HAUSP as a therapeutic target for hematopoietic tumors (Review)

KANG WOO CHEON and KWANG-HYUN BAEK

Graduate School of Life Science and Biotechnology, Cell and Gene Therapy Research Institute,
Pochon CHA University, CHA General Hospital, Seoul 135-081, Korea

Abstract. p53, one of the most important tumor suppressor proteins, plays an essential role in regulating the cell cycle and apoptosis by sensing the integrity of genome. Therefore, the level of p53 protein is critical for normal cellular homeostasis, and is known to be subtly regulated by ubiquitination and deubiquitination systems. Numerous genetic alterations of p53 have been reported in all types of tumors. In hematopoietic tumors, the mutations of *p53* gene are rare compared with solid tumors, which showed more than 50% frequency for *p53* mutations. According to this characteristic feature of hematological tumors, the therapeutic strategy for targeting the level of p53 may be valuable in anti-cancer treatment of hematological tumors. Herein, we deal with the posttranslational regulation of p53 via its specific ubiquitinating enzymes (Mdm2, Mdmx, COP1, Pirh2, ARF-BP1/Mule, and CHIP) and a deubiquitinating enzyme, herpesvirus-associated ubiquitin-specific protease (HAUSP). In this article, we review the regulatory mechanism of p53 via ubiquitination and deubiquitination system and suggest the several possible therapeutic strategies of targeting HAUSP, a deubiquitinating enzyme for p53, for treating hematopoietic tumors.

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Correspondence to: Dr Kwang-Hyun Baek, Cell and Gene Therapy Research Institute, Pochon CHA University, CHA General Hospital, 605 Yeoksam 1-Dong, Kangnam-Gu, Seoul 135-081, Korea
E-mail: baek@cha.ac.kr

1. The mechanism of the ubiquitination/deubiquitination system

In view of cell biology, the regulation of selective proteolysis is largely mediated by the ubiquitin and proteasome system. In eukaryotes, most proteins, including transcription factors, cell-cycle regulators, signal transducers, and misfolded proteins, are processed and degraded by this mechanism (1,2). Therefore, the regulation of the ubiquitination system plays a crucial role in pathological cellular physiology (3,4). This regulated proteolysis occurs in the 26S proteasome, which is composed by the association of the 20S proteasome with two particles of the PA700 proteasome activator (5,6). For 26S proteasomal proteolysis, polyubiquitination of target proteins is necessary (Fig. 1).

Ubiquitin is a well-conserved 76-amino acid protein and is covalently attached to the amino group of internal Lys residue of target proteins by an enzymatic cascade of ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), ubiquitin ligases (E3), and additional novel ubiquitination factors (E4), if necessary. In general, polyubiquitination is mediated by E1, E2, and E3 enzymes. However, recent reports demonstrated that the polyubiquitination of certain proteins essentially requires an additional ubiquitination factor, E4 (1,7,8). For example, the parkin E3 ligase can monoubiquitinate Pael-receptor and further polyubiquitination requires the U-box-type E4 enzyme, CHIP (the C-terminus of Hsc70-interacting protein), which was originally known as an E3 ligase for unfolded CFTR and glucocorticoid receptor (9). Another example of human E4 ligase is p300 (non-U-box-type E4), which is known to have several biological activities, including transcriptional cofactor and histone acetyltransferase (10,11). Following the monoubiquitination of target proteins, the second ubiquitin is attached to the Lys6, Lys11, Lys29, Lys48, or Lys63 amino acid residue of the first ubiquitin (12). This reaction can produce variable lengths of polyubiquitin chains, and the linkage type along with the length of chain may determine the fate of the target protein (13). Mono-ubiquitination and Lys63-linked polyubiquitination seem to have non-proteolytic roles, whereas Lys48-linked polyubiquitination plays a role in proteasomal degradation of target proteins (14).

The conjugation of ubiquitin to target proteins is reversible and the removal of ubiquitins is catalyzed by the deubiquitinating enzymes. Deubiquitinating enzymes hydrolyze polyubiquitin chains, resulting in rescue of target proteins from proteasomal degradation and release of free ubiquitins (15). In this ubiquitin-dependent proteolysis system, specific
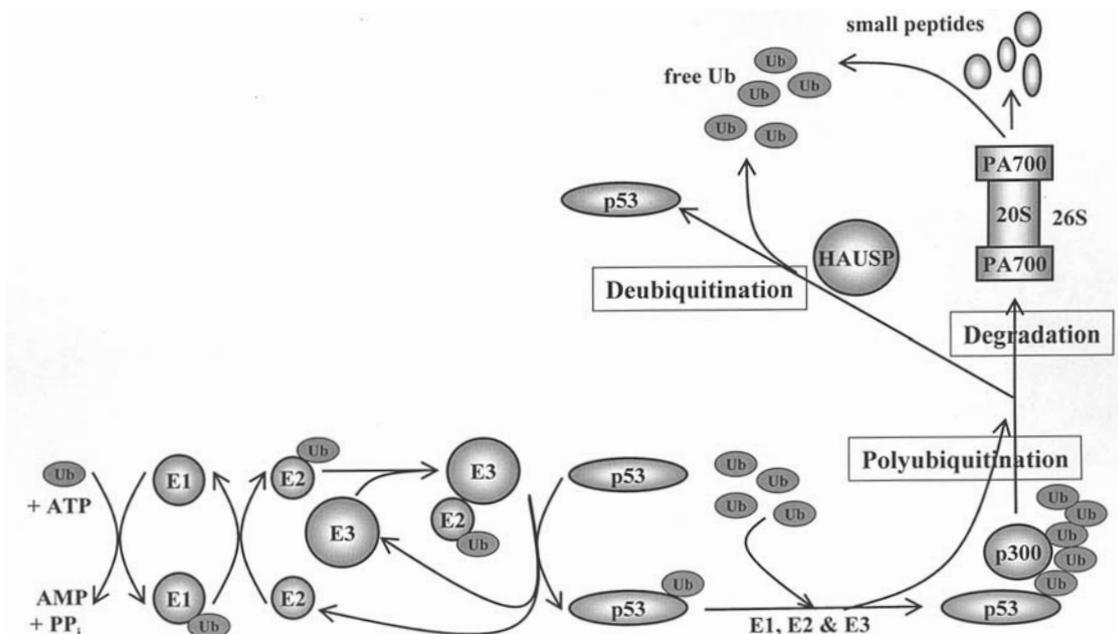
Figure 1. Schematic overview of the ubiquitination and deubiquitination system for p53. Monoubiquitination of p53 is mediated by E1, E2, and E3 enzymes with ubiquitin (Ub) and ATP. p300, an E4 enzyme, is known to be involved in polyubiquitination and the polyubiquitinated p53 is degraded by the 26S proteasome. When the Mdm2 E3 ligase is highly expressed, p53 is polyubiquitinated by Mdm2 without p300. When a deubiquitinating enzyme, HAUSP, rescues the polyubiquitinated p53 before degradation, p53 recovers its protein functions, and free ubiquitins are recycled. Mdm2, COP1, Pirh2, Mule, and CHIP are known to be E3 ligases for p53.

recognition of substrate is necessary to prevent indiscriminate removal of essential proteins. To our knowledge, this specificity is determined by the interactions among substrates and their respective E2, E3, E4, and deubiquitinating enzyme by specific protein-protein interactions (8,16). However, the precise mechanisms for these interactions are still undiscovered. The PEST motif has been known as a common feature of proteins that become ubiquitinated. This conserved hydrophilic motif is enriched in proline (P), glutamine (E), serine (S), and threonine (T) and is involved in the ubiquitination for rapid degradation of proteins (17). In addition, some of the substrates have to be post-translationally modified before they are recognized for ubiquitination. These post-translational modifications of substrates are commonly achieved by phosphorylation/dephosphorylation (16,18), hydroxylation (19), or displacement of masking factors (20).

2. The role of p53 in hematopoietic tumors

p53 is well known as a tumor suppressor which leads to cell cycle arrest, cell senescence or apoptosis upon stress signals including DNA damage and oncogenic conditions (21). For example, γ-irradiation activates the ATM (ataxia-telangiectasia) kinase and the CHK-2 kinase, both of which can phosphorylate p53. Phosphorylated p53 acts as a transcription factor for inducing the $p21^{KIP}$ family, which binds to the cyclin D and E complex and inhibits the release of E2Fs from the Rb:E2Fs complex (22). In addition, phosphorylated p53 induces the transcription of apoptosis-related genes, including *Bax*, *NoxA*, and *Fas*, which have the p53 binding site in their regulatory regions (23).

   More than 50% of solid tumors have loss of wild-type *p53* expression because of deletions or point mutations (24,25). A

previous report showed that out of 118 lung tumors, 57 (48.3%) had single (53 tumors) or double mutations (4 tumors) (25). Of these 57 tumors with *p53* mutations, 36 had missense mutations and 21 had null mutations that caused an aberrant-sized p53 protein (26). In contrast, hematopoietic tumors are less likely to have mutant *p53* in spite of their pathological diversity. In version R5 of the database for *p53* mutations at the International Agency for Research on Cancer, only 14% of 672 hematopoietic tumors have *p53* mutations (27). A recent meta-analysis demonstrated that only 11.9% of 4388 hematological tumors showed *p53* mutations (Table I). In addition, 1482 tumors of acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) showed only 10% of *p53* mutations. Chronic phase chronic myeloid leukemia (CP-CML) and Hodgkin's disease (HD) showed no *p53* mutation (124 and 13 tumors, respectively). 690 tumors of acute lymphoblastic leukemia (ALL) showed 5% of *p53* mutations and multiple myeloma (MM) showed 3% (1 of 37 tumors) of *p53* mutation. In contrast to the *p53* mutations, the altered level of *p53* expression was more frequent in hematopoietic tumors. Decreased expression of *p53* was 30.4% of 23 hematological tumors (AML/MDS). Moreover, hematopoietic tumors with *Rb* point mutations or deletions were found in only 7.7% of 388 hematological malignancies, but 27.8% of 777 tumors have decreased expression of Rb (22). Collectively, these reports suggest that the majority of hematopoietic tumors may have wild-type p53 and its downstream of signal transduction pathway.

3. Regulation of p53 by ubiquitination and deubiquitination

p53 is a short-lived protein and its amount is maintained at a low level in normal cellular conditions due to its effect on cell cycle arrest and apoptosis. One mechanism of regulating

Table I. A meta-analysis of alterations for *p53* and *Rb* genes in hematological tumors.

Gene	Type of alteration	Number of samples	Incidence (%)
p53	Mutation	524/4388	11.9
	Deletion or rearrangement	194/1378	14.1
	Decreased expression	7/23	30.4
Rb	Mutation	6/118	5.1
	Deletion or rearrangement	24/270	8.9
	Decreased expression	216/777	27.8

p53 is post-translational modification, including ubiquitination, phosphorylation, and acetylation (28). Key molecules involved in the process of p53 ubiquitination are E3 ligase Mdm2, and Mdmx (Fig. 2). It has been reported that Mdm2 has capacity for polyubiquitination and degradation of p53 when Mdm2 is highly expressed (29). Interestingly, p300 can also recognize monoubiquitinated p53 for ubiquitin chain elongation (11). It has been suggested that the Mdm2-mediated monoubiquitination of p53 results in nuclear export of p53, whereas poly-ubiquitination of p53 is crucial for degradation by the 26S

proteasome (30). Another important regulator of p53 function is Mdmx, a structural homologue of Mdm2 without E3 activity. Mdmx binds p53 and inhibits transcriptional activity by p53. Because Mdmx is a substrate for Mdm2-dependent ubiquitination, it can stabilize Mdm2 by the inhibition of self-ubiquitination. Therefore, in normal cells, Mdmx plays a role in stabilizing Mdm2 and inhibiting p53 activity, which regulates normal cellular growth against apoptosis (31). In addition to Mdm2, additional E3 ligases for p53, Pirh2, COP1, ARF-BP1/Mule, and CHIP, have been identified (32-35). ARF-BP1/Mule also has E3 ligase activity for Mcl-1 (36) and CHIP has previously known to possess both E3 activity for CFTR and glucocorticoid receptor and E4 activity for Pael-receptor (9). However, these additional E3 ligases for p53 have not been well studied yet. The precise cellular roles and regulation of COP1, Pirh2, ARF-BP1/Mule, and CHIP are not elucidated with regard to their cell- or tissue-specificity, and developmental stages. Therefore, the further investigation for synergistic interaction effects of these E3 ligases along with Mdm2 is required to disclose the precise regulatory mechanism of p53 ubiquitination.

p53 is ubiquitinated by Mdm2, resulting in the basal amount of p53 protein for preventing uncontrolled apoptosis. In normal status, Mdm2 binds to the transactivation domain of p53 to prevent its interaction with transcriptional machinery, and ubiquitinates p53 for degradation by the 26S proteasome (37).
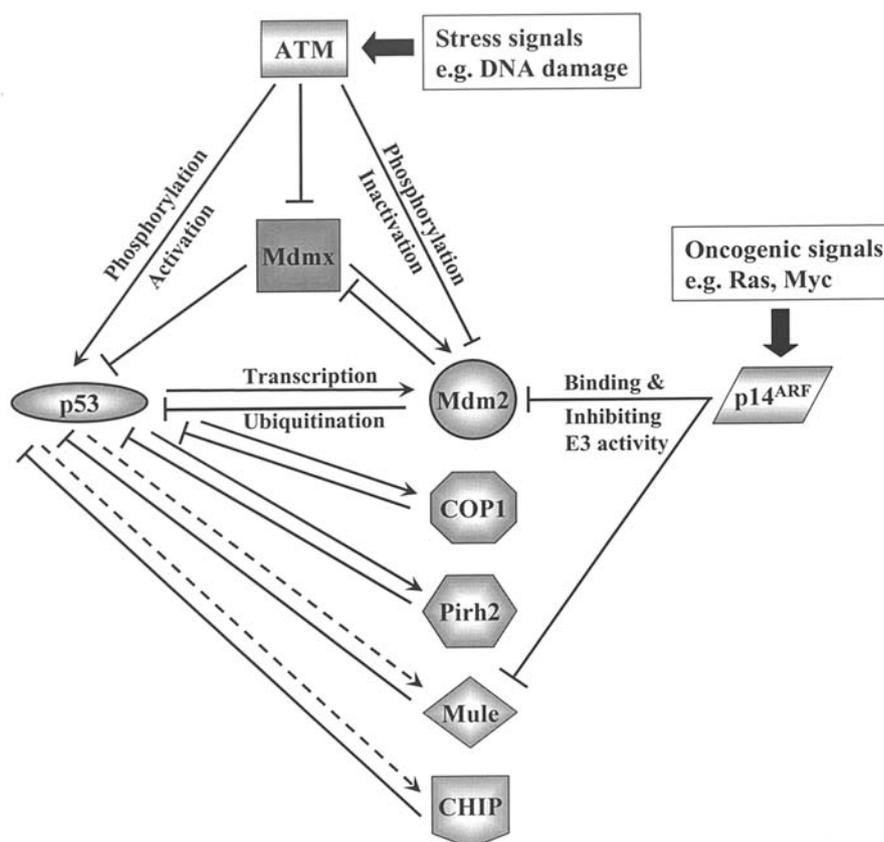

Figure 2. The regulatory model of p53 by the ubiquitination system. Phosphorylated Mdm2 inhibits the ubiquitination of p53. Phosphorylated p53 is activated as a transcription factor and induces the transcription of p53-specific E3 enzymes including Mdm2, COP1 and Pirh2, resulting in a negative feedback loop but this transcriptional regulation by Mule and CHIP (dots) has not been determined yet. Mdmx binds p53 and inhibits the transcriptional activity of p53. Mdmx also stabilizes Mdm2 by the inhibition of self-ubiquitination, and the ATM-dependent phosphorylation of Mdmx plays a crucial role in the ubiquitination and degradation of Mdmx by Mdm2. Arrows denote stimulatory effects, whereas horizontal bars indicate inhibitory influences.
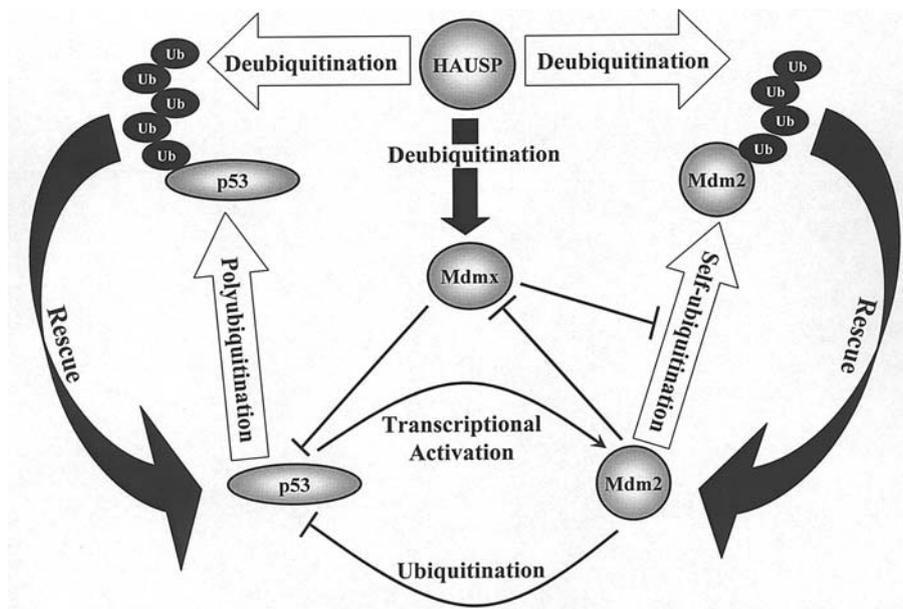
Figure 3. A regulatory model controlling the stability of p53 and Mdm2 by HAUSP. HAUSP can deubiquitinate p53, Mdm2, and Mdmx. The self-ubiquitination activity of Mdm2 is important to regulate both Mdm2 and p53 at opposite levels. Mdmx stabilizes Mdm2 by inhibiting self-ubiquitination. HAUSP plays a crucial role for regulating the levels of p53, Mdm2, and Mdmx.

Consequently, the activity of p53 is maintained at a very low level in normal cells. In addition, activated p53 serves as a transcription factor for the *Mdm2* gene, and up-regulation of Mdm2 eventually decreases the level of p53 by a negative feedback loop (38). Under stress conditions, such as DNA damage, the function of Mdm2 is inhibited by p14ARF and ATM. p14ARF is induced by oncoproteins (e.g. Ras and Myc) and binds both Mdm2 and ARF-BP1/Mule, resulting in inhibition of E3 ligase activity (34,39). ATM is activated by stress signals and phosphorylates several target proteins, including p53, Mdm2 and Mdmx. ATM directly and indirectly induces the phosphorylation of Mdm2 and Mdmx, leading to decreased activity and stability of these proteins (40). Phosphorylated p53 is resistant to ubiquitination mediated by Mdm2, and the phosphorylation of Mdm2 by ATM inhibits the ubiquitination of p53. In addition, ATM-dependent phosphorylation of Mdmx plays a role in regulation of ubiquitination and degradation mediated by Mdm2 (41-43) (Fig. 2).

Deubiquitination is mediated by cysteine protease activity of deubiquitinating enzymes which possess highly conserved domains of the catalytic Cys, His, and Asp residues (6). HAUSP, one of the deubiquitinating enzymes, binds and stabilizes p53 by deubiquitination. HAUSP-overexpressed human lung carcinoma cells (H460) showed stabilization of p53 against polyubiquitination, resulting in p53-dependent cell cycle arrest and apoptosis (44). We also found the growth retardation and apoptosis of HAUSP-overexpressed cervical adenocarcinoma cells (HeLa) by transfection with a mouse orthologue of HAUSP (mHAUSP) (45,46). Interestingly, disruption of HAUSP in human colorectal cancer (HCT116) cells and human osteosarcoma (U2OS) cells also showed the same results as shown in HAUSP-overexpressed cancer cells (47,48). It is interesting that HAUSP can also deubiquitinate Mdm2 and Mdmx, resulting in stabilization of these proteins (31,40). Therefore, it is possible that the disruption of HAUSP

makes Mdm2 extremely unstable and fails to ubiquitinate p53, because Mdm2 is constitutively self-ubiquitinated and degraded (Fig. 3). When HAUSP is overexpressed, both p53 and Mdm2 are deubiquitinated and rescued, resulting in the increasing level of these proteins. In this situation, both self-ubiquitination of Mdm2 and HAUSP-dependent rescue of p53 may lead to dominant function of p53 in the balance of p53 and Mdm2. When HAUSP is disrupted, both p53 and Mdm2 failed to be deubiquitinated. In this case, the absence of HAUSP-mediated Mdm2 deubiquitination and self-ubiquitination of Mdm2 have a synergistic effect on the degradation of Mdm2. Consequently, ubiquitination of p53 is suppressed by an extremely low level of Mdm2, and p53 becomes stable and functional (37,45,47,48).

4. HAUSP as an evolutionarily conserved protein in mammals

Human HAUSP (hHAUSP, also known as USP7) was originally identified by its binding activity for two herpesviral proteins, ICP0 (infected cell protein 0) and EBNA1 (Epstein-Barr nuclear antigen 1) (49,50). hHAUSP consists of 1102 amino acids and its molecular weight is approximately 135 kDa. Recently, we isolated the orthologues of hHAUSP in mouse (mHAUSP) and rat (rHAUSP) (45,51). The open reading frame of both mHAUSP and rHAUSP consists of 3312 bp and encodes a predicted protein of 1103 amino acids with a molecular weight of approximately 135 kDa. HAUSP is a well-conserved protein in mammals. hHAUSP shows 98.6% amino acid identity with both mHAUSP and rHAUSP. Also, the amino acid identity is 99.6% between rHAUSP and mHAUSP (51). Using a partial proteolysis of HAUSP with MALDI-TOF/MS, four structural domains were found (52). Of these, three functional domains important for protein-protein interaction and enzymatic activity have been identified. The
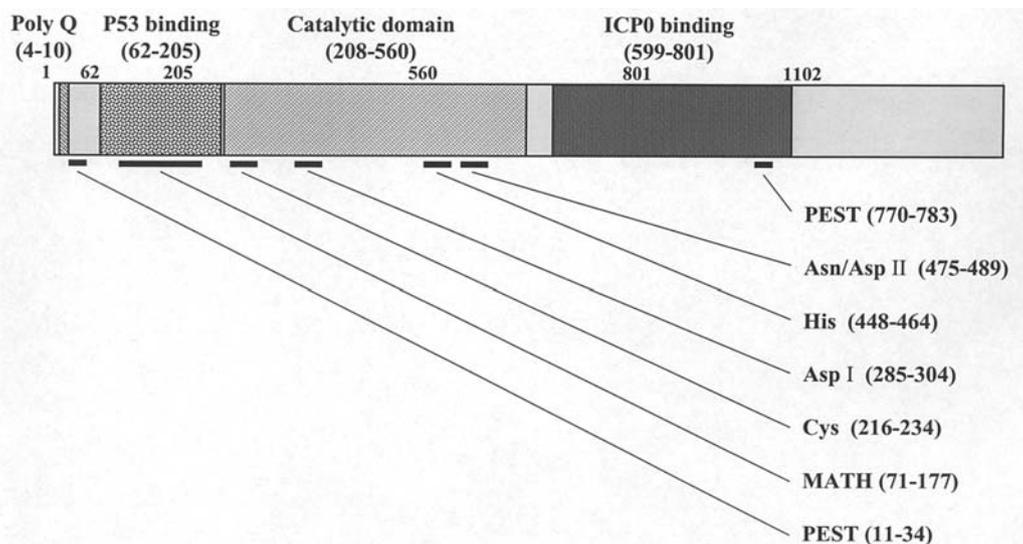
Figure 4. Schematic structure of the HAUSP protein. The domains of human HAUSP (PEST, MATH and catalytic domains) are illustrated with boxes and bold lines. The numbers of amino acid residues are in parentheses.

N-terminal domain (residues 62-205) is responsible for binding both EBNA1 and p53. Moreover, this domain includes the MATH (meprine and TRAF homology) domain, which is known to be critical for protein-protein interaction (45). The catalytic domain consists of approximately 350 amino acids (residues 208-560) and conserved three-domain architectures (Fingers, Palm, and Thumb) were found by crystal structure analysis (53). The catalytic domain has highly conserved Cys, Asp (I), His, and Asn/Asp (II) domains, which are one of the characteristic features of the deubiquitinating enzyme family and the amino acid sequences of these conserved domains are entirely identical in mouse, rat, and human (51). The domains of Cys, His, and Asn/Asp (II) are involved in the catalytic cleft and the domain of Asp (I) is located in the Thumb domain (53). ICP0 binding domain is located in the C-terminal 200 amino acids (residues 599-801) (52). The remaining C-terminal domain of 300 amino acids (residues 802-1102) has not been characterized. In addition, the domain responsible for interacting with Mdm2 has not been identified yet (Fig. 4). We recently found that rHAUSP is dimerized (51). However, the domain responsible for dimerization has not been identified yet and whether the dimerization of HAUSP is essential for its enzymatic activity remains to be investigated.

One of the interesting features of HAUSP is the N-terminal polyglutamine (poly Q) region which is conserved among mouse, rat, and human. hHAUSP contains 7 Glu residues (residues 4-10) and 8 Glu residues (residues 4-11) in both mHAUSP and rHAUSP, respectively (51). Abnormal expansion of CAG repeat encodes poly Q tract. This mutational expansion of the polyglutamine tract in certain proteins results in neuro-degenerative diseases, including spinocerebellar ataxia 1, 2, 3, 6, 7, and 17, Huntington's disease, spinobulbar muscular atrophy and dentatorubral-pallidoluysian atrophy (54). Therefore, it remains to be elucidated whether the mutant HAUSP with the expanding poly Q tract exists and results in neurodegenerative diseases.

Interestingly, two conserved hydrophilic PEST motifs were found in the HAUSP proteins in human, mouse and rat using the PESTfind algorithm (http://www.at.ambnet.org/embnet/tools/bio/PESTfind). One is located just after the poly Q tract in the N-terminus (24 amino acids: 11-34; KAGEQQLSEPEDMEMEAGDTDDPP) and the other is located in the C-terminus (14 amino acids: 770-783; KDDPE NDNSELPTA). It has been suggested that the PEST motif is involved in ubiquitination for the rapid degradation of proteins (17). Even though it is not known whether the ubiquitination of target proteins is directly mediated by the PEST motif, USP36 and HAUSP containing the PEST motif have been recently identified to be ubiquitinated (51,55).

5. Therapeutic approaches for targeting HAUSP

As mentioned above, hematopoietic tumors are less likely to have mutations of *p53* and *Rb*. This characteristic feature of hematopoietic tumors makes the hypothesis possible that the induction of p53 pathway via up- or down-regulation of HAUSP can force certain hematopoietic tumors into apoptosis as well as cell cycle arrest. HAUSP-targeted therapy is based on the proposition that the endogenous level of p53 expression (both transcription and translation) is not altered but the post-translational regulation of p53 protein can be achieved by targeting HAUSP. We suggest that the amount of p53 protein has to be maintained within a normal range to prevent un-wanted cell proliferation or growth retardation with the balance between p53 and its regulatory proteins, including Mdm2, Mdmx and HAUSP. According to this regulation, the strategy for targeting HAUSP is a valuable method for regulating the balance among these proteins. This therapeutic possibility regulating the endogenous level of p53 was already proven in non-hematopoietic tumor cells *in vitro* (44-48). A possible therapeutic strategy for targeting HAUSP includes the knock-down of HAUSP via siRNA, gene therapy for increasing expression of HAUSP, or delivery of HAUSP proteins into the cells. For example, HAUSP-CS, a catalytic mutant of HAUSP, in which a highly conserved Cys residue at the catalytic Cys domain was replaced by Ser (C224S in both mHAUSP and rHAUSP, C223S in hHAUSP), retained its strong binding ability to p53, but is functionally defective

in deubiquitinating p53 *in vitro* (44,45,51). Also, this catalytic mutant hHAUSP failed to deubiquitinate Mdm2 (31). In our study performed with mHAUSP-transfected HeLa cells, D296A and H465Q mutations also showed no enzyme activity, as identical with D295A and H464Q mutations in hHAUSP (45,46). There are two possible models of how mutant HAUSP plays an inhibitory role for the endogenous intact HAUSP. One possibility is that a mutant HAUSP without deubiquitinating enzyme activity competitively binds p53 and Mdm2, resulting in failure of both p53 and Mdm2 deubiquitination. The other possibility is that mutant HAUSP acts as a dominant negative form to block the intact HAUSP function. We recently found that exogenously transfected HAUSP proteins can dimerize with endogenous HAUSP proteins in HeLa cells (51). This finding is concordant with our previous observation that B-lymphocyte-specific murine DUB-1 is dimerized (Baek, unpublished data). Our two observations imply that the dimerization of HAUSP may be required for its enzymatic functions. Therefore, the introduction of mutant HAUSP into cells may cause the formation of an inactive dimer complex between endogenous and mutant HAUSP enzymes.

We recently reported that deubiquitinating enzymes, USP36 and DUB-1A, are polyubiquitinated (55,56). In addition, HAUSP is also polyubiquitinated, indicating the existence of HAUSP-specific E3 ligases and deubiquitinating enzymes (51). Therefore, investigation of HAUSP-specific E3 ligases and DUB enzymes may provide a novel strategy for targeting HAUSP and therapeutic tools for hematopoietic tumors. A recent report revealed that ICP0, a herpes simplex virus type 1 (HSV-1) E3 ligase, can ubiquitinate HAUSP, suggesting that HAUSP has the potential to be regulated by the ubiquitin-proteasome pathway (57).

Finally, it has been suggested that HAUSP may contain several distinct binding sites, including p53, Mdm2, Mdmx, unidentified E3 and deubiquitinating enzymes for HAUSP, even though these binding regions and their specificity have not been investigated yet. This indicates that the high-throughput screening of chemical and peptide libraries for searching HAUSP binding molecules, which specifically interferes with binding of interacting proteins for HAUSP, may be valuable in finding binding site-specific drugs for HAUSP. The nutlin, as a small inhibitor molecule, is a good example of a p53 protein regulator. The nutlins bind Mdm2 in the p53 binding pocket with high selectivity and inhibit the p53-Mdm2 interaction, resulting in p53 stabilization (58).

For the application of HAUSP-targeting therapy in cancer treatment, its safety must be proven in normal cells, as with all types of anti-cancer therapy. Overexpression of HAUSP in mouse embryo fibroblasts (MEF) caused cell growth repression (44) and disruption of HAUSP in telomerase-immortalized normal retinal-pigment epithelial cells (RPE) also arrested cell proliferation (48). In contrast to previous results, we observed that the overexpression of HAUSP did not affect the cell proliferation of normal fibroblasts (Baek, unpublished data). Therefore, further investigations and solutions for side effects of HAUSP-targeting therapies, including cell cycle arrest and possible apoptosis of normal cells, are preferentially required to provide a successful therapy for hematopoietic tumors.

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