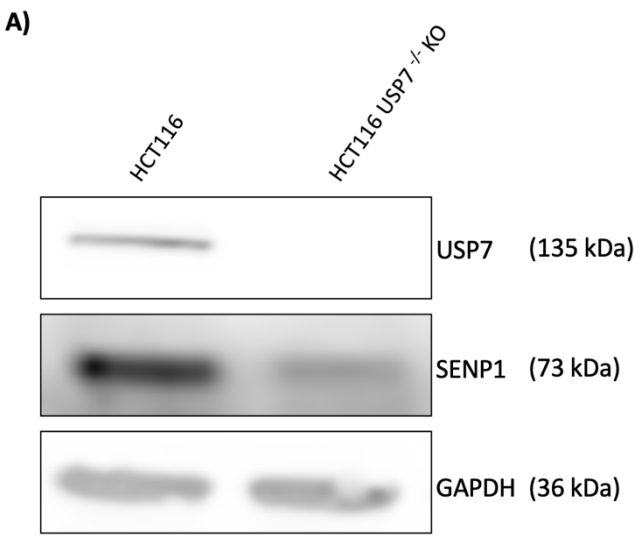


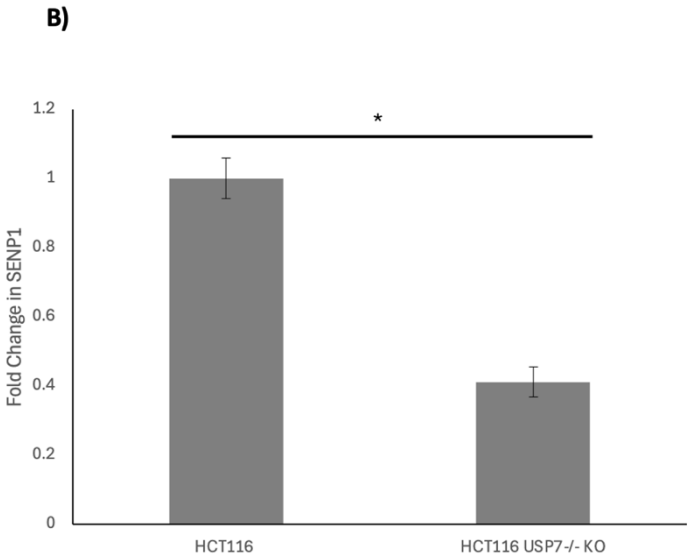
Figure 11. USP7 deubiquitinates SENP1 in vivo.
A, HA-Ub and FLAG-SENP1 were transfected in HEK293T cells. The ubiquitination of exogenous SENP1 was analyzed by co-IP using FLAG Magnetic M2 beads and Western blotting using an anti-HA antibody (*top panel*).
B, HEK293T cells were transfected with FLAG-SENP1, Myc-USP7 or Myc-USP7 (C223S) mutant. Cells were treated with MG132 (40 μM) for four hours prior to harvesting. The ubiquitination of SENP1 was detected by co-IP using FLAG Magnetic M2 beads and Western blotting for endogenous ubiquitin (Ub) with an anti-Ub antibody (*top panel*) (n=2).
C, HA-Ub, FLAG-SENP1, Myc-USP7 or Myc-USP7 (C223S) mutant were transfected into HCT116 cells. Cells were treated with MG132 (40 μM) for four hours prior to harvesting. The ubiquitination of SENP1 was detected by co-IP using FLAG Magnetic M2 beads and Western blotting for HA with an anti-HA antibody (*top panel*) (n=2).

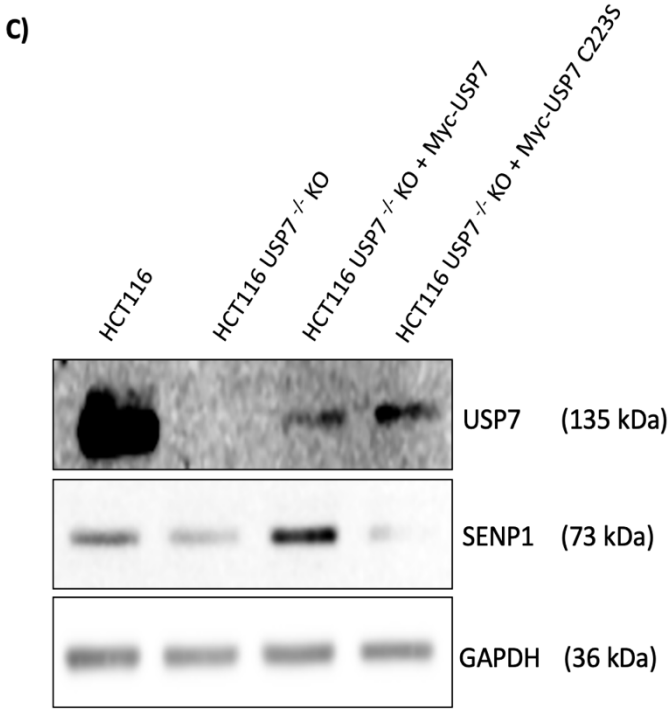
3.3 USP7 reconstitution in HCT116 USP7$^{-/-}$ KO cells rescue SENP1 protein level

To investigate whether USP7 regulates endogenous SENP1 protein levels, whole cell lysates from HCT116 cells and HCT116 USP7$^{-/-}$ KO cells, a genetically engineered version of the human colon carcinoma cell line which have the USP7 gene knocked out were extracted to assess SENP1 protein levels in the presence and absence of USP7. As shown in Figure 12A, protein levels of SENP1 in the absence of USP7 are notably reduced in comparison to HCT116 cells, which exhibited higher levels of SENP1. Blots were quantified using ImageJ and the mean fold change ± standard error of the mean (SEM) of four biological replicates is illustrated in Figure 12B. Quantification from four biological replicates suggests that this result is significant with p p-value of 0.03. SENP1 protein levels had a mean fold change of 0.38 ± 0.06 in the HCT116 cell condition and 0.16 ± 0.04 in the HCT116 USP7$^{-/-}$ KO cell condition.

Reintroduction of wild-type USP7 restored SENP1 protein levels in HCT116 USP7$^{-/-}$ KO cells (Figure 12C, lane 3), whereas the catalytically inactive mutant USP7 (C223S) failed to rescue SENP1 protein levels (Figure 12C, lane 4). Quantification from three biological replicates is depicted in Figure 12D. In the parental HCT116 cell condition, SENP1 protein fold change was 0.70 ± 0.14 which decreased to 0.44 ± 0.07 in the HCT116 USP7$^{-/-}$ KO cell condition. Expression of Myc-USP7 in the HCT116 USP7$^{-/-}$ KO cells increased SENP1 fold change to 0.95 ± 0.07, whereas in the HCT116 USP7$^{-/-}$ KO cells expressing the catalytic mutant of USP7 (C223S), reduced the fold change of SENP1 to 0.30 ± 0.17. The difference between the HCT116 USP7$^{-/-}$ KO cells transfected with Myc-USP7 and Myc-USP7 C223S is statistically significant with a p-value of 0.02. Thus, these results revealed that USP7 regulates the protein stability of SENP1 in the HCT116 cell line.


A)
HCT116
HCT116 USP7 -/- KO
USP7 (135 kDa)
SENP1 (73 kDa)
GAPDH (36 kDa)


B)
Fold Change in SENP1
1.2
1
0.8
0.6
0.4
0.2
0
*
HCT116
HCT116 USP7-/- KO


C)
HCT116
HCT116 USP7 -/- KO
HCT116 USP7 -/- KO + Myc-USP7
HCT116 USP7 -/- KO + Myc-USP7 C223S
USP7 (135 kDa)
SENP1 (73 kDa)
GAPDH (36 kDa)

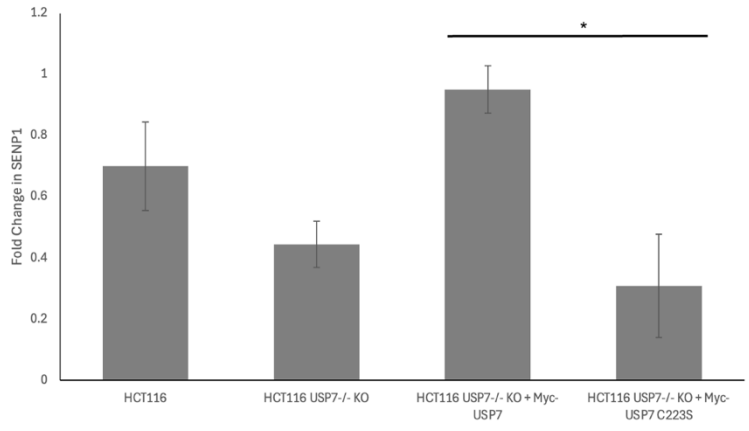

D)
Fold Change in SENP1
1.2
1
0.8
0.6
0.4
0.2
0
*
HCT116
HCT116 USP7-/- KO
HCT116 USP7-/- KO + Myc-USP7
HCT116 USP7-/- KO + Myc-USP7 C223S

Figure 12. USP7 regulates SENP1 protein levels in HCT116 cells.
A, Protein levels of endogenous SENP1 from whole cell lysate of HCT116 and HCT116 USP7$^{-/-}$ KO cells was detected using an anti-SENP1 antibody.
B, Western Blots from four biological replicates were quantified using ImageJ software. A student's t-test was conducted to evaluate statistical significance in the level change of SENP1.
C, HCT116 USP7$^{-/-}$ KO cells were transfected for 48 hours with either Myc-USP7 or Myc-USP7 (C223S) mutant. Protein levels of SENP1 were measured by Western blotting using an anti-SENP1 antibody.
D, Western Blots from three biological replicates were quantified using ImageJ software. A one-way ANOVA followed by Tukey's HSD test was conducted to evaluate statistical significance in the level change of SENP1.
Statistical significance was defined as $p<0.05$=significant and is indicated by an asterisk (*). Error bars represent the Standard Error of mean (SEM).

3.4 SENP1 protein levels are dependent on the catalytic activity of USP7

To further investigate USP7 function on SENP1 protein levels, HCT116 cells were transfected with Myc-USP7 or the catalytic mutant Myc-USP7 C223S. As depicted in Figure 13A, SENP1 protein levels increased upon Myc-USP7 expression (lane 2) compared to the control, empty vector transfected lane (lane 1). In contrast, SENP1 protein levels decreased with the catalytically inactive mutant (lane 3) compared to the wild-type USP7 transfected lane. Although not statistically significant, quantification of three biological replicates indicates that SENP1 levels are rescued with USP7 and not the catalytically inactive USP7 (Figure 13B).

To further assess SENP1 stability by USP7, FLAG-SENP1 was co-expressed along with either wild-type USP7 or C223S mutant in HCT116 cells to measure the changes in FLAG-SENP1 expression. As shown in Figure 13C, wild-type USP7 resulted in increased FLAG-SENP1 levels (lane 2), compared to control (lane 1), whereas the C223S mutant had no such effect (lane 3). Quantification of three biological replicates is illustrated in Figure 13D. FLAG-SENP1 fold change in the control empty vector (EV) condition was 0.38 ± 0.10 which significantly increased to 0.93 ± 0.7 (p-value: 0.049) upon myc-USP7 expression. In response to the inactive catalytic mutant, the fold change of FLAG-SENP1 was significantly lower at 0.35 ± 0.16 (p-value = 0.04). These findings provide additional evidence that USP7 positively regulates SENP1 protein levels through its catalytic activity.

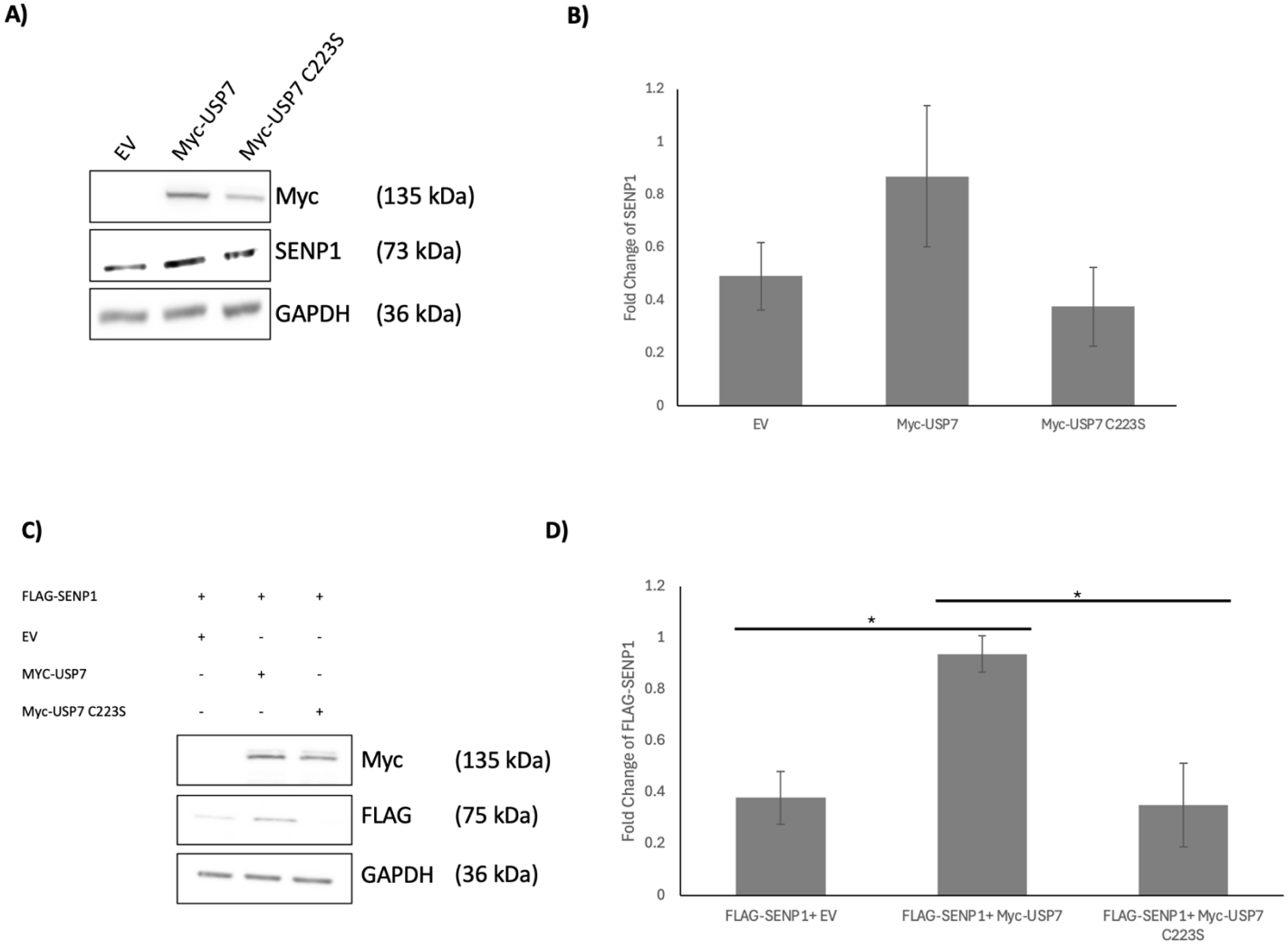


Figure 13. USP7 regulates SENP1 protein levels in HCT116 cells through its catalytic activity.

A, Endogenous SENP1 protein levels were monitored in HCT116 cells transfected with either Myc-USP7 or Myc-USP7 (C223S) mutant.

C, HCT116 cells were transfected with FLAG-SENP1 along with either Myc-USP7 or Myc-USP7 (C223S) mutant. Exogenous SENP1 protein levels were assessed using an anti-FLAG antibody.

GAPDH served as a loading control for all experiments.

B & D, Blots from three biological replicates from (A) or (C) were quantified using ImageJ software. A one-way ANOVA followed by Tukey's HSD test was conducted to evaluate statistical significance in the level change of SENP1 or FLAG, respectively.

Statistical significance was defined as $p<0.05$=significant and is indicated by an asterisk (*). Error bars represent SEM.

3.5 USP7 regulates SENP1 protein stability

To study the effect that USP7 has on the protein stability of SENP1, the half-life of SENP1 was measured in the presence and absence of USP7 by treating HCT116 and HCT116 USP7$^{-/-}$ KO cells with cycloheximide (CHX). Cycloheximide is a protein synthesis inhibitor which functions to block the elongation phase of eukaryotic translation by binding to the 60S ribosome.[77] As shown in Figure 14A, treatment with cycloheximide led to a marked reduction in SENP1 protein levels in the absence of USP7. Notably, SENP1 protein levels at 16- and 24-hours post-treatment in HCT116 USP7$^{-/-}$ KO cells are hardly detectable compared to the same time points in HCT116 cells. Additionally, SENP1 levels were consistently lower in HCT116 USP7$^{-/-}$ KO cells across all time points. Additionally, the protein half-life of SENP1 was 60.1 ± 21.2 hours in the presence of USP7 compared to 15.7 ± 5.6 hours without USP7, indicating a nearly fourfold decrease in SENP1 stability with the loss of USP7. The levels of USP7 remained relatively stable over the 24-hour treatment period compared to SENP1, which exhibited a marked reduction, as evident in Figure 14B. These results show that USP7 protects SENP1 from proteasomal degradation, thus extending the half-life of SENP1. Collectively, these findings indicate that USP7 plays a critical role in maintaining SENP1 protein stability.

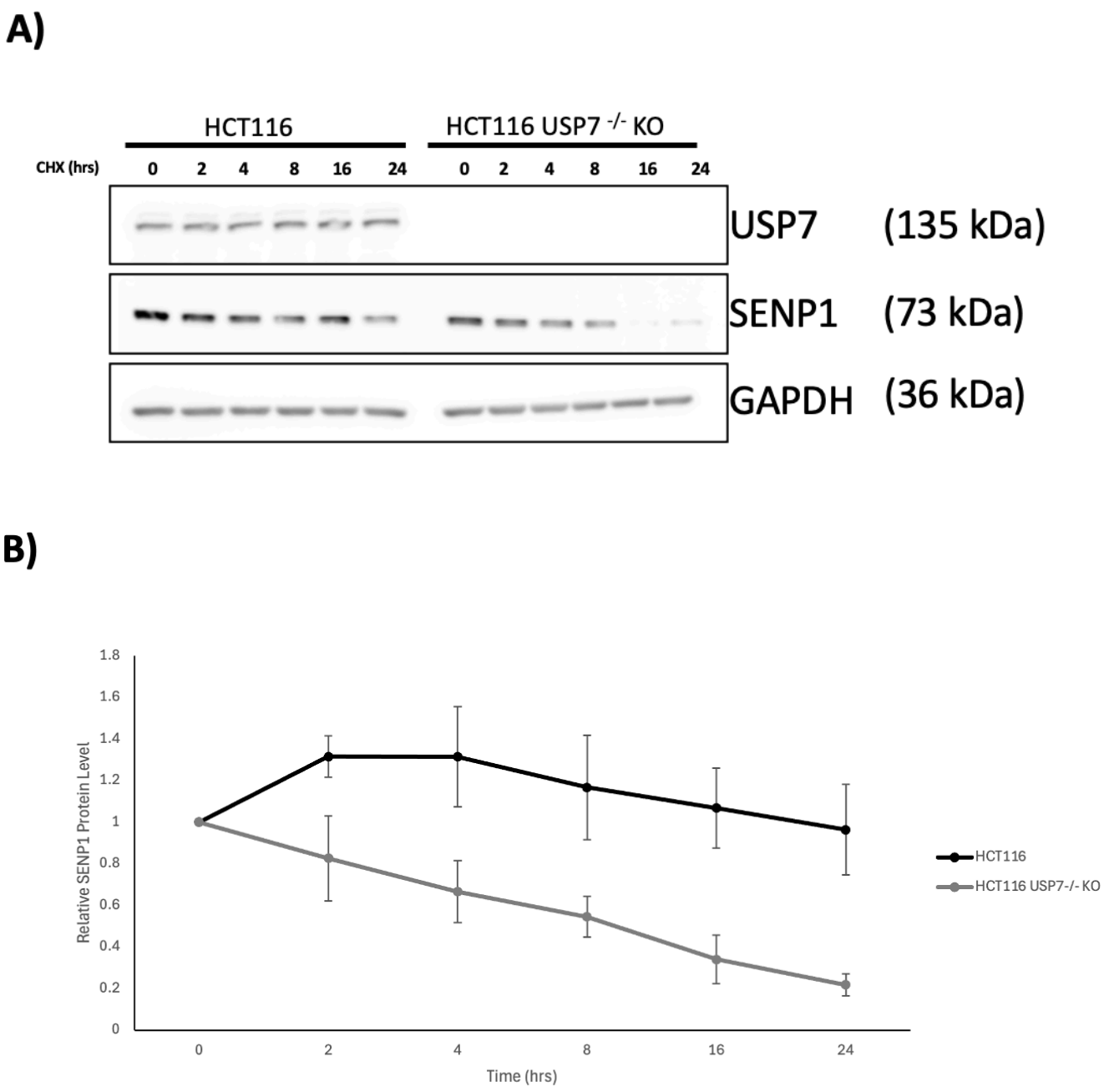


Figure 14. USP7 regulates SENP1 protein stability.
A. HCT116 and HCT116 USP7$^{-/-}$ KO cells were treated with cycloheximide (CHX) for various time points. The protein levels were assessed by Western blotting using the indicated antibodies. *B*. Time course decay curve of SENP1 protein levels with and without USP7 following cycloheximide (CHX) treatment. Data represent mean ± SEM from 5 biological replicates, normalized to time 0.

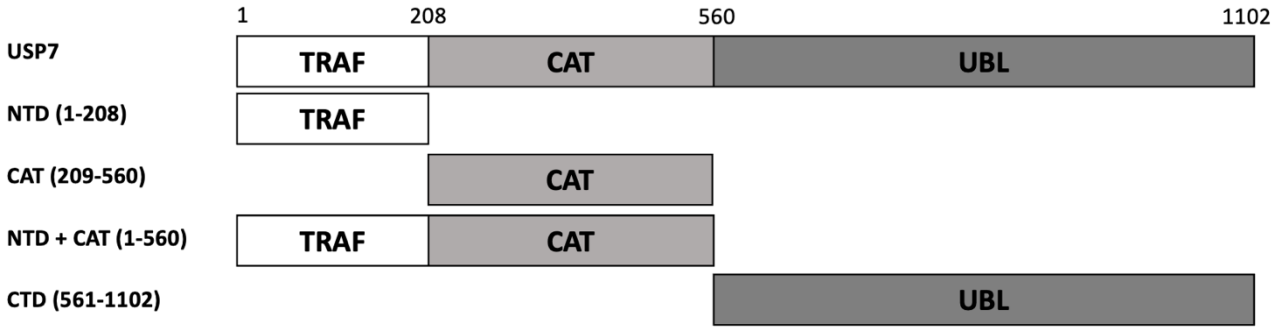
3.6 Domain-Specific Effects of USP7 on SENP1

To evaluate the domain-specific effects of USP7 on SENP1, different truncated constructs of USP7 (Figure 15A) were expressed and their effects on FLAG-SENP1 protein levels were examined in HCT116 cells. Expression of the N-terminus of USP7 (Myc-NTD) reduced FLAG-SENP1 protein levels (Figure 15B), an effect that is similar when expressing the N-terminus plus the catalytic domain construct together (Myc-NTD + CTD (1-560)) (Figure 15D). On the other hand, overexpression of the USP7 C-terminus (Myc-CTD) led to an increase in FLAG-SENP1 protein levels (Figure 15F). Quantification of two biological replicates for each condition is depicted in Figures 15C, 15E and 15G. Overall, this data indicates that there is a USP7 domain-specific effect where the N- and C-terminus is vital in regulating SENP1 protein stability in HCT116 cells.

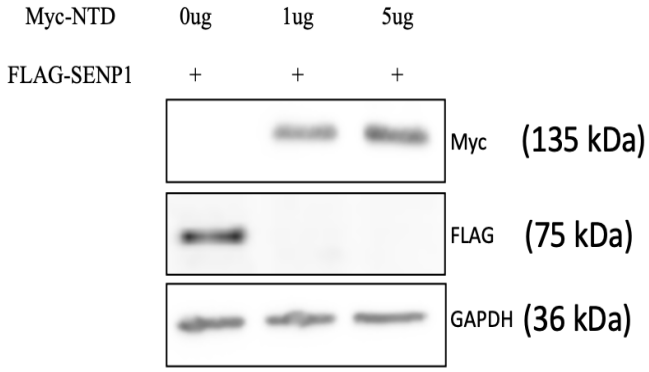
Based on the previous data, we sought to determine whether FLAG-SENP1 levels could be restored when wild-type USP7 is co-transfected with either the N-terminus or the N-terminus plus the catalytic domain of USP7 in HCT116 cells. To do this, equal amount of FLAG-SENP1 was transfected in all conditions with either wild-type USP7 alone, wild-type USP7 plus the N-terminus or wild-type USP7 plus the N-terminus and catalytic domain of USP7. As shown in Figure 15H, expression of wild-type USP7 alone increased FLAG-SENP1 levels (lane 2) as demonstrated previously. Surprisingly, wild-type USP7 plus the N-terminus (lane 3) or wild-type USP7 plus the N-terminus and catalytic domain of USP7 (lane 4) failed to rescue FLAG-SENP1 levels. Notably, there were no FLAG bands detected in the last two lanes which had the N-terminal construct, even with the presence of wild-type USP7, suggesting a dominant-negative effect. It is noteworthy to point out that the overexpression of the N-terminus of USP7 (Myc-NTD) resulted in a reduction in wild-type USP7 (lane 3), as there is no band present around 135

kDa. These findings suggest that the N-terminal of USP7 exerts a dominant-negative effect on SENP1 stability and its co-expression with wild-type USP7 prevents wild-type USP7 from stabilizing SENP1, resulting in its degradation. Additionally, the N-terminal of USP7 may inhibit and destabilize wild-type USP7 expression, further reinforcing its inhibitory role.

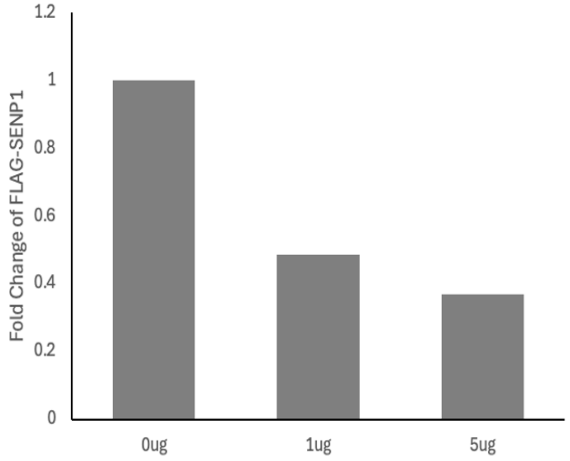
A)


1
208
560
1102
USP7
TRAF
CAT
UBL
NTD (1-208)
TRAF
CAT (209-560)
CAT
NTD + CAT (1-560)
TRAF
CAT
CTD (561-1102)
UBL

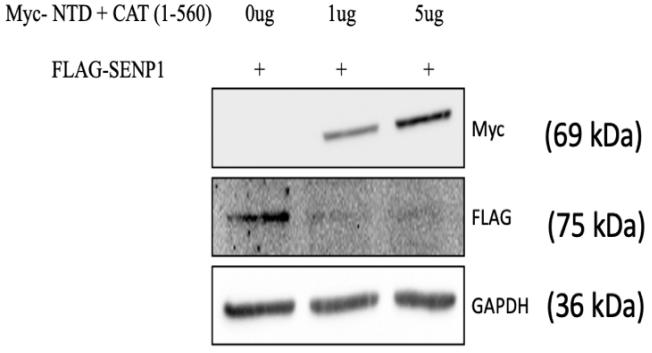
B)


Myc-NTD
0ug
1ug
5ug
FLAG-SENP1
+
+
+
Myc
(135 kDa)
FLAG
(75 kDa)
GAPDH
(36 kDa)

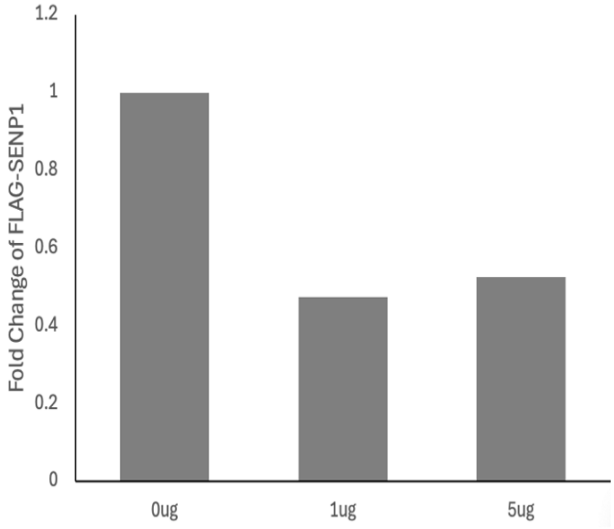
C)


Fold Change of FLAG-SENP1
1.2
1
0.8
0.6
0.4
0.2
0
0ug
1ug
5ug

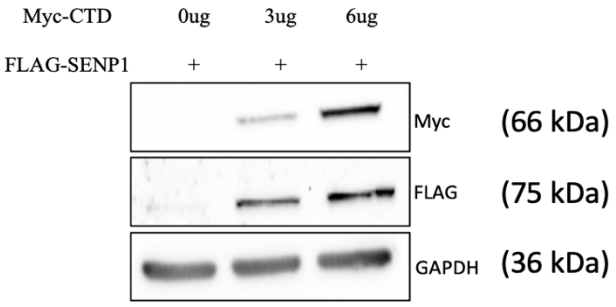
D)


Myc- NTD + CAT (1-560)
0ug
1ug
5ug
FLAG-SENP1
+
+
+
Myc
(69 kDa)
FLAG
(75 kDa)
GAPDH
(36 kDa)

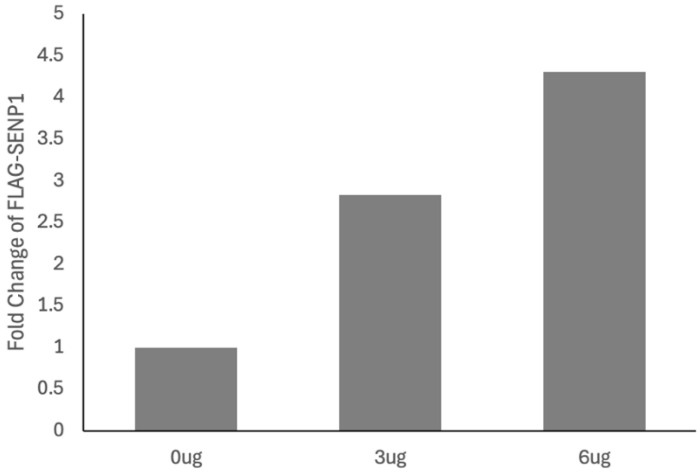
E)


Fold Change of FLAG-SENP1
1.2
1
0.8
0.6
0.4
0.2
0
0ug
1ug
5ug

F)


Myc-CTD
0ug
3ug
6ug
FLAG-SENP1
+
+
+
Myc
(66 kDa)
FLAG
(75 kDa)
GAPDH
(36 kDa)

G)


Fold Change of FLAG-SENP1
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0ug
3ug
6ug

H)

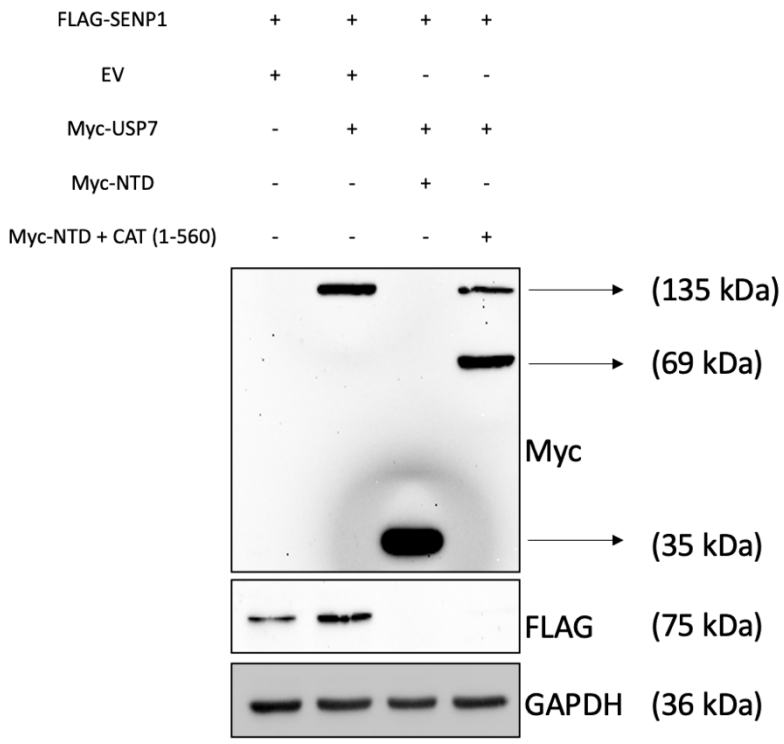

FLAG-SENP1 + + + +
EV + + - -
Myc-USP7 - + + +
Myc-NTD - - + -
Myc-NTD + CAT (1-560) - - - +
(135 kDa)
(69 kDa)
Myc
(35 kDa)
FLAG
(75 kDa)
GAPDH
(36 kDa)

Figure 15. Domain-Specific Effect of USP7 on SENP1.
A, Schematic representation of USP7 domains and truncation constructs.
B, HCT116 cells were transfected with FLAG-SENP1 and increasing amounts of Myc-NTD.
C, Western Blots from two biological replicates from (B) were quantified using ImageJ software. The band intensities were normalized to GAPDH. (n=2)
D, HCT116 cells were transfected with FLAG-SENP1 and increasing amounts of Myc-NTD+CTD (1-560).
E, Western Blots from two biological replicates from (D) were quantified using ImageJ software. The band intensities were normalized to GAPDH. (n=2)
F, HCT116 cells were transfected with FLAG-SENP1 and increasing amounts of Myc-CTD.
G, Western Blots from two biological replicates from (F) were quantified using ImageJ software. The band intensities were normalized to GAPDH. (n=2)
H, HCT116 cells were transfected with FLAG-SENP1 along with either wild-type Myc-USP7, Myc-NTD or Myc-NTD+ CTD (1-560). Exogenous SENP1 protein levels were assessed using an anti-FLAG antibody (n=3).

Chapter 4: Discussion

The purpose of this research project was to identify and characterize a novel substrate of the deubiquitinating enzyme, USP7. Bioinformatics analysis for potential USP7 interactors highlighted SENP1, an enzyme responsible for the removal of SUMO modifications from target proteins, as a potential USP7 interactor. Using co-immunoprecipitation studies, we confirmed that USP7 interacts with SENP1. We further demonstrated that USP7 is critical in regulating the protein stability of SENP1 through its deubiquitinase activity.

4.1 USP7 Interacts with SENP1

To assess if SENP1 is a novel interactor of USP7, we performed a series of exogenous co-immunoprecipitation assays in HEK293T cells. We found that exogenously expressed FLAG-SENP1 is co-immunoprecipitated with Myc-USP7 (Figure 10B) and reciprocally, Myc-USP7 is co-immunoprecipitated with FLAG-SENP1 (Figure 10C). Moreover, endogenous USP7 was pulled down with exogenously expressed FLAG-SENP1 (Figure 10D). Similarly, when conducting the reciprocal reaction, endogenous SENP1 was immunoprecipitated with exogenously expressed Myc-USP7 (Figure 10E). However, we were unable to detect an interaction between the two proteins under fully endogenous conditions (data not shown), likely due to low endogenous levels of SENP1. Additionally, there is a possibility that USP7 interaction with SENP1 could represent a weak interactor therefore, it was unable to be detected by endogenous co-immunoprecipitation. Given these limitations, our combined results nonetheless, provide strong evidence that USP7 and SENP1 can interact and associate with each other.

4.2. USP7 Regulates the Stability of SENP1

To determine if USP7 regulates the stability and protein levels of SENP1, we first examined the ubiquitination status of SENP1. Ubiquitination assays revealed that SENP1 is ubiquitin-conjugated, consistent with previous literature (Figure 11A).[78] Given that SENP1 is ubiquitinated and interacts with USP7, a deubiquitinating enzyme, we hypothesized that USP7 may regulate and stabilize SENP1 through deubiquitination. Indeed, deubiquitination assays demonstrate that USP7 removes both endogenous and exogenously expressed HA-tagged ubiquitin from SENP1, whereas the catalytically inactive mutant of USP7, which harbours a missense mutation at the cysteine residue that changes it to a serine (C223S) failed to do so (Figure 11B & C).

Given that SENP1 is a novel substrate that is deubiquitinated by USP7, we next examined whether SENP1 protein levels are dependent on USP7. Using HCT116 parental cells and a corresponding USP7 knockout cell line (HCT116 USP7$^{-/-}$ KO), we found that SENP1 levels were significantly reduced in the knockout cell line. The reduction in the knockout cell line was rescued with the re-expression of Myc-USP7, whereas re-expression of the catalytic mutant of USP7 failed to do so (Figure 12). Therefore, collectively these results suggest that USP7 stabilizes the levels of SENP1 by deubiquitinating them, a function that is dependent on an active catalytic core, thus preventing SENP1 degradation via the 26S proteasome.

Considering a knockout of USP7 significantly reduces SENP1 levels, we next sought to investigate whether overexpression of Myc-USP7 in the parental HCT116 cells could increase SENP1 protein levels. Although not statistically significant, our data led to a trend suggesting that the re-expression of Myc-USP7 did increase endogenous SENP1 levels in comparison to the catalytic mutant, which could not. Additional replicates will be required to confirm this effect

(Figure 13A). However, upon overexpressing Myc-USP7 alongside exogenous FLAG-SENP1, we observed a clear and significant increase in FLAG-SENP1 levels. In contrast, the catalytic mutant failed to stabilize FLAG-SENP1 (Figure 13B). This could likely be due to the increased FLAG-SENP1 levels which could potentially make detecting changes easier. Also, it is possible that endogenous SENP1 could be engaged with other regulatory proteins making it less accessible to USP7, while overexpressed FLAG-SENP1 remains more available for deubiquitination. Finally, it is likely that endogenous SENP1 is more stable in comparison to overexpressed FLAG-SENP1, making USP7's catalytic role more pronounced in the overexpression system.

Finally, we showed that USP7 regulates the half-life of SENP1 via a cycloheximide chase (Figure 14). Comparing the stability of SENP1 with and without USP7 revealed that USP7 contributes to maintaining SENP1 protein levels by slowing its degradation through deubiquitination. SENP1 stability is reduced nearly fourfold in the absence of USP7, whereas its half-life is substantially longer when USP7 is present.

4.3 Domain-Specific Contribution of USP7 on SENP1

Interestingly, we found that the N-terminal and C-terminal domains of USP7 have opposing effects on FLAG-SENP1 protein levels. The N-terminal domain caused a decrease in FLAG-SENP1 protein levels, whereas the C-terminal domain caused an increase (Figure 15B & F). This is surprising, given USP7's well-characterized role in stabilizing its substrate proteins through deubiquitination.[79] One explanation for this result could be that USP7, through its N-terminal domain, is known to interact with and stabilize many proteins, one of which could be an E3 ubiquitin ligase that causes the degradation of SENP1. This is probable since under some circumstances, USP7 can promote the degradation of p53 by stabilizing Mdm2, an E3 ubiquitin

ligase.[27] Furthermore, some substrates have a higher affinity for the N-terminal domain of USP7. For example, it is well known that many viral proteins outcompete cellular substrates by binding to USP7 with high affinity.[80,81] Similarly, cellular E3 ligases could bind USP7 more strongly than SENP1, preventing SENP1 binding and deubiquitination, thus targeting SENP1 for degradation. Another possibility is that the N-terminal domain of USP7 recruits a negative regulator such as an E3 ubiquitin ligase, forming a complex that degrades SENP1. Alternatively, the N-terminal domain of USP7 could be interacting with SENP1 directly, but since it lacks the catalytic domain, it fails to deubiquitinate and stabilize SENP1, therefore causing its degradation. However, our data indicate that when the N-terminal and catalytic domain of USP7 are expressed in mammalian cells, FLAG-SENP1 levels still decrease, similar to the N-terminus alone (Figure 15D). Therefore, there must be another reason for SENP1 downregulation when the N-terminal domain of USP7 is overexpressed. Interestingly, we found that when the N-terminal domain of USP7 is overexpressed, full-length Myc-USP7 is also downregulated (Figure 15H), with a milder effect seen when the N-terminal and catalytic domain construct of USP7 is expressed. This raises the possibility that the N-terminal domain is inhibiting full-length Myc-USP7, thereby indirectly reducing SENP1 stability. An additional explanation for the observed reduction in the full-length Myc-USP7 levels when co-expressed with Myc-NTD is competition for the shared cellular machinery. Although both constructs are driven by the same CMV promoter, the truncated Myc-NTD is smaller, more easily translated and may exhibit greater protein stability compared to full-length Myc-USP7. As a result, the translational machinery may engage with Myc-NTD and lead to its disproportionately higher production or stabilization relative to full-length Myc-USP7. The imbalance could contribute to the domain-negative effect observed in SENP1 regulation. Further

investigation will be required to fully elucidate how truncated USP7 fragments influence the expression, stability, and function of full-length USP7.

On the other hand, the C-terminus of USP7 could also be interacting with SENP1 more directly, possibly through a weak interaction, therefore stabilizing it. It has been reported that the C-terminus of USP7, particularly Ubl4 and Ubl5 are necessary to activate USP7 and promote ubiquitin binding and increase catalytic activity 100-fold.[29] It is possible that the C-terminal construct is mimicking the activation signal thus enhancing the deubiquitinating activity of endogenous USP7, therefore stabilizing SENP1. Alternatively, the C-terminal domain might disrupt the N-terminus-driven degradation pathway, therefore stabilizing SENP1. Collectively, these results suggest that there are opposing effects of USP7 domains in regulating SENP1.

4.4 Coordinated Regulatory Network targeting SENP1

Previous literature has shown that SENP1 is a ubiquitination target of the Polycomb Repressive Complex-1 (PRC-1) which includes E3 ubiquitin ligases RING1A or RING1B, as well as Polycomb group RING finger (PCGF) proteins such as BMI-1, and MEL-18.[78] Interestingly, USP7 is also known to regulate the same proteins involved in the PRC-1 complex. It has been shown that USP7 interacts with and deubiquitinates RING1B, MEL18 and BMI1, thereby stabilizing the PRC1 complex.[82,83] Given that SENP1 undergoes ubiquitination, it is likely subject to deubiquitination as well. Our data indicate that USP7 functions as a novel deubiquitinase for SENP1. This dual role highlights a complex regulatory balance in which USP7 can either protect SENP1 from degradation through direct deubiquitination or indirectly target it for degradation through the regulation of PRC1. Nevertheless, it is well-known that SENP1 is also regulated by other USP family members.

Previous studies have suggested that USP28 interacts with SENP1, an interaction that is critical for regulating hypoxia-inducible transcription factor-1α (HIF-lα).[84] HIF-lα has a central role in regulating angiogenesis, carcinogenesis and cell adaptation to hypoxia.[84] Under hypoxic conditions, HIF-lα induces SENP1 expression, which in turn deSUMOylates HIF-lα, forming a positive feedback loop that stabilizes HIF-lα. Additionally, it was found that USP28 itself is SUMOylated under normoxia and deSUMOylated during hypoxic conditions.[84] This deSUMOylation is mediated by SENP1 which promotes the subsequent deubiquitination and stabilization of HIF-lα under hypoxic conditions.[84] It is unclear whether USP28 also deubiquitinates SENP1, but given its function as a deubiquitinase, it is a plausible possibility.

In addition, USP51 regulates HIF-lα both indirectly, by binding to Elongin C (ELOC), a subunit of the VHL E3 ubiquitin ligase complex (VHL/ CUL2/ ELOC/ ELOB/RBX1), and directly through deubiquitination of HIF-lα itself.[85] ELOC is subject to SUMOylation which prevents its interaction with USP51. SENP1 mediates the deSUMOylation of ELOC, thereby facilitating ELOC-USP51 binding.[85] This, in turn, enhances USP51-mediated deubiquitination and stabilization of HIF-lα. Thus, SENP1 also indirectly promotes HIF-lα stability by enabling USP51 function.

Interestingly, USP7 has also been reported to be involved in regulating HIF-lα by directly deubiquitinating and stabilizing it.[86] Our data suggest that USP7 interacts with and deubiquitinates SENP1, providing a novel mechanism by which SENP1 function, and thus the hypoxia response may be regulated. Importantly, when considered alongside USP28 and USP51, this highlights that SENP1 is a central protein that is modulated by multiple USP family members, underscoring its pivotal role in regulating HIF-lα and thus the hypoxia response.

4.5 Potential USP7-ICP0-SENP1 Mechanism to Regulate PML Nuclear Bodies

SENP1 has been associated with early viral replication in HSV-1 infections. Previous studies have indicated that ICP0, an E3 ubiquitin ligase, promotes the loss of PML nuclear bodies (NBs) in a proteasome-dependent manner, a crucial interaction that promotes early viral replication.[87] These NBs are areas within the nucleus that are enriched in proteins that are modified at specific lysine residues by SUMO-1, a requirement for PML-NB formation.[79,87] They control processes such as p53 activation, apoptosis, senescence, DNA damage repair and innate antiviral responses.[79] It has been suggested that a loss of PML-NBs is associated with the development and progression of a variety of cancers.[79]

Immunofluorescence co-localization experiments have demonstrated that the expression of ICP0 causes a significant reduction in SUMO-1 levels.[87] This loss appears to be facilitated through ICP0-mediated recruitment of SENP1 during the early stages of viral infection.[87] However, during later stages of viral infection, ICP0 and SENP1 are no longer co-localized and exhibit a more diffused distribution throughout the nucleus and cytoplasm.[87] Interestingly, USP7 has been shown as a negative regulator of PML proteins and PML NBs and also interacts with and deubiquitinates ICP0.[88,89] This interaction is critical in regulating the ICP0-mediated degradation of SUMOylated PML-NBs. Our data highlights a new pathway by which PML NBs can potentially be disrupted through the USP7-SENP1 axis. USP7 mediates deubiquitination and stabilization of SENP1, potentially leading to enhanced deSUMOylation of PML NBs thus disrupting their formation, and causing the dysregulation of key cellular processes as mentioned above. Although we show that SENP1 is stabilized by USP7, whether it enhances the deSUMOylation process remains to be investigated.

4.6 Limitations of the Study and Future Directions

Many downstream targets of SENP1 are also regulated by USP7, making it difficult to determine whether the changes observed on those targets are mainly due to USP7, SENP1 or their combined activity. As a result, it would be worth examining the changes in gene expression by measuring the mRNA levels of both SENP1 and its downstream targets in the presence of USP7. Additionally, SENP1 removes SUMO modifications from target proteins, so it will be interesting to investigate whether the deSUMOylation process is enhanced in the presence of USP7. It would be expected that overexpression of USP7, by stabilizing SENP1, would lead to a decrease in global SUMO-conjugated proteins and a corresponding increase in free SUMO monomers. Moreover, it would be valuable to further investigate the opposing, domain-specific effects on SENP1. To our knowledge, this is the first report showing that USP7 domains exert opposing effects on a protein, highlighting a previously unrecognized mode of regulation. In line with this, it would be critical to confirm the domain-specific interaction of SENP1 with USP7. In particular, given that the ^{164}DWGF^{167} motif within the N-terminus of USP7 recognizes the P/A/ExxS consensus motif, validating the N-terminal interaction with SENP1 represents an important next step. SENP1 contains a PSSS motif that fits this consensus. However, the present study did not examine whether this motif mediates an interaction with the DWGF residues of USP7. Therefore, confirming this interaction through future pulldown and binding assays will provide deeper insight into how USP7 recognizes and regulates its substrate proteins. Additionally, the scope of this work was restricted to a few cell lines. To broaden the impact of this interaction, it will be important to determine if this interaction is conserved in other cell types, animal models or patient samples. Also, to date, no other SENP family member has been reported to interact with USP7. It will therefore be important to determine whether USP7 also

interacts with other SENPs or if this interaction is unique to SENP1. Another limitation within this study is the potential for off-target effects resulting from USP7 knockout on SENP1 protein levels. Given that USP7 interacts with many proteins, a complete knockout could trigger compensatory or unintended pathways that indirectly influence SENP1 stability. A related limitation is the absence of siRNA-mediated USP7 knockdown experiments. While knockout models provide clear evidence of USP7 loss, siRNA-mediated USP7 knockdown would provide a complementary approach to assess SENP1 stability over a shorter time frame without potential long-term compensatory changes. Additionally, as mentioned earlier, an endogenous interaction was not detected between USP7 and SENP1, which may reflect a weak or transient nature of this interaction. It is also worth noting that the endogenous co-immunoprecipitation experiment was performed using an anti-USP7 antibody. Given the large number of USP7 interactors, SENP1 may be a relatively weak interactor. Therefore, future experiments could improve detection by increasing protein input or using an anti-SENP1 antibody for co-immunoprecipitation.

4.7 Conclusion

In conclusion, we have demonstrated that USP7 interacts with and deubiquitinates SENP1, thereby maintaining its protein stability within mammalian cells. Any changes in USP7 result in changes in SENP1 stability. When the concentration of USP7 is increased, the protein levels of SENP1 are also increased, whereas if USP7 is knocked out, SENP1 levels decrease, suggesting that USP7 regulates its stability within cells. Interestingly, we found domain-specific effects of USP7 on SENP1, where the N- and C-terminal domains result in contrasting effects. The N-terminal domain reduces the abundance of SENP1 while the C-terminal domain increases it. Given that SENP1 expression is upregulated in many cancer types, including breast and lung cancer and that its stability is regulated by USP7, provides a new way to target and limit this

interaction. Together, these findings not only deepen our understanding of USP7 but also highlight new avenues for therapeutically modulating its effects on SENP1.

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