USP7: structure, substrate specificity, and inhibition

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ABSTRACT

Turnover of cellular proteins is regulated by Ubiquitin Proteasome System (UPS). Components of this pathway, including the proteasome, ubiquitinating enzymes and deubiquitinating enzymes, are highly specialized and tightly regulated. In this mini-review we focus on the de-ubiquitinating enzyme USP7, and summarize latest advances in understanding its structure, substrate specificity and relevance to human cancers. There is increasing interest in UPS components as targets for cancer therapy and here we also overview the recent progress in the development of small molecule inhibitors that target USP7.

1. Introduction

Living cells constantly synthetize proteins, and dispose proteins that are misfolded, aggregated and no longer needed. Maintenance of proper protein homeostasis is essential for cell growth and survival. In eukaryotes, protein degradation is carried out by the ubiquitin–proteasome system (UPS). In this multi-step pathway, a polyubiquitin chain is conjugated to a protein substrate and serves as a signal for the substrate recognition and degradation by the 26S proteasome (1). Protein ubiquitination can be reversed by action of deubiquitinating enzymes (DUBs) (2) that remove ubiquitin moieties from their substrates (**Figure 1**).

Human ubiquitin-specific protease 7 (USP7) also known as Herpes virus associated protease (HAUSP) is a cysteine peptidase that belongs to the largest USP family of DUBs (**Figure 2**) (3). Located primarily in the nucleus, USP7 regulates the stability of multiple proteins involved in diverse cellular processes including DNA damage response, transcription, epigenetic control of gene expression, immune response, and viral infection (**Table 1**). USP7 has been extensively studied for its ability to regulate the cellular level of tumor suppressor p53 affected in the majority of solid tumors (4-7). USP7 knockout was shown to be lethal in mice (8,9). However, several children have been recently identified carrying USP7 mutations and deletions. The 46 individuals identified so far suffer from neurodevelopmental disorders such as autism spectrum disorder, intellectual disability, and speech/motor impairments (10) (www.usp7.org).

In this review, we discuss recent advances in understanding of the USP7 function, structure, regulation and its pharmaceutical relevance.

2. USP7 and Cancer

Given involvement of USP7 in multiple cellular pathways, it is not surprising that its expression is often dysregulated in human malignancies. USP7 overexpression contributes to tumor progression through changes in DNA damage response, apoptosis and cell cycle control. In particular, USP7 is upregulated in chronic lymphocytic leukemia (11) and its overexpression in human prostate cancer correlates with the tumor aggressiveness (12). USP7 expression level was shown to gradually increase with the tumor progression from grade I to grade IV in glioma

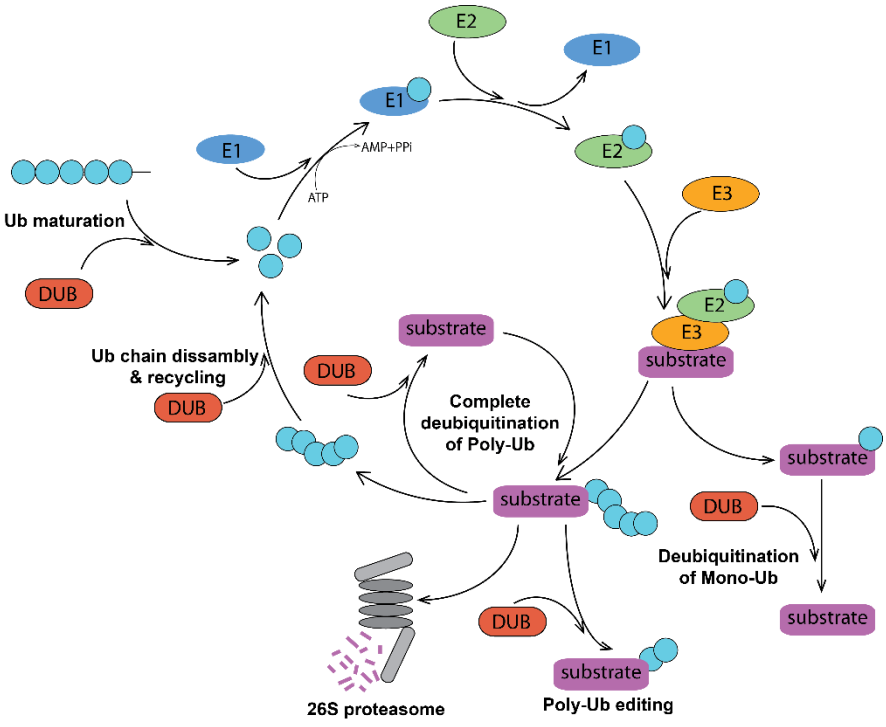


Figure 1. Ubiquitin-Proteasome System and DUBs.

Mono- and polyubiquitination of a protein substrate is catalyzed by consecutive action of E1, E2 and E3 ubiquitinating enzymes. The K48-linked polyubiquitin tag targets the substrate for proteasomal degradation, while mono-ubiquitination and other ubiquitin linkages result in a different functional outcome. DUBs deubiquitinate both poly- and monoubiquitinated proteins and thus change their fate. DUBs can edit polyubiquitin chains architecture and recycle ubiquitin. They also participate in maturation of the free ubiquitin.

***Ub** – ubiquitin; **E1** – ubiquitin activating enzyme; **E2** – ubiquitin conjugating enzyme; **E3** – ubiquitin Ligase; **DUB** – deubiquitinating enzyme.*

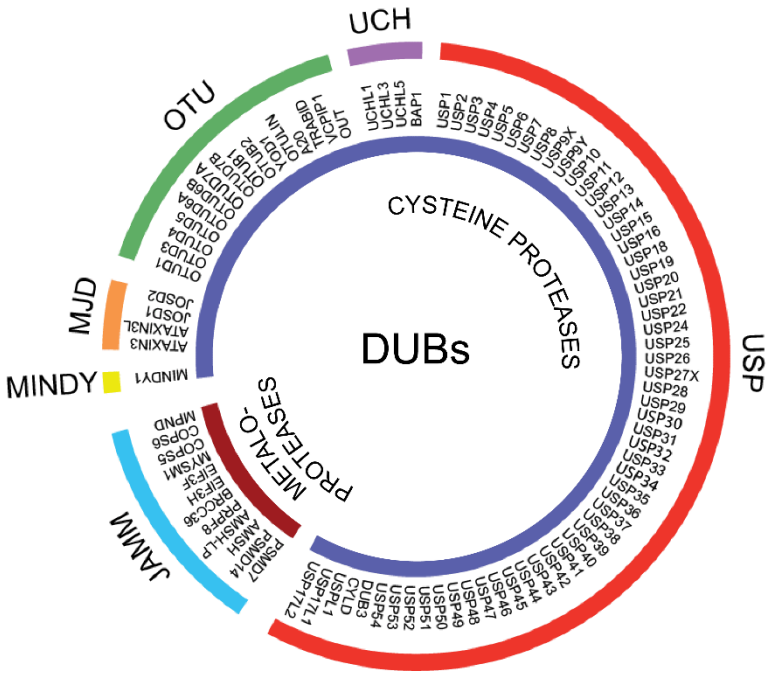


Figure 2. Human DUBs.

Human DUBs consist of two classes of enzymes: cysteine proteases and metalloproteases. Cysteine proteases are further subdivided into five families, including ubiquitin-specific proteases (USPs), ubiquitin carboxyl-terminal hydrolases (UCHs), ovarian tumor proteases (OTUs), Machado-Joseph (Josephin) domain containing (MJD) proteases, and MINDY (motif interacting with Ub-containing novel DUB family). The JAB1/MPN/MOV34 domain containing proteases (JAMMs) family is the only representative of the metalloproteases class of DUBs. Adapted from (2).

patients (13). The enzyme is also overexpressed in breast carcinomas (14), as well as in lung squamous cell carcinoma and large cell carcinoma (15). Its dysregulation in non-small cell lung cancers leads to disruption of the HDM2–p53 axis and is associated with induced cell epithelial mesenchymal transition (EMT), metastasis and overall poor prognosis (15). Similarly, USP7 overexpression in patients with epithelial ovarian cancer was found to induce cell invasion and correlate with poor survival (16,17). Because of the USP7 aberrant expression in many human cancers and its role in important cell signaling pathways, this enzyme has emerged as a promising target for cancer therapy.

3. Cellular Function of USP7

3.1. *USP7 in DNA damage response*

USP7 controls cellular levels of several key proteins involved in DNA damage response. The enzyme has been extensively studied due to its ability to regulate the level of tumor suppressor p53, a central regulator of the cell fate during DNA damage response. Interestingly, USP7 can deubiquitinate both p53 and its negative regulator HDM2, an E3 ubiquitin ligase responsible for polyubiquitination and subsequent degradation of p53 (18-20). Under normal conditions, USP7 preferentially interacts with HDM2 and prevents its degradation (4). In turn, HDM2 ubiquitinates p53, which results in low cellular levels of p53 during normal homeostasis. DNA damage causes ATM-dependent dephosphorylation of USP7 by PPM1G that decreases the affinity of the enzyme towards HDM2 (21). This allows USP7 to associate with p53, protecting it from ubiquitination. This interaction results in p53 stabilization and initiation of the p53-dependent DNA damage response.

USP7 is involved in regulation of the nucleotide excision repair (NER) pathway. Specifically, it prevents degradation of the XPC protein that recognizes helix-distorting DNA lesions and initiates the repair (22). USP7 stabilizes both RNF168 and RNF169 that control access of DNA damage response proteins to chromatin at the sites of double stranded DNA breaks (23,24). USP7 also deubiquitinates the cell cycle checkpoint CDC25A upon DNA damage in the BRCA-deficient cells and contributes to cell survival despite the loss of BRCA-mediated genome integrity (25).

USP7 plays a significant role in the ATR-Chk1 branch of DNA damage response where it deubiquitinates Chk1, an important checkpoint kinase (26), and an adapter protein claspin (27). Another important substrate of USP7 is UVSSA, one of initiating factors of the UV-induced

transcription-coupled DNA repair (28). Additionally, in response to genotoxic stress, USP7 stabilizes HLTF, which regulates error-free bypass replication over DNA lesions (29).

Furthermore, USP7 is involved in DNA damage tolerance through controlling the stability of the E3 ubiquitin ligase Rad18 that monoubiquitinates DNA sliding clamp PCNA in response to replication forks stalled at DNA lesions (30). PCNA monoubiquitination facilitates the recruitment of specialized translesion synthesis (TLS) DNA polymerases, which can bypass DNA lesions in an error-prone manner, thereby allowing replication to proceed (31). The stability of at least one TLS DNA polymerase, polη, is also controlled by USP7 (32).

3.2. USP7 in DNA Replication

In addition to DNA damage repair, USP7 plays an important role in bulk DNA replication by stabilizing several key proteins in this process. For example, USP7 was shown to be a part of the minichromosome maintenance (MCM) complex, which is loaded onto chromatin following mitosis and remains on chromatin until the completion of DNA synthesis. USP7 is required for efficient unloading of the MCM complex from chromatin at the end of S-phase (33). Recently, USP7 was shown to be enriched at the replication forks, where it counteracts ubiquitination of SUMO and SUMOylated proteins and, thus, creates SUMO-rich environment at sites of DNA replication, which promotes replication fork progression (34,35). USP7 also stabilizes Geminin, an inhibitor protein involved in prevention of DNA re-replication (36).

3.3. USP7 in Epigenetics

USP7 is known to associate with chromatin and participates in epigenetic control of gene expression. It stabilizes multiple proteins responsible for maintenance of epigenetic modifications of chromatin, including "readers" and "writers" of epigenetic modifications of DNA and histones. For example, tissue-specific DNA methylation patterns are maintained by replisome components UHRF1 and methyltransferase DNMT1. Both proteins are the substrates of USP7 (37,38). Monoubiquitination of histones is another common epigenetic modification that control gene expression. USP7 can modulate ubiquitination status of histones. It associates with and stabilizes the components of the Polycomb Repressive Complexes 2 and 1 (PRC2, PRC1), resulting in monoubiquitination of histone H2A(K119), which serves as a marker for gene repression (39-41).

USP7 stabilizes several other histone-associated proteins including acetyltransferase TIP60 (42), demethylase PHF8 (14), methyltransferase MLL5 (43), lysine-specific demethylase 1 (LSD1/KDM1) (44), and directly deubiquitinates histone H3 (45).

Although USP7 is mostly known for its role in substrate stabilization, it has multiple other cellular functions. For example, deubiquitination of monoubiquitinated transcription factors PTEN and FOX(O)4 (12,46) by USP7 serves to negatively regulate transcription activity of these proteins. Deubiquitination of both PTEN and FOX(O)4 causes their translocation from the nucleus to cytoplasm leading to their inactivation. In addition, USP7 was shown to remove Lys-63-linked polyubiquitin chain from the SIRT7 histone deacetylase, which leads to inhibition of its activity.

3.4. USP7 in viral infections

In addition to numerous cellular processes, USP7 is known to play a role in viral infection. Originally, USP7 was discovered as an enzyme associated with ICP0, an immediate-early protein from Herpes Simplex Virus-1 (47). ICP0 is an E3 ubiquitin ligase required for efficient lytic infection (48). Association with USP7 prevents auto-ubiquitination and proteasomal degradation of ICP0 (49). Later studies revealed that USP7 interacts with proteins from three other viruses of the *Herpesviridae* family. Thus, binding of USP7 to LANA from Kaposi's sarcoma-associated herpes virus (KSHV) and its functional homologue EBNA-1 from Epstein-Barr virus (EBV) has regulatory effect on latent viral replication (50,51). Furthermore, EBNA-1–USP7 interaction prevents the binding of USP7 to p53 and thereby diminishes p53 stabilization (52). Similar viral mechanism that disrupts the p53-mediated antiviral response was proposed for USP7 substrates vIRF1 and vIRF4 (viral interferon regulatory factors 1 and 4) from KSHV (53,54), while the significance of interaction between USP7 and UL35 from human cytomegalovirus (HCMV) remains elusive (55). Finally, besides its role in infections mediated by

Herpesviruses, USP7 also promotes adenoviral replication *via* interaction with viral multifunctional protein E1B-55K (56) and enhances HIV viral production by deubiquitinating its Tat protein (57).

4. Regulation of USP7 in the cell

USP7 is an important component of UPS and its activity in the cell is tightly regulated to avoid uncontrolled stabilization of its multiple substrates. There are several levels of USP7 regulation including intramolecular mechanisms, post-transcriptional modifications, and protein–protein interactions. Intramolecular mechanisms include domain reorganization required for the enzyme activation and active site rearrangement (58-64). Post-transcriptional modifications can further tune the activity of USP7. In particular, the enzyme was shown to be phosphorylated at Ser18, Ser963, and ubiquitinated at Lys869 (65). Phosphorylation at Ser18 by the protein kinase CK2 alters affinity of USP7 towards its substrates HDM2 and p53 in a way that the phosphorylated enzyme preferably binds to HDM2, while its dephosphorylation results in the higher affinity to p53 (21). USP7 ubiquitination at Lys869 by E3 ligase TRIM27 promotes the TNF-α-induced apoptosis through deubiquitination of RIPK1 (66) and the role of phosphorylation at Ser963 remains to be determined. In addition, USP7 is aberrantly phosphorylated at Tyr243 by chimeric protein p210 BCR-ABL in chronic myeloid leukemia (CML) cells. This post-translational modification enhances deubiquitinase activity of the enzyme towards the tumor suppressor PTEN whose dysregulation is linked to CML pathogenesis (67). Efficient USP7 regulation is mediated by several proteins that interact with the enzyme and mediate its stability and/or activity. Thus, Trip12 was recently identified as an E3 ligase for USP7 ubiquitination (68). In the absence of DNA damage, the DAXX protein binds to USP7 and HDM2, facilitating the HDM2 deubiquitination (69). DAXX–USP7 complex also regulates stability of the E3 ligase CHFR and Aurora-A kinase involved in mitosis (70). In the Wnt/β-catenin signaling pathway, the USP7 mediated stabilization of β-catenin depends on the E3 ligase RNF220 that forms a ternary complex with both USP7 and β-catenin and promotes deubiquitination of the latter (71). Another protein, MBD4, recruits USP7 to heterochromatin foci where it co-localizes with UHRF1 and DNMT1 promoting their interaction (72). Finally, at least one protein, TRAF4, negatively regulates USP7 activity. It binds to the same region of USP7 as p53 blocking p53 accesses to the enzyme. This inhibition mechanism was shown to play an important role in breast cancers where TRAF4 overexpression prevents USP7-mediated p53 stabilization and diminishes cytotoxic stress response (73).

5. Structure of USP7

Schematic representation of the USP7 domain architecture is shown in **Figure 3B**. USP7 is a 135 kDa protein that consists of seven domains, including the N-terminal TRAF-like (Tumor necrosis factor Receptor–Associated Factor) domain, followed by the catalytic core domain and the five C-terminal ubiquitin-like domains, UBL1-5, (Figure 3B) (50,59). The TRAF-like and the UBL domains recognize various USP7 substrates (4,7,33,52-54,74,75), and the regulatory C-terminus of the protein is thought to be crucial for enhancement of the USP7 catalytic activity (5,59,64). The domains of this dynamic DUB are connected by linkers that allow for flexibility of interdomain arrangement (**Figure 3B** and **Supplementary Movie 1**). Such flexibility is likely important for regulation of the enzyme's activity (59,63,64,76).

5.1. Substrate recognition by the TRAF-like domain

The N-terminal TRAF-like domain spans residues 53-205 and has a characteristic eight-stranded, antiparallel β-sandwich fold (52) (purple in **Figure 3B**, **D**). The function of TRAF-like domain is to bind USP7 substrates. Tumor suppressor p53 is the first characterized substrate of the TRAF-like domain (58) and later studies revealed many others (**Table 2**) (4,7,33,52-54,74,75). All these substrates share common P/AxxS motif that is recognized by the same shallow grove on the surface of the TRAF-like domain, in which the USP7 residues D164 and W165 anchor the conserved motif of a substrate (**Figure 3D)** (4,7,33,75). A novel ExxS motif that binds to the same site was recently reported (54). This common mode of interaction suggests that the substrates compete for USP7 binding. Such competition between the tumor suppressor p53 and

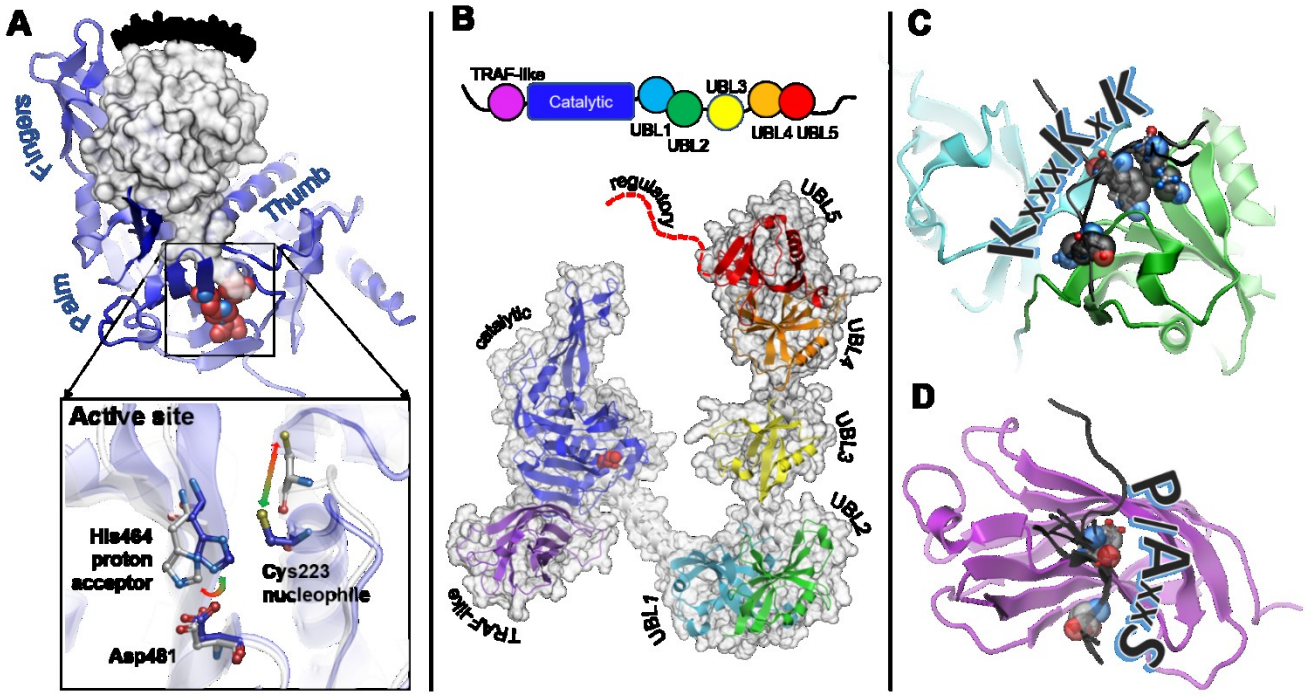


Figure 3. Structural features of USP7.

A. *Structure of the catalytic core of USP7 (blue) has a overall right-hand shape formed by Palm, Thumb and Fingers regions. The Fingers grasp ubiquitin molecule, shown as a gray surface. The C-terminal tail of ubiquitin enters the narrow catalytic cleft and extends towards the catalytic triad (shown as red spheres). The inset shows conformational rearrangements of the active site switching between "active" (blue, PDB 1NBF) and "inactive" (grey, PDB 1NB8) conformations.* ***B.*** *Schematic representation of the protein domain organization is shown on top. A structural model of the full length USP7 assembled based on overlapping structures of USP7 fragments including PDB IDs 2F1Z, 5FWI and 2YLM. The individual domains are labeled and color-coded: TRAF-like (purple), catalytic (blue), UBL1 (cyan), UBL2 (green), UBL3 (yellow), UBL4 (orange), UBL5 (red), the disordered extreme C-terminus is shown as red dotted line.* ***C.*** *Structure of UBL12 domains (cyan and green, respectively) in complex with its known substrates (black) including DNMT1 (PDB 4YOC), ICP0 (PDB 4WPH) and UHRF1 (PDB 5C6D), The lysine residues of the shared KxxxKxK motif are shown as spheres. The ICP0 peptide has a reversed RxKxxxK motif and binds in the opposite direction.* ***D.*** *Structure of the TRAF-like domain in complex with its known substrates including p53 (PBD 2F1X), HDM2 (PDB 2FOP), HDMX (PDB 3MQR), UbE2E1 (PDB 4JJQ), MCM-BP (PDB 4KG9), vIRF1 (PDB 4YSI), vIRF4 (PDB 2XXN) and EBNA1 (PDB 1YY6). The TRAF-like domain is shown as purple ribbon, and the substrate peptides are black. The P/AxxS motif shows the direction of the peptides. The conserved P/A and S substrate residues are shown as spheres.*

its E3 ubiquitin ligase HDM2, for example, serves as a switch to fine-tune the level of p53 in a cell (4).

5.2. Unique structure of the USP7 catalytic core

The function of the DUB catalytic core domain is to bind ubiquitin and cleave the isopeptide bond between ubiquitin and a substrate. The USP7 catalytic core is the largest domain within the protein (residues 208-560) centrally located between the TRAF-like and UBL domains. It adopts characteristic for all USP enzymes fold resembling the right hand. Residues C223, H464 and D481 form the catalytic triad of the enzyme that are located in a cleft between the two protein regions referred to as Thumb and Palm. The third

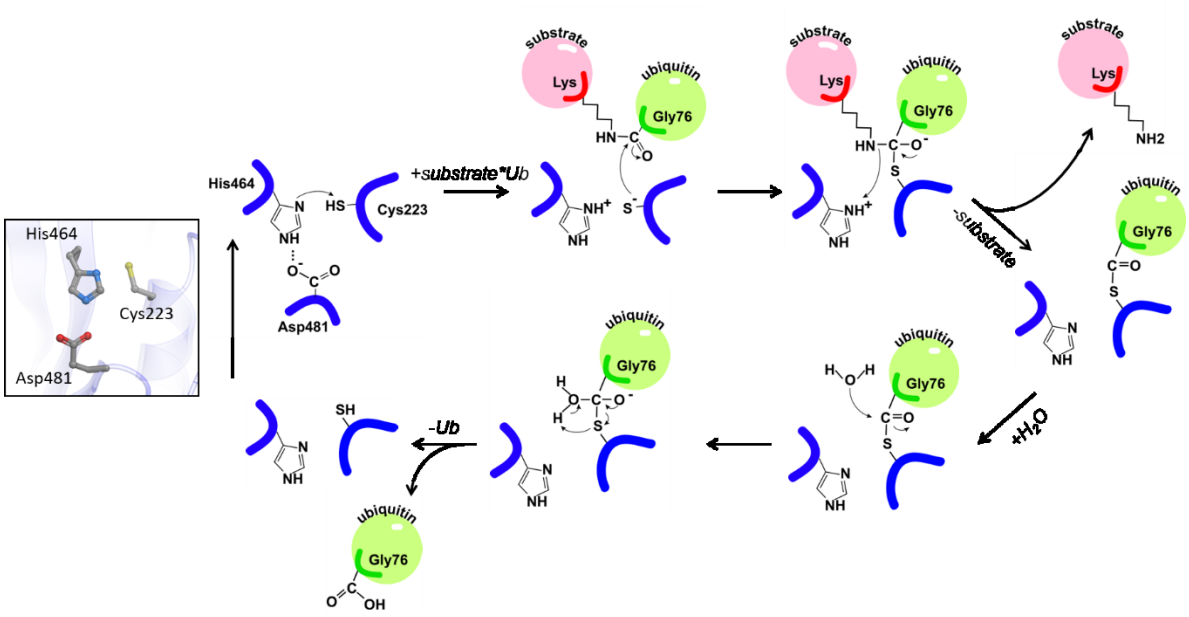


Figure 4. Chemical reaction mechanism of catalysis by USP7.

The catalytic triad of USP7 is shown on the left. Cleavage of ubiquitinated substrate is shown on the right. The substrate and ubiquitin are shown in pink and green, respectively, and labeled. The nucleophilic attack of the isopeptide bond is carried out by C223 of the catalytic triad. H464 serves as a proton acceptor and D481 restricts the side-chain rotation of the histidine.

region, Fingers, is responsible for binding to ubiquitin (58,77) (**Figures 3A, 3B**).

The catalytic mechanism of USP7 and other cysteine proteases is similar to that of well-studied plant enzymes papains (78) (**Figure 4**). The role of the catalytic histidine is to deprotonate the thiol group of the cysteine and initiate its nucleophilic attack on the isopeptide bond, while the aspartate restricts the side-chain rotation of the histidine leading to its polarization. Remarkably, active site conformation of the isolated USP7 catalytic domain differs from those observed in the available structures of other USPs. While in USP4, USP8, USP14 and CYLD the catalytic residues are properly positioned for catalysis (79-82), the USP7 catalytic triad is found in an unproductive conformation (**Figure 3A inset** and **Supplementary Movie 1**). The catalytically competent active site conformation was captured in the crystal structure of USP7 in complex with ubiquitin covalently attached to the catalytic cysteine (58,64). Based on the conformational changes seen in crystal structures, it was postulated that ubiquitin binding triggers activation of the enzyme (**Figure 3A, inset**).

Remarkably, recent NMR studies revealed that structural realignment into productive conformation is nit induced by interaction with free ubiquitin in solution. Although ubiquitin binds to the Fingers region of USP7 (K_D of 106 ± 16 μM), its C-terminal tail does not enter the catalytic cleft and does not affect the active site conformation (83). These results suggest that the substrate-conjugated rather than free ubiquitin is necessary to activate USP7. This is not surprising considering that the C-terminus of free ubiquitin is negatively charged and unlikely to enter the hydrophobic catalytic cleft, while the conjugated ubiquitin lacks such negative charge. Upon the cleavage of ubiquitin moiety from the substrate the negative charge is restored, thus, causing free ubiquitin to leave the active site of the enzyme. Furthermore, interaction with other domains (64) and a substrate (59,60) may also be necessary to achieve the full activation of the enzyme.

5.3. USP7 specificity for polyubiquitin chain type:

Ubiquitin contains seven lysine residues, including K6, K11, K27, K29, K33, K48, and K63, and all of them can be utilized during polyubiquitin

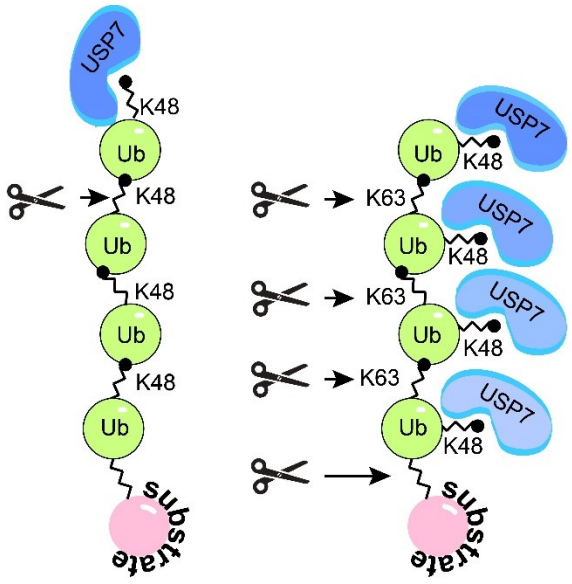


Figure 5. USP7 polyubiquitin cleavage specificity.

USP7 preferentialy binds free K48 side chains, which directs USP7 to the distal ubiquitin subunit of K48-polyubiquitin and promotes sequential exo-cleavage of K48-linked poly-ubiquitin. Whereas USP7 can bind all subunits of K63-polyubiquitin and promotes exo-, endo-, and base-cleavage. Adapted from (85).

chain formation, thus, determining the type of a polyubiquitin chain formed. USP7 deubiquitinates both mono- and poly-ubiquitinated substrates. Similar to other members of the USP family, USP7 is fairly promiscuous towards the type of poly-ubiquitin chains. While it cannot cleave the linear M1-linked poly-ubiquitin, USP7 cleaves K6, K11, K33, K48, and K63-linked chains connected by iso-peptide bonds, and less efficiently K27 and K29 chains (84). However, a recent study (85) revealed a marked difference between the way USP7 cleaves the two most common linkage types, a degrative K48 and a non-degrative K63 (**Figure 5**). The study suggests that USP7 requires free K48 ubiquitin side chain for the efficient binding, which means that the K48-linked chains can only be de-polymerized by one ubiquitin moiety at a time starting from the most distal ubiquitin in a chain (exo-cleavage), while the K63-linked chains can be cleaved between any two consecutive ubiquitin moieties (exo- and endo-cleavage) as well as between the substrate and the proximal ubiquitin (base-cleavage). This preference results in significantly faster depolymerization of K63- compared to K48-linked ubiquitin chains.

5.4. Five UBL domains act as an additional substrate-binding platform

The C-terminal region of USP7 (C-USP7) spans residues 564-1102. Its five UBL domains are separated from the catalytic core by a 39 Å long α-helix. The extended nature of this rigid "connector" helix is important for the USP7 activity (62). The UBL domains are arranged in the following manner: UBL1 closely interacts with UBL2, UBL4 shares the extensive interface with UBL5, and UBL3 is separated from UBL2 and UBL4 by flexible linkers (**Figure 3B**) (59,76,86). UBL domains are not unique to USP7; 18 other DUBs of the USP family also contain UBLs (87). Interestingly, the number of the domains varies from one in USP9X/Y, USP19, USP31 and several others to five in USP7, USP40 and USP47. Position of the UBLs within different USPs also varies, with some domains located at either N- or C-terminus, and others embedded in the catalytic core. Although such divergence within the USP protein family is surprising, the UBL domains appear to share a common regulatory function. They regulate catalytic activity of USPs by either inhibiting, or enhancing the enzyme's activity, or localizing it to proteasome (79,88-90).

UBLs of USP7 can serve as a substrate-binding platform. Interestingly, despite the overall structural similarity, sequences of USP7 UBLs significantly vary from that of ubiquitin and each other resulting in unique surface charge distributions for each domain. The presence of differently charged surfaces implies that several distinct protein interaction sites may be harbored within the C-USP7. Mounting biochemical evidence suggests that different USP7 substrates are recognized by distinct UBL domains in its C-terminal region. Thus, UBL12 binds human XPC, RNF168, RNF169, DNMT1, UHRF1 and viral ICP0 (22-24,50,63,76,86,91), UBL3 contains secondary binding site for HDM2 (5), and UBL45 was shown to recognize p53 (5) and FOX(O)4 transcription factors (46). The distinct substrate-specific binding sites in the C-terminus on USP7 provide attractive targets for potential therapeutic intervention by manipulating USP7 activity toward a specific substrate or a group of substrates.

Recent structural studies of the USP7 UBL domains uncovered a mechanism of substrate recognition by UBL12 (24,63,76,86,91,92) (**Table 2**). A viral E3 ubiquitin ligase ICP0 from Herpes

Simplex Virus 1 was identified as the first USP7 substrate that binds exclusively to its UBL domains. Several structures of UBLs in complex with ICP0 has now been reported (63,86,92) and revealed that the positively charged region of ICP0 interacts with the negatively charged patch on UBL2 surface. Furthermore, solution NMR studies showed that the ICP0-binding stabilizes the interface between UBL1 and UBL2 domains (92). In addition, crystal structures of the UBL domains in complexes with UHRF1, DNMT1 and RNF169 were recently published (24,76,91). Interestingly, structural analysis showed that all three proteins recognize UBL12 in a manner similar to ICP0, but in reverse orientation (**Figure 3C**). Specifically, these proteins share a positively charged KxxxKxK motif in their sequence that binds to the same negatively charged surface of UBL2 as ICP0, which has a RxKxxxK motif (61,63). The presence of such motif in a USP7 substrate can now be used to predict its interaction with the UBL12 tandem in a way P/A/ExxS motif has been used to predict binding to the N-terminal TRAF-like domain.

5.5. Auto-regulation of USP7activity by its C-terminus

A conserved disordered peptide (residues 1080-1102) at the extreme C-terminus of USP7 is required for the enzyme's activation. Its deletion significantly decreases the deubiquitinase activity of the DUB (5,59). The molecular mechanism of activation, however, remains unknown and raised controversy in the literature. According to the crystal structure of the UBL1-5 tandem, UBL domains are found in the extended conformation (**Figure 3B**), which positions the extreme C-terminus about 80 Å away from the enzyme's active site. Furthermore, the isolated USP7 C-terminal peptide fails to directly interact with the catalytic core and only partially restores the enzymatic activity *in trans* (59). On the other hand, the catalytic domain of USP7 fused to the C-terminal peptide *via* a 10 amino residue linker has deubiquitinase activity comparable to that of the full-length protein (64). A crystal structure of the fusion complex engaged with ubiquitin offered a potential molecular mechanism for such stabilization. It shows that ubiquitin trapped in the active site of USP7 causes rearrangements of the catalytic triad and the adjacent "switching" loop of USP7. The conformational change in the "switching" loop, in turn, creates a binding site for the regulatory peptide, resulting in stabilization of catalytically competent enzyme (64).

The apparent discrepancy between the current structural models of the full-length USP7, which places the C-terminus 30-80 Å away from the catalytic site, and evidence of direct interaction between the catalytic and C-terminal domains suggests that a major conformational change is taking place during protein activation (**Supplementary Movie 2**). Flexible inter-domain linkers of USP7 may provide the means for domain rearrangement (62,63,86). Therefore, structural characterization of the USP7 conformations and dynamic exchange between them is necessary to fully understand the mechanism of its action.

6. Inhibition of USP7

Manipulating stability of proteins that are mutated, overexpressed or downregulated in human malignancies represents a promising therapeutic strategy for cancer treatment. Between the two protein degradation pathways, lysosomal proteolysis and UPS, the latter is highly selective and, therefore, its inhibition provides a strategy for the development of highly specific targeted therapies (93). E3 ligases and DUBs are of special interest since they determine the selectivity of UPS.

USP7 is a promising pharmaceutical target because of (i) its role in cellular pathways involving regulators of DNA damage, oncogenes and tumor suppressors and (ii) growing evidence of its aberrant expression in various cancer cell lines. Despite several USP7 inhibitors have been reported in the literature (94,95), the lack of co-crystal structures of USP7 with small-molecule compounds until recently has been a limiting factor in the development of potent and selective USP7 inhibitors. In the past year, several groups reported structures of USP7 in complexes with small molecule inhibitors (**Figure 6**, **Supplementary Table 1**) (83,85,96-98) , which triggered a rapid structure-based design of a number of potent and highly specific analogues (96-100).

Solution NMR and mass spectrometry studies of interactions between the USP7 catalytic domain and P22077 (101) and P50429 (102) inhibitors uncovered the molecular mechanism of action of these thiophene-based compounds (**Figure 6**, green, and **Supplementary Table 1**). Both inhibitors bind to the active site of USP7 and

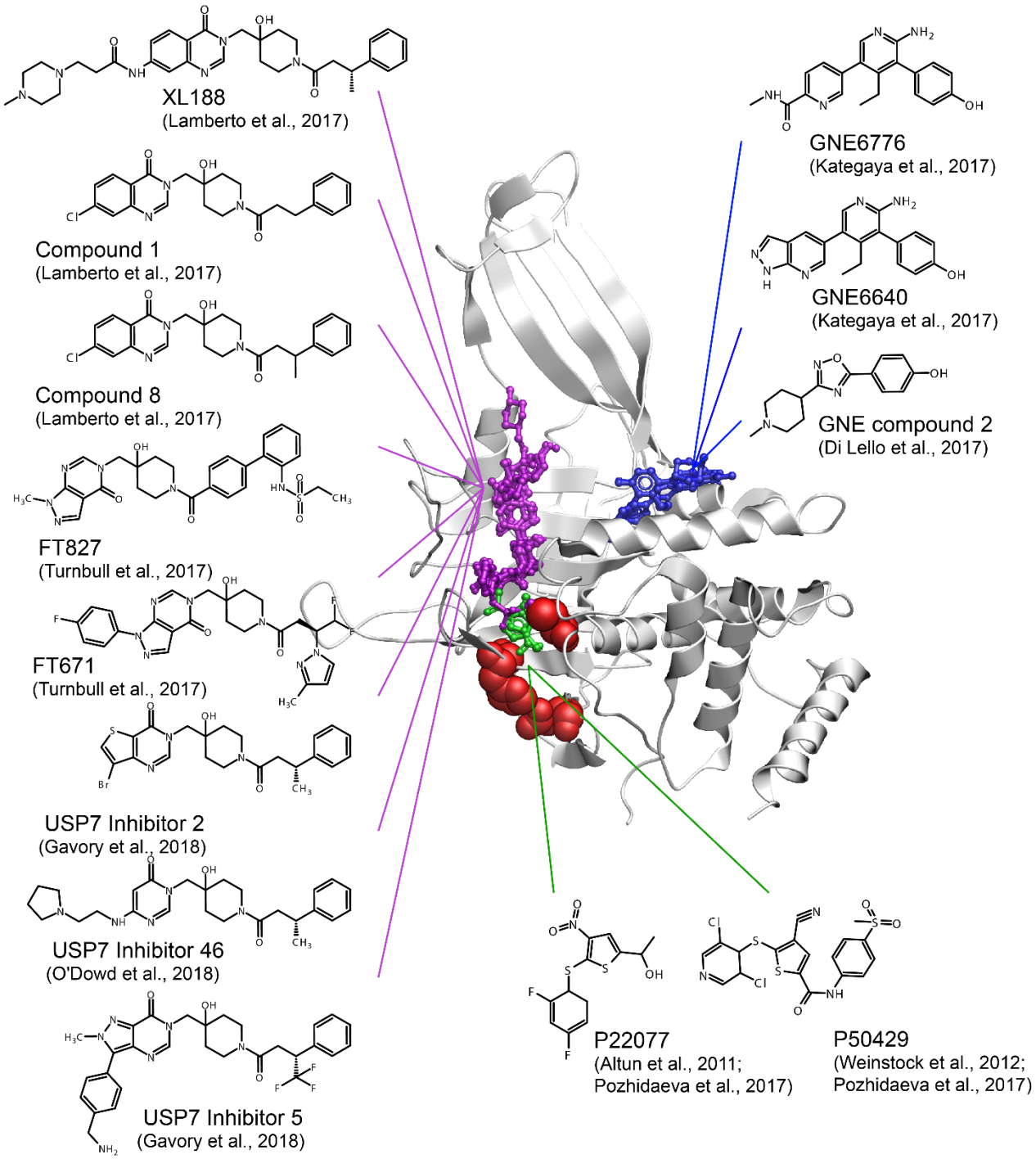


Figure 6. Small molecule inhibitors of USP7.

USP7 inhibitor complexes are overlayed and colored according to the region of the catalytic domain they target. Residues of the catalytic triad is shown as red spheres. The compounds that bind to the catalytic cleft are shown in purple, alosteric inhibitors that bind outside of the catalytic cleft are shown in blue, and those directly targeting the catalytic triad are shown in green. FT827 targers the catalytic cleft and extends to the catalytic triad. P22077, P50429 and FT827 covalently modify catalytic cystein C223. PDB 5UQX was used to display USP7 catalytic domain.

covalently and irreversibly modify the catalytic cysteine C223 with remarkable specificity (83).

Co-crystal structures of several pyrimidinone based USP7 inhibitors were reported by several independent groups (96-99) and shown to bind the same narrow and long grove of the catalytic site normally occupied by the C-terminal tail of ubiquitin (**Figure 6**, purple). One of the inhibitors, FT827, carries vinylsulfonamide moiety, which reaches the catalytic triad and covalently modifies the catalytic cysteine. All other reported USP7 inhibitors are non-covalent.

Another class of allosteric inhibitors, GNE-6640, GNE-6776 (85) and USP7 inhibitor 2 (103), were shown to bind 12 Å away from the catalytic cysteine and impede ubiquitin binding (85) (**Figure 6**, blue). Using a different approach, Zhang *et al*. reported ubiquitin variants that selectively interact with USP7 blocking its interaction with ubiquitin (104). All these USP7 inhibitors exhibit cytotoxic activity in several cancer cell lines making them promising leads for further development and optimization that through rational structure-based design.

7. Concluding Remarks and Perspectives

USP7 plays an essential role in regulation of p53 pathway and, therefore, is a promising target for development of novel therapies for cancer treatment. Research efforts of recent years brought significant breakthroughs in our understanding of USP7 structure, regulation and inhibition. However, many questions remain to be answered. What is the structure of the full length enzyme and what conformations can it sample? What role does conformational dynamics play in USP7 activation and substrate recognition? How does USP7 achieve specificity to its many diverse substrates, and can it be pharmaceutically manipulated to change affinity to a particular substrate or a group of substrates?

Future studies of structure and dynamics of USP7 will undoubtedly provide detailed understanding of regulation of its activity and specificity, leading to development of new approaches to cancer treatment.

Table 1. USP7 substrates

Substrate name	*Reference*	*Substrate name*	*Reference*
Transcription factors		***Epigenetic control of gene expression***	
p53	(18)	RING1B	(39)
HDM2	(19)	Bmi1	(40)
Rb	(105)	Histone H2B	(106)
FOX(O)3	(46)	DNMT1	(107)
FOX(O)4	(46)	UHRF1	(37)
PTEN	(12)	RNF168	(23)
β-catenin	(71)	MLL5	(43)
PPARγ	(108)	Tip60	(42)
N-Myc	(109)	UbE2E1	(75)
DNA replication/Cell cycle control		PHF8	(14)
CHFR	(110)	***Telomere proteins***	
Bub3	(111)	TPP1	(112)
SUMO	(34)	***Immune response***	
DNA damage response/DNA repair		NFκB	(113)
DAXX	(114)	TRAF6	(115)
Polymerase η	(32)	IKKγ	(115)
HLTF	(29)	TRIM27	(116)
Rad18	(30)	***Viral proteins***	
Clapsin	(27)	EBNA1 from EBV	(117)
Chk1	(26)	ICP0 from HSV-1	(49)
UVSSA	(28)	vIRF1 from KSHV	(54)
Mule/ARF-BP1	(118)	vIRF4 from KSHV	(53)
XPC	(22)	LANA from KSHV	(51)
Epigenetic control of gene expression		UL35 from HCMV	(55)
MEL18	(40)	E1B from Adenovirus	(56)

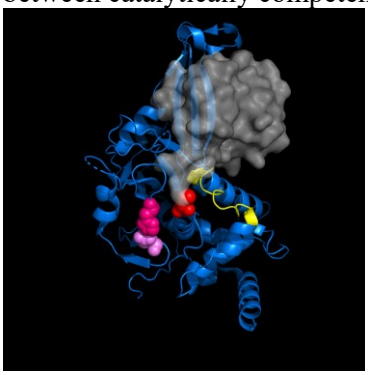
Table 2. Structures of USP7

pdb ID	*Ligand*	*Reference*	*pdb ID*	*Ligand*	*Reference*
TRAF-like domain			***Catalytic domain***		
2F1W	no	(4)	2F1Z	no	(4)
2F1Y	no	(4)	5J7T	no	(64)
1YZE	no	(52)	1NBF	Ubiquitin-aldehyde	(58)
2F1X	p53	(4)	5JTV	Ubiquitin-bromoethylamine	(64)
2FOO	p53	(7)	5JTJ	Ubiquitin-bromoethylamine	(64)
2FOJ	p53	(7)	***USP7 UBL domains***		
2FOP	HDM2	(7)	5J7T	no	(64)
3MQS	HDM2	(74)	5FWI	no	(62)
3MQR	HDMX	(74)	2YLM	no	(59)
4KG9	MCM-BP	(33)	4WPI	no	(63)
4JJQ	UbE2E1	(75)	4PYZ	no	unpublished
1YY6	EBNA1	(52)	4YOC	DNMT1	(76)
4YSI	vIRF1	(54)	4Z96	DNMT1	unpublished
2XXN	vIRF4	(53)	5C56	ICP0	(86)
Catalytic domain			4Z97	DNMT1(K1115Q)	unpublished
1NB8	no	(58)	4WPH	ICP0	(63)
4M5W	no	(77)	5GG4	RNF169	(24)
4M5X	no	(77)	5C6D	UHRF1	(91)

Supplementary Movie 1 caption:

Structural rearrangements of the catalytic core of USP7 during activation.

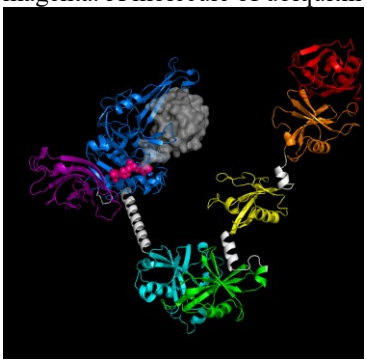
The catalytic domain is shown as a blue ribbon. The catalytic triad is shown in red and the "switching" loop is highlighted in yellow. Ubiquitin moiety is shown as a transparent surface. The molecule switches between catalytically competent (1NBF) and inactive (1NB8).



Supplementary Movie 2 caption:

A model of conformational changes associate with USP7 activation.

A structural model of the full length USP7 assembled based on overlapping structures of USP7 fragments including 2F1Z, 5FWI and 2YLM (inactive conformation) and 2F1Z, 1NBF, 5FWI and 4YOC (active conformation). The individual domains of USP7 are color-coded: TRAF-like (purple), catalytic (blue), UBL1 (cyan), UBL2 (green), UBL3 (yellow), UBL4 (orange), UBL5 (red). The linkers are shown in white. The catalytic triad is shown in magenta. A molecule of ubiquitin is shown as a transparent surface. The extreme C-terminal peptide is not shown.



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