# USP13 regulates stability and localization of HMGB1

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# USP13 regulates stability and localization of HMGB1

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The Master's Thesis
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Jaemin Shin

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This certifies that the Master's Thesis
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## ABSTRACT

**USP13 regulates**
**stability and localization of HMGB1**

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High Mobility Group Box 1 (HMGB1) is a damage-associated molecular pattern (DAMP) molecule, which plays a central role in innate immunity. HMGB1 acts as a late mediator of inflammation when actively secreted in response to inflammatory stimuli. Several post-translational modifications (PTMs), such as acetylation, phosphorylation, and oxidation are known to regulate HMGB1 secretion. However, how HMGB1 is modulated by deubiquitination is unknown. Here, we report ubiquitin-specific protease 13 (USP13) is a novel binding partner of HMGB1. We demonstrated that USP13 stabilizes HMGB1 through deubiquitination. USP13 also regulates nucleocytoplasmic localization and extracellular secretion. Additionally, we found that a triple mutant form of USP13, which catalytic inactive (C345A) and ubiquitin-associated (UBA) domain mutant (M664/739E), keeps promoting HMGB1

nucleocytoplasmic translocation and secretion, even though the deubiquitinase (DUB) activity is abolished. These data suggest that USP13 modulates subcellular localization and secretion of HMGB1 in a DUB activity-independent manner. Taken together, we report that USP13 regulates HMGB1 stability and subcellular localization followed by secretion. Further investigation seems to be required about how HMGB1 translocation and secretion are regulated in a DUB activity-independent manner.

Key words: HMGB1, deubiquitination, USP13, secretion

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## I. INTRODUCTION

High mobility group box 1 (HMGB1) is a nuclear protein highly conserved in most of eukaryotic cells[1]. HMGB1 is known for dual roles according to its subcellular location. In the nucleus, HMGB1 regulates gene transcription, DNA repair, and functions as a DNA chaperone to modulate nucleosome stability[2-4]. In the extracellular space, HMGB1 also acts as a damage-associated molecular pattern (DAMP), which plays a late mediator of pro-inflammatory response[5]. HMGB1 is actively secreted reacting to various stimuli, such as endotoxin (LPS), interleukin (IL)-1, TNF-α, and hydrogen peroxide[5-8]. Following stimuli, post-translational modifications (PTMs) including oxidation, acetylation, methylation, and phosphorylation occur to regulate secretion of HMGB1[9-12]. Therefore, it is important to identify secretion mechanisms of HMGB1 as a DAMP.

Ubiquitination, one of the post-translational modifications, plays key roles to

regulate protein stability and signal transduction pathways. Ubiquitination is a series of process to conjugate ubiquitin to target substrate in reversible and ATP dependent manners. This process is achieved by E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzyme), and E3 (ubiquitin ligases) enzymatic cascade. E1 initiates reaction by activating ubiquitin consuming ATP, and then activated ubiquitin is transferred to the E2. E2 interacts with E3, and E3 conjugates ubiquitin to target protein[13]. Ubiquitination is categorized according to the type of chains, which are linked by isopeptide bond between carboxyl-terminal glycine of ubiquitin and seven internal lysine residues (K6, K11, K27, K29, K33, K48 and K63) of ubiquitin[14]. A single lysine can be attached to substrate protein resulting in monoubiquitination. Substrate protein is usually conjugated by several ubiquitins forming polyuibiquitination. All seven lysines can form polyubiquitin chain, however only K48 and K63-linked ubiquitinations are well characterized. K48-linked ubiquitination leads target proteins to proteasomal degradation, whereas K63-linked ubiquitination is involved in signal transduction or protein trafficking[15].

Deubiquitination is a reverse process of ubiquitination[16]. The human genome encodes about 100 deubiquitinases (DUBs), which are opposed to E3 ligases. DUBs are classified into five families based on sequence homology and mechanism of action. They are 1) ubiquitin-specific proteases (USPs), 2) ubiquitin C-terminal hydrolases (UCHs), 3) ovarian tumor proteases (OTUs), 4) Machado-Joseph disease proteases, and 5) JAB1/MPN/Mov34 metalloenzymes (JAMMs)[17]. Functions of DUBs are divided into several categories. First, ubiquitin precursors are processed by DUBs to generate free ubiquitins. Second, DUBs remove K48-linked ubiquitin chains, which are related with proteasomal degradation, leading to stabilize substrate protein. Lastly, DUBs are also involved in non-degradative ubiquitin signal by trimming ubiquitin chains while they switch from certain type of ubiquitin signal to another. In conclusion, all these

functions of DUBs are contributed to regulate ubiquitin homeostasis for maintaining cellular ubiquitin pools[18].

Ubiquitin-specific protease 13 (USP13), one of the USP family, modulates cellular signaling by interacting with multiple proteins. For example, USP13 regulates antiviral responses by deubiqitinating STAT1 and STING[19,20]. USP13 also deubiquitinates and stabilizes PTEN and MITF for modulation of tumorigenesis[21,22]. USP13 is also identified to regulate RAP80-BRCA1 complex in respond to DNA damage[23].

Although several PTMs are reported to modulate HMGB1, ubiquitination of HMGB1 is poorly understood. Our previous study reveals that defective HMGB1 glycosylation regulates stability of HMGB1 through K48-linked ubiquitination[24]. Currently, E3 ligases of HMGB1 are not identified yet as well as deubiquitinases. Here, we first report USP13 is a novel interacting protein of HMGB1. Also, we revealed that USP13 regulates not only HMGB1 stability but also, subcellular localization of HMGB1 followed by its secretion.

# II. MATERIALS AND METHODS

## 1. Cell cultures and transfection

Human embryonic kidney (HEK) 293T and RAW 264.7 cells were cultured in DMEM media supplemented with 10% FBS (Life Technologies, Camarillo, CA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μl/ml streptomycin at 37°C with 5% $CO_2$. The plasmids were transfected into HEK293T using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) on the following instructions of manufacturer.

## 2. Plasmid construction

Myc- and GFP-tagged HMGB1 were inserted into pCMV vector[24]. Flag-tagged USP13 and Flag-HA-tagged USP10 plasmid were used (Addgene, #61741, #22543, respectively). Flag-HA-tagged USP10 was subcloned into pFLAG-CMV-TOPO vector (MGmed, Seoul, Korea) to generate Flag-tagged USP10 plasmid. Flag-tagged USP15 plasmid was also used. HA-tagged Ubiquitin (Ub) plasmid was purchased from addgene (#17608) to overexpress ubiquitin. Site-directed mutagenesis was performed to generate USP13 C345A/M664/739E mutant using QuickChange Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA). To generate mutated USP13, the primer sequences we used are shown in Table 1. To transiently knock down of USP13, we purchased human USP13 shRNAs in the GIPZ lentiviral vector (Dharmacon Inc, Lafayette, CO, USA), which clone number is V2LHS_47077 (5'-TTTCCAGTGAAGTACACAG-3').

**Table 1. Primers used for site-directed mutagenesis**

| Mutant | Primer |
|---|---|
| $USP13^{C345A}$ | For: 5'-GAACCTGGGCAACAGCGCCTATCTCAGCTCTGTC-3' |
| | Rev: 5'- GACAGAGCTGAGATAGGCGCTGTTGCCCAGGTTC-3' |
| $USP13^{M664E}$ | For: 5'-GTGATGCAGCTGGCCGAGGAGGGTTTCCCGCTGGAAGCA-3' |
| | Rev: 5'- TGCTTCCAGCGGGAAACCCTCCTCGGCCAGCTGCATCAC-3' |
| $USP13^{M739E}$ | For: 5'-GAAATCGTAGCTATCATCACCTCCGAGGGATTTCAGCGAAATCAGGCT-3' |
| | Rev: 5'- AGCCTGATTTCGCTGAAATCCCTCGGAGGTGATGATAGCTACGATTTC-3' |

## 3. Antibodies and chemicals

Antibodies we used are as follows: antibodies against HMGB1 (Abcam, Cambridge, UK, ab18256), USP13 (Bethyl, Montgomery, TX, USA, A302-762A), β-actin (Cell Signaling Technology, Danvers, MA, USA, #4967), Ub (Millipore, Billerica, MA, USA, MAB1510), Ub-K48 (#05-1307), K63 (#05-1308), c-Myc (Invitrogen Technology, #13-2500), Flag (Sigma-Aldrich, St. Louis, MO, USA, F7425), HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-805), CRM1 (sc-5595), Lamin B1 (Abcam, ab133741), and GAPDH (Abfrontier, Seoul, Korea, YF-MA10022) were purchased.

10 μM spautin-1 (Tocris, Bristol, UK) was used to inhibit deubiquitinase activity of USP13[25]. Proteasome inhibitor, 10 μM MG132 (Sigma-Aldrich), and protein biosynthesis inhibitor, 100 μg/ml cycloheximide (CHX) (Sigma-Aldrich) were used. To induce secretion of HMGB1, 100 ng/ml LPS (Sigma-Aldrich, L4391) was treated.

## 4. Immunoblotting and immunoprecipitation

HEK293T cells were transfected with various plasmids and washed with PBS after harvest. For immunoblotting, cells were lysed with 1x RIPA buffer (GenDEPOT, Barker, TX, USA) containing protease inhibitor (GenDEPOT). After sample buffer (100 mM Tris-HCl pH 6.8, 25% glycerol, 5% β-mercaptoethanol, 2% SDS, and 0.1% bromophenol blue) was added to whole cell lysates (WCLs), it was heated at 94$^{o}$C for 5 min. Quantitative proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (GE Healthcare, Little Chalfont, UK), which blocked with 5% skim milk in TBS with 0.1% Tween-20. Indicated antibodies were incubated and ECL solution (GenDEPOT) was reacted for detection. To detect HMGB1 secretion, cell culture supernatants were harvested after treatments, and methanol and chloroform mixture at a 4:1 ratio was added to supernatants for precipitating proteins, and then immunoblotted.

For immnunoprecipitation, pre-cleared Protein G magnetic beads (Bio-Rad, Hercules, CA, USA) were reacted with indicated antibodies for 1 h at RT and WCLs were incubated with beads for overnight at 4$^{o}$C. After beads were washed four times with PBST buffer (0.001% Tween-20 in PBS), immunocomplexes were eluted by sample buffer and separated by SDS-PAGE.

## 5. *In vivo* ubiquitination assay

HEK293T cells were co-transfected with Myc-tagged HMGB1, HA-tagged Ub, and Flag-tagged wild-type (WT) or mutated USP13. After transfection for 24 h, 10 μM MG132 was treated for 18 h. Cells were lysed with 1x RIPA

buffer and Protein G magnetic beads conjugated with anti-Myc antibody were added to sample and then, rotated for overnight. Eluted samples were resolved on SDS-PAGE, and anti-Ub and HA antibodies were incubated overnight at 4$^{o}$C for immunoblotting.

## 6. Immunofluorescence

To observe subcellular translocation of HMGB1, GFP-tagged HMGB1 and Flag-tagged USP13 or USP13$^{C345A/M664/739E}$ were co-transfected into HEK293T cells, which were cultured on cover glasses in a six-well plate. After 24 h, 10 μM Spautin-1 was treated for 18 h. Cells were fixed with 4% paraformaldehyde-PHEM buffer for 30 min at RT and permeabilized with 1 % Triton X-100 for 10 min at RT. After washing with cold PBS, cells were blocked with 1% BSA in PBST buffer (0.02% Tween-20 in PBS) for 30 min at 37$^{o}$C, and then incubated with antibody against Flag for overnight at 4$^{o}$C, followed by incubation with Alexa Fluor 594 goat anti-mouse IgG (Life Technologies, A11005) for 45 min at 37$^{o}$C. After mounting with DAPI, a confocal FV1000 microscope (Olympus, Shinjuku, Tokyo, Japan) was used to obtain images.

## 7. Nuclear and cytosolic fractionation

Nuclear and cytosolic proteins were fractionated using a nuclear and cytosolic fractionation kit (Bio Vision) according to instructions of manufacturer. HEK293T cells were transfected with Flag-tagged USP13 for 24 h, and treated with 10 μM spautin-1 for 18 h. Immunoblotting was performed to confirm subcellular localization of HMGB1.

## 8. Proximity ligation assay (PLA)

To confirm interaction between HMGB1 and USP13, PLA was performed using Duolink In Situ-Fluorescence kit (Sigma-Aldrich). HEK293T cells were co-transfected with Myc-tagged HMGB1 and Flag-tagged USP13, and treated with 10 μM spautin-1 for 18 h. After fixing and permeabilization as above, cells were blocked with blocking solution. Cells were incubated with anti-Myc and anti-Flag antibodies for overnight at 4°C. Next, PLA probes and ligation solution were reacted. After treatment with amplification solution, cells were mounted with DAPI. To measure cell fluorescence, a confocal microscope was used.

## 9. LC-MS/MS for peptides analysis

HEK293T cells were co-transfected with Myc-tagged HMGB1 and HA-tagged Ub plasmids. Cells were lysed with 1x RIPA buffer, and incubated with anti-Myc affinity gel (Biotool, Houston, TX, USA, B23401) for overnight at 4°C. Resin was washed with cold PBS three times, and 2x sample buffer added to elute the sample. After boiling at 94°C for 5 min, sample was separated by SDS-PAGE followed by Coomassie Blue Staining. The band which located in HMGB1 size was extracted for analysis.

Nano LC–MS/MS analysis was performed with a nano HPLC system (Agilent, Wilmington, DE). The nano chip column (Agilent, Wilmington, DE, 150 mm × 0.075 mm) was used for peptide separation. The mobile phase A for LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 3% B to 40% B in 80 min, 40% B to 60% B in 10 min, 95% B in 10 min, and 3% B in 20 min. The

flow rate was maintained at 400 nL/min. Product ion spectra were collected in the information-dependent acquisition (IDA) mode and were analyzed by Agilent 6530 Accurate-Mass Q-TOF using continuous cycles of one full scan TOF MS from 350-1200 m/z (1.0 s) plus three product ion scans from 100-1700 m/z (1.5 s each). Precursor m/z values were selected starting with the most intense ion, using a selection quadrupole resolution of 4 Da. The rolling collision energy feature was used, which determines collision energy based on the precursor value and charge state. The dynamic exclusion time for precursor ion m/z values was 30 s.

## 10. Database searching

The mascot algorithm (Matrixscience, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria was *HMGB1* (CAG 33144.1, High Mobility Group Box 1), *homo sapiens* (downloaded Sep 21 2016) fixed modification; carbamidomethylated at cysteine residues; variable modification; oxidized at methionine residues, maximum allowed missed cleavage; 2, MS tolerance; 100 ppm, MS/MS tolerance; 0.1 Da. Only peptides resulting from trypsin digests were considered.

## 11. Statistical analysis

The data were analyzed by one-way ANOVAs or Student's *t*-test. Average values were shown as graphs. All values were represented as the mean and ± standard error of the mean (SEM). P value $< 0.05$ was regarded as significant.

# III. RESULTS

## 1. Identification of HMGB1 binding proteins

HMGB1 has diverse roles depending on its various interactors[26]. Therefore, it is crucial to identify the binding partners of HMGB1. Our previous study showed that defective glycosylation of HMGB1 is regulated by K48-linked ubiquitination[24]. In order to investigate binding proteins related with ubiquitination, we performed LC-MS/MS. HEK293T cells were co-transfected with Myc-tagged HMGB1 and HA-tagged Ub plasmids, and treated with MG132. Myc-tagged HMGB1 proteins were immunoprecipitated with anti-Myc affinity gel, and then separated by SDS-PAGE (Figure 1A). The extracted band was analyzed by LC-MS/MS. A few candidate proteins, such as elongation factor 2 (EF2), heat shock protein 90 (HSP90), and mitochondrial heat shock protein 75 (MTHSP75) were revealed to interact with HMGB1 (Table 2). LC-MS/MS results also identified 13 matched peptides of USP13, also called isopeptidase T-3 (Figure 1B). Next, we focused on USP13 protein for its correlation with deubiquitination.

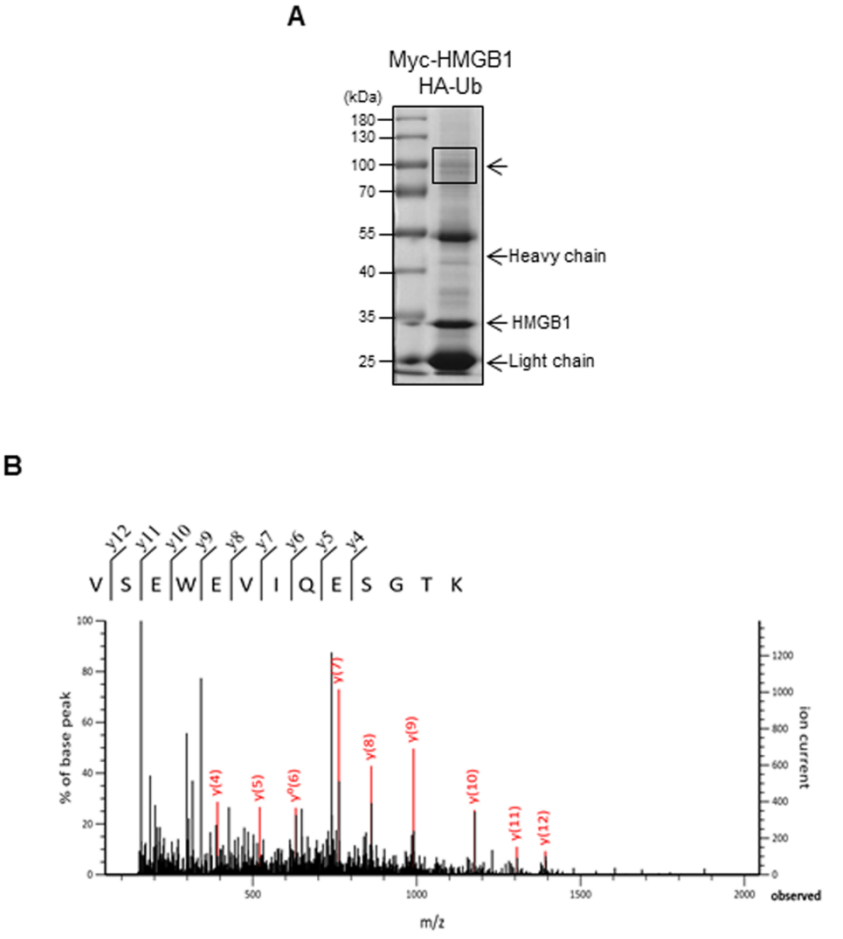


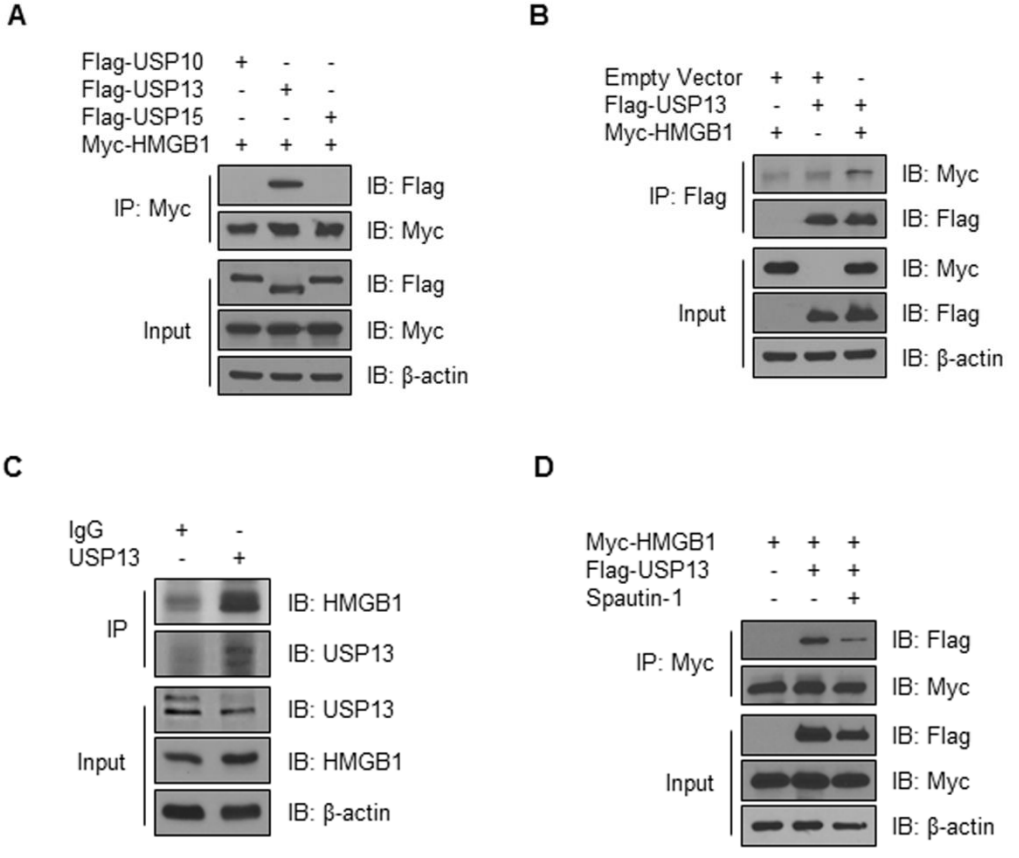

**Figure 1. Identification of HMGB1 binding proteins.** (A) HEK293T cells were co-transfected with Myc-HMGB1 and HA-Ub plasmids, and treated with 10 μM MG132 for 18 h prior to harvest. WCLs were immunoprecipitated with anti-Myc affinity gel, and separated by SDS-PAGE. Indicated band was extracted for analysis. (B) Base peak in the LC-MS/MS analysis of digested USP13 by trypsin was shown. Peptide sequence was identified by the mascot algorithm.

**Table 2. HMGB1 binding proteins**

| gi number (NCBI databse) | Protein identification | Mascot score | MW | PI value | Matched peptides number | Matched peptides |
|---|---|---|---|---|---|---|
| gi\|31108 | Elongation factor 2 | 103 | 96,246 | 6.41 | 26 | TFC QLILDPIFK, ETVSEESNVL CLSK |
| gi\|225608 | Heat shock protein 90 | 64 | 83,584 | 4.97 | 11 | EQVANSAFV ER |
| gi\|292059 | Mitochondrial heat shock protein 75 (MTHSP 75) | 60 | 74,019 | 5.97 | 12 | AQ FEGIVTDLIR |
| gi\|1658463 | Isopeptidase T-3 (USP13) | 57 | 97,978 | 5.33 | 13 | VSEWEVI QESGTK |
| gi\|189306 | Nucleolin | 55 | 76,355 | 4.59 | 22 | TLVL SNLSYSATEE TLQEVFEK |

## 2. USP13 interacts with HMGB1

Based on the LC-MS/MS results, co-immunoprecipitation (IP)-immnoblot assays were performed to confirm whether USP13 physically interacts with HMGB1. To determine if USP13 binds to HMGB1 among other USPs, we overexpressed Myc-tagged HMGB1 plasmid, along with Flag-tagged USP10, USP13, or USP15 plasmid in HEK293T cells. USP10 was subjected to test since specific and potent autophagy inhibitor-1 (spautin-1) inhibits USP10 and USP13. As shown in Figure 2A, HMGB1 specific binding to USP13 was determined by immunoprecipitating with anti-Myc antibody. Myc-tagged HMGB1 was also detected in Flag-tagged USP13 overexpressed cells when immunoprecipitated with anti-Flag antibody (Figure 2B). Furthermore, endogenous HMGB1 was detected in the anti-USP13 immunoprecipitates (Figure 2C). On the other hand, spautin-1 is a small molecule inhibitor of autophagy. It inhibits USP10 and USP13 that regulate the deubiquitination of Beclin1 in Vps34 complexes, resulting in promoting the degradation of Vps34 complexes[25]. We tested whether spautin-1 has effect on interaction between HMGB1 and USP13. Spautin-1 decreased the binding of HMGB1 to USP13, suggesting that inhibition of USP13 deubiquitinase activity affects their binding (Figure 2D). Interaction between HMGB1 and USP13 was also confirmed by a proximity ligation assay (PLA). In parallel with a co-IP-immunoblot assay, spautin-1 significantly decreased interaction between HMGB1 and USP13 (Figure 2E). HMGB1 is composed of two homologous DNA-binding domains, the A box and B box. We mapped which domain is essential for binding to USP13. HEK293T cells were co-transfected with Myc-tagged full length HMGB1, A box or B box plasmids, together with Flag-tagged USP13 plasmid. We found that USP13 dominantly binds to B box through co-IP-immunoblot assay (Figure 2F). These results indicate that USP13 physically interacts with HMGB1.



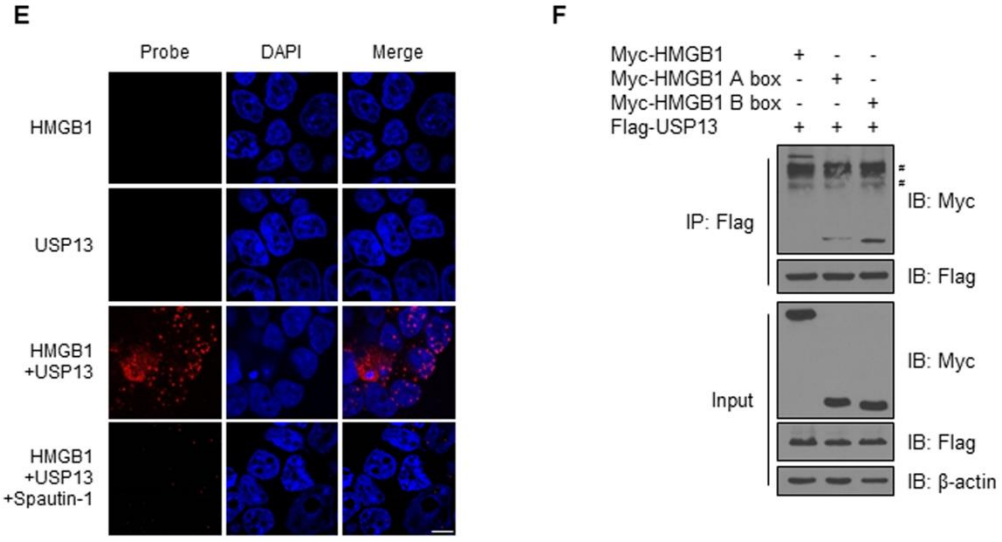
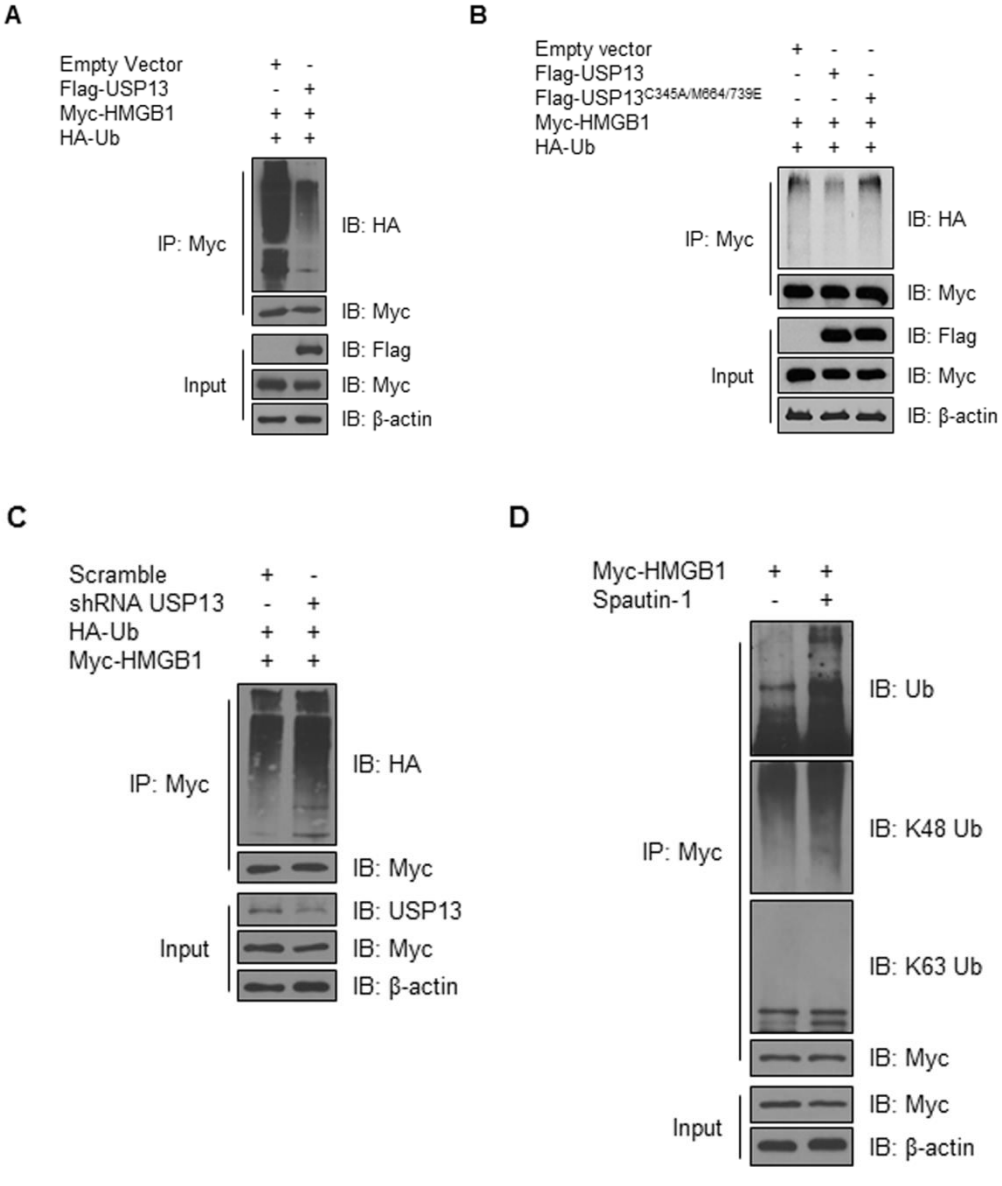



**Figure 2. USP13 interacts with HMGB1.** (A) HEK293T cells were co-transfected with Myc-HMGB1 plasmid and Flag-USP10, USP13, or USP15 plasmid. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-Flag antibody. (B) Myc-HMGB1 and Flag-USP13 were co-overexpressed in HEK293T cells. Cells were immnunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody. (C) WCLs of HEK293T cells were immunoprecipitated with anti-IgG or USP13 antibody and immunoblotted with anti-HMGB1 antibody. (D) HEK293T cells were co-transfected with Myc-HMGB1 and Flag-USP13 plasmids, and treated with 10 μM spautin-1 for 18 h. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-Flag antibody. (E) PLA was performed on HEK293T cells co-expressing Myc-HMGB1 and Flag-USP13 with or without 10 μM spautin-1 treatment for 18 h. Scale bar: 10 μm. (F) HEK293T cells were co-transfected with Myc-HMGB1, A box or B box plasmid and Flag-USP13 plasmid. WCLs were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-Myc antibody. #: Non-specific band.

### 3. USP13 deubiquitinates HMGB1

Since USP13 interacts with HMGB1, we assumed that USP13 deubiquitinates HMGB1. HEK293T cells were co-transfected with Myc-tagged HMGB1, Flag-tagged USP13 plasmids, along with HA-tagged Ub plasmid, and treated with MG132. As shown in Figure 3A, overexpression of USP13 reduced ubiquitination of HMGB1. Conserved cysteine motif and ubiquitin-associated (UBA) domain are known to be essential for the function of USP13[27,28]. Therefore, we generated USP13 DUB inactive mutant (C345A/M664/739E) to test whether USP13 mutant affects HMGB1 ubiquitination. HEK293T cells were co-transfected with Myc-tagged HMGB1, HA-tagged Ub and Flag-tagged USP13 or USP13 C345A/M664/739E, and treated with MG132. HMGB1 ubiquitination level was reduced by USP13, but not USP13 C345A/M664/739E (Figure. 3B). Contrary to overexpression of USP13, knockdown of USP13 enhanced HMGB1 ubiquitination (Figure 3C). We further tested whether spautin-1 regulates HMGB1 ubiquitination. Endogenous ubiquitination of HMGB1 was increased by spautin-1 treatment. Moreover, spautin-1 also increased K48-linked ubiquitination of HMGB1, not K63-linked ubiquitination of that (Figure 3D). To further confirm USP13 deubiquitinates K48-linked ubiquitin chains from HMGB1, Myc-tagged HMGB1, Flag-tagged USP13 plasmids, together with HA-tagged WT-Ub or K48-Ub plasmid were co-transfected into HEK293T cells. Overexpression of USP13 reduced WT and K48-linked ubiquitination of HMGB1. In parallel with co-IP-immunoblot assay, mono- and di-ubiquitination of exogenous HMGB1 were also decreased by overexpression of USP13 (Figure 3E). In conclusion, we demonstrated that USP13 deubiquitinates HMGB1.



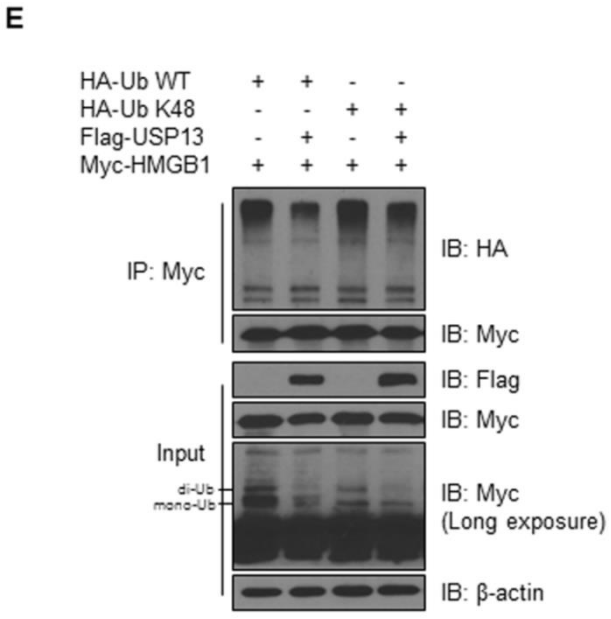
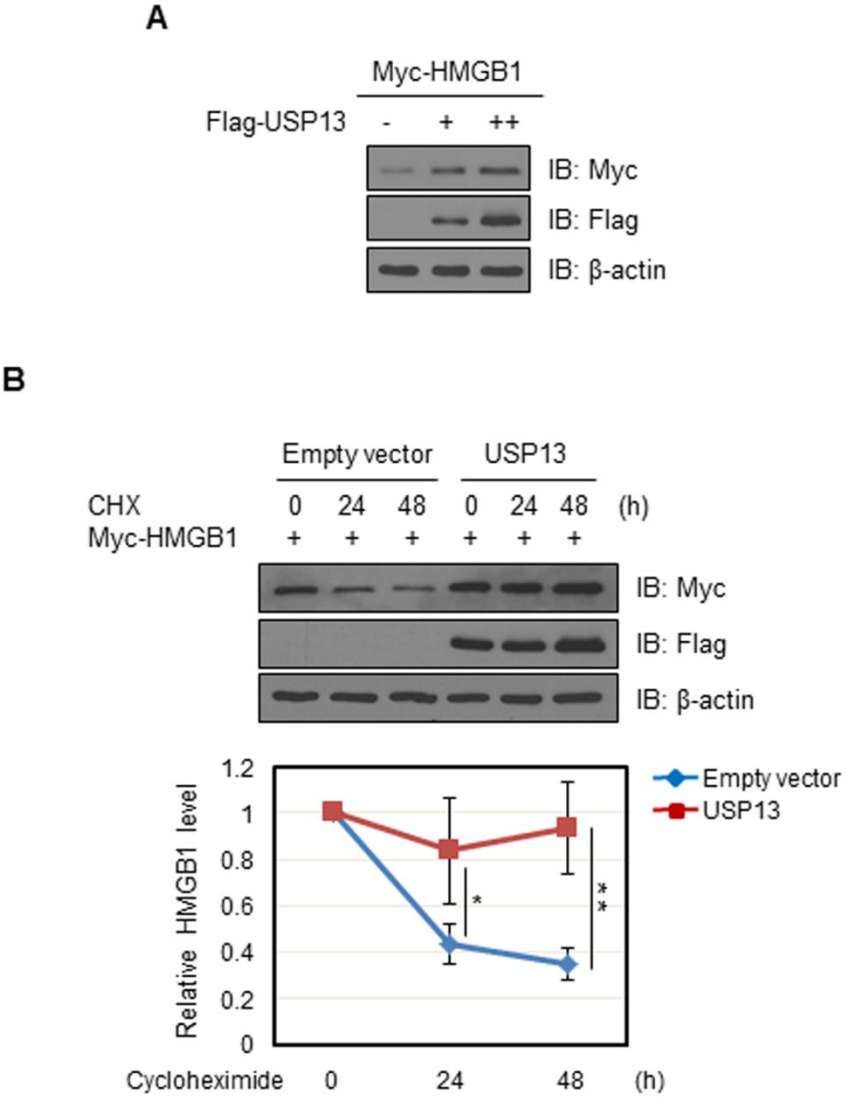



**Figure 3. USP13 deubiquitinates HMGB1.** (A) HEK293T cells were co-transfected with Myc-HMGB1, Flag-USP13, and HA-Ub plasmids and treated with 10 μM MG132 for 18 h before harvest. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody. (B) HEK293T cells were co-transfected with Myc-HMGB1, HA-Ub plasmids, and Flag-USP13 or $USP13^{C345A/M664/739E}$ plasmid and treated with 10 μM MG132 for 18 h. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody. (C) HEK293T cells were co-transfected with Myc-HMGB1, HA-Ub, and shRNA USP13 plasmids and treated with 10 μM MG132 for 18 h prior to collection. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody. (D) Myc-HMGB1 plasmid was overexpressed in HEK293T cells. After 24 h, 10 μM spautin-1 was treated for 18 h. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with indicated antibodies. (E) HEK293T cells were co-transfected with Myc- HMGB1, Flag-USP13 plasmids, and HA-WT-Ub or HA-K48-Ub plasmid, and treated with 10 μM MG132 for 18 h. Co-IP-immunoblot assay was performed to observe ubiquitination.

### 4. USP13 stabilizes HMGB1

To determine whether USP13 regulates stability of HMGB1, We overexpressed Myc-tagged HMGB1 plasmid, together with increasing amounts of Flag-tagged USP13 plasmid. The level of HMGB1 increased in a dose-dependent manner of Flag-tagged USP13 plasmid (Figure 4A). Also, we investigated the stability of HMGB1 when cells were treated with protein translation inhibitor, cycloheximide (CHX). The level of exogenous HMGB1 was significantly upregulated by overexpression of USP13 (Figure 4B). Conversely, knockdown of USP13 reduced the stability of HMGB1 (Figure 4C). Next, spautin-1 was used to measure the level of HMGB1. Spautin-1 also reduced HMGB1 level, suggesting that inhibition of USP13 deubiquitinase activity also decreased HMGB1 stability (Figure 4D). These results show that USP13 stabilizes HMGB1.



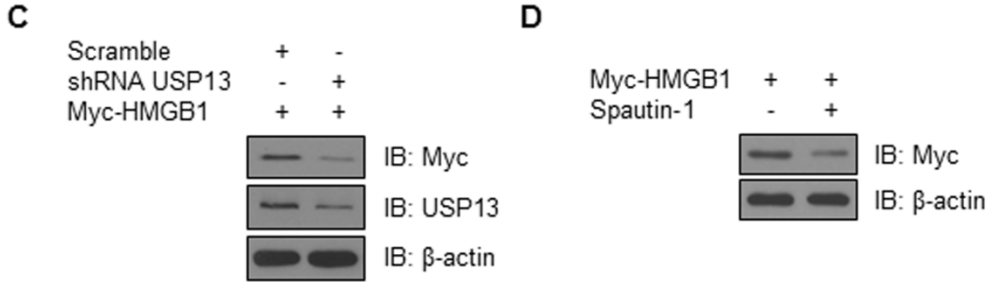
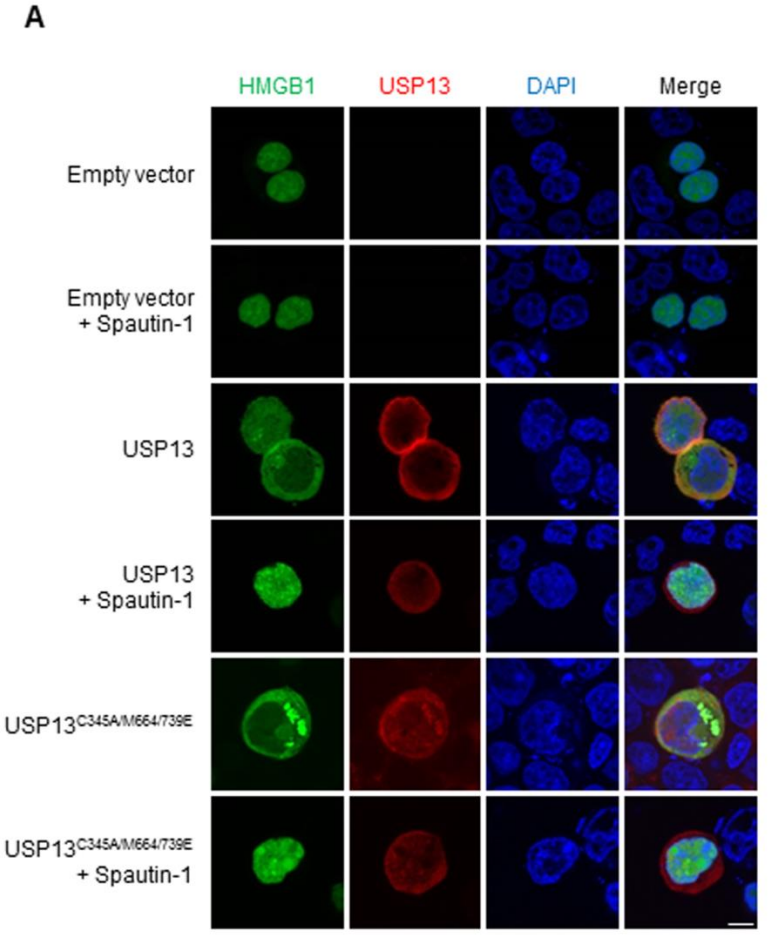
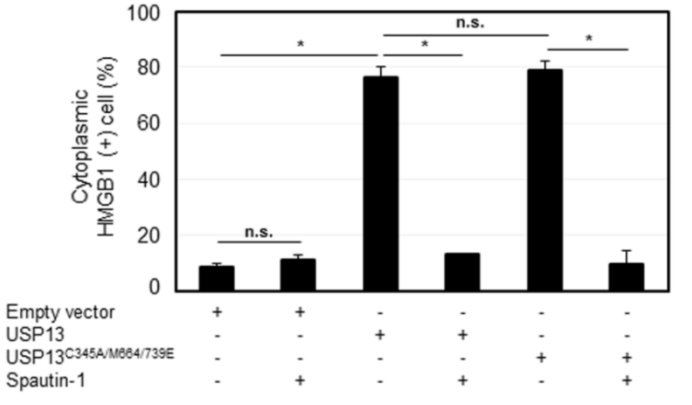



**Figure 4. USP13 stabilizes HMGB1**. (A) HEK293T cells were co-transfected with Myc-HMGB1 plasmid and increasing dose of Flag-USP13 plasmid. WCLs were immunoblotted with anti-Myc and Flag antibodies. (B) HEK293T cells were co-transfected with Myc-HMGB1 and Flag-USP13 plasmids, treated with 100 μg/ml CHX for the indicated times. WCLs were immunoblotted with anti-Myc and Flag antibodies. (C) HEK293T cells were co-transfected with Myc-HMGB1 and shRNA USP13 plasmids to transiently knock down of USP13, and then WCLs were immunoblotted with indicated antibodies. (D) HEK293T cells were transfected with Myc-HMGB1 plasmid, treated with 10 μM spautin-1 for 18 h. WCLs were immunoblotted with anti-Myc antibody. *P<0.05, **P<0.01.

### 5. USP13 regulates subcellular localization of HMGB1

According to previous reports, DUBs regulate the subcellular localization of target molecules[29,30]. Therefore, we examined whether USP13 controls nucleocytoplasmic translocation of HMGB1 depending on its DUB activity. HEK293T cells were co-transfected with GFP-tagged HMGB1 plasmid, along with Flag-tagged USP13 or USP13 C345A/M664/739E plasmid, and treated with spautin-1. As shown in Figure 5A, the majority of HMGB1 is located in nucleus under resting state. Interestingly, translocation of HMGB1 from nucleus to cytosol was significantly increased by overexpression of USP13. Spautin-1 treatment, however, reduced cytoplasmic localization of HMGB1. This tendency was also observed when mutant form of USP13 overexpressed, suggesting that USP13 regulates nucleocytoplasmic translocation of HMGB1 in a DUB activity-independent manner. Also, nuclear and cytosolic fractionation was performed to confirm this phenomenon (Figure 5B). CRM1, a nuclear export protein, is associated with transport nuclear HMGB1 to cytoplasm[10]. To investigate whether USP13 affects interaction between HMGB1 and CRM1, HEK293T cells were co-transfected with Myc-tagged HMGB1 and Flag-tagged USP13 plasmids, and treated with spautin-1. Overexpressed USP13 increased the binding of HMGB1 and CRM1, however, spautin-1 decreased its interaction (Figure 5C). Collectively, these results suggest that USP13 regulates HMGB1 localization via CRM1-dependent nuclear export pathway.



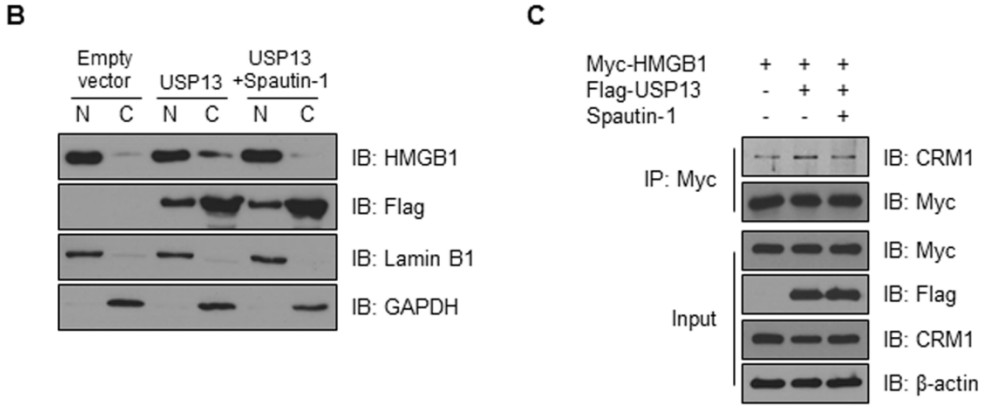



**Figure 5. USP13 regulates subcellular localization of HMGB1.** (A) HEK293T cells were co-transfected with GFP-HGMB1 plasmid and Flag-USP13 or USP13$^{C345A/M664/739E}$ plasmid. After 24 h, cells were treated with 10 μM spautin-1 for 18 h. Immunofluorescence staining was performed to count cytoplasmic HMGB1 cells. Scale bar: 10 μm. *p<0.001. (B) HEK293T cells were transfected with Flag-USP13 for 24 h, and spautin-1 were treated for 18 h. WCLs were immunoblotted with anti-HMGB1 antibody. (C) HEK293T cells were co-transfected with Myc-HMGB1 and Flag-USP13 plasmids, and treated with 10 μM spautin-1 for 18 h. WCLs were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-CRM1 antibody..

### 6. USP13 promotes HMGB1 secretion

Since USP13 regulates subcellular localization of HMGB1, we further studied whether HMGB1 is secreted following cytoplasmic translocation. HEK293T cells were transfected with Flag-tagged USP13 plasmid, and then treated with spautin-1. Secretion of HMGB1 was increased by overexpressed USP13. Spautin-1, however, reduced secretion of HMGB1 (Figure 6A). Several PTMs, such as acetylation, phosphorylation, and oxidation are involved in secretion of HMGB1[9-12]. Therefore, we examined whether deubiquitinase activity of USP13 affects HMGB1 secretion. As shown in Figure 6B, either USP13 or USP13 C345A/M664/739E mutant promotes HMGB1 secretion. Conversely, knockdown of USP13 decreased HMGB1 secretion under starvation condition (Figure 6C). Spautin-1 also reduced HMGB1 secretion in LPS-stimulated cells (Figure 6D). Therefore, USP13 regulates HMGB1 secretion parallel with HMGB1 translocation in a DUB activity-independent manner.

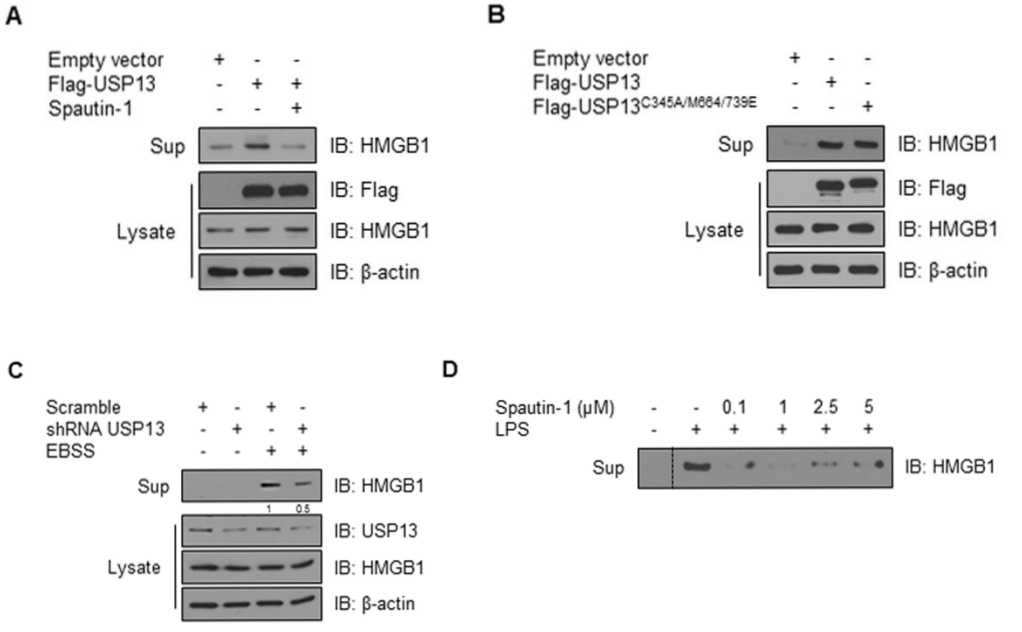



**Figure 6. USP13 promotes HMGB1 secretion.** (A) HEK293T cells were transfected with Flag-USP13 plasmid and treated with 10 μM spautin-1 for 18 h. Cell culture supernatants were precipitated and immunoblotted with anti-HMGB1 and Flag antibodies. (B) HEK293T cells were transfected with Flag-USP13 or USP13$^{C345A/M664/739E}$ plasmids for 48 h, and culture supernatants were immunoblotted with anti-HMGB1 antibody. (C) HEK293T cells were transfected with shRNA USP13 plasmid for 24 h and cultured in EBSS for 6 h. Collected supernatants were immunoblotted with anti-HMGB1 antibody. (D) RAW264.7 cells were pre-treated with 10 μM spautin-1 for 1 h and 100 ng/ml LPS was treated for 18 h. Immunblotting was performed to detect HMGB1 secretion.

## IV. DISCUSSION

In this study, we identified that USP13 deubiquitinates HMGB1. Our previous study showed that HMGB1 glycosylation modulates HMGB1 stability by K48-linked ubiquitination. Additionally, HMGB1 glycosylation is crucial for nucleocytoplasmic translocation and secretion of HMGB1[24]. Currently, ubiquitination of HMGB1 is poorly understood as well as deubiquitination. This is the first report that USP13 regulates HMGB1 stability and subcellular localization, which results in secretion.

HMGB1 is actively secreted when modified by several PTMs, including acetylation, oxidation, phosphorylation, and methylation[9-12]. DUBs were also reported to regulate subcellular localization of target proteins. For example, HAUSP (USP7) is identified as an essential enzyme for PTEN deubiquitination and translocation from cytoplasm to nucleus[29]. Additionally, USP10 regulates p53 stability and localization counteracted by Mdm2 action[30]. Interestingly, we found that USP13 regulates subcellular localization of HMGB1. However, we couldn't clarify the distinction between wild-type USP13 and deubiquitinating enzymatic inactive mutant USP13. It suggests that USP13 regulates translocation and secretion of HMGB1 in a DUB activity-independent manner.

These findings have allowed us to offer a few explanations. First, upregulated HMGB1 stability by USP13 could subsequently increase HMGB1 quantities, further leading to translocation from nucleus to cytoplasm and secretion of HMGB1. Second, USP13 has diverse binding partners, so overexpressed USP13 could regulate the stability of other interactors, which lead to activate other signal transduction pathways. For example, USP13 physically interacts with Beclin1, autophagy initiator, thus deubiquitinates and stabilizes it[25]. HMGB1 binds to Beclin1, which results in dissociation of Beclin1-Bcl-2 complex and induction of autophagy[31]. So, HMGB1 secretion promoted by USP13 could be related with autophagy-medicated secretion pathway. Third, considered that USP13 increased

interaction between CRM1 and HMGB1, USP13 might act as a co-factor to promote HMGB1 translocation. Lastly, it has been reported that USP13 directly regulates deubiquitination of USP10, so USP13 could act as a scaffold protein to regulate other DUBs.

We demonstrate that USP13 is a novel binding protein of HMGB1. Furthermore, it regulates HMGB1 stability and subcellular localization, leading to secretion. How USP13 promotes HMGB1 nucleocytoplasmic translocation and secretion in a DUB activity-independent manner is unclear. Further studies will focus on which genes related with HMGB1 secretion are upregulated by USP13. We also found that spautin-1 reduces HMGB1 secretion in USP13 overexpressed or LPS-stimulated cells. Therefore, spautin-1 could be used as an autophagy inhibitor but also as an anti-inflammatory drug. In conclusion, we revealed the dual roles of USP13 to regulate HMGB1 stability and localization.

## V. CONCLUSION

Here, we revealed that HMGB1 interacts with USP13. USP13 deubiquitinates HMGB1 and regulate stability. Interestingly, USP13 also modulate subcellular localization of HMGB1. This phenomenon is accomplished in a DUB activity-independent manner. How USP13 modulates HMGB1 translocation is unclear, therefore further studies will be needed. Moreover, USP13 promotes HMGB1 secretion following translocation. To sum up, USP13 regulates stability and subcellular localization of HMGB1.

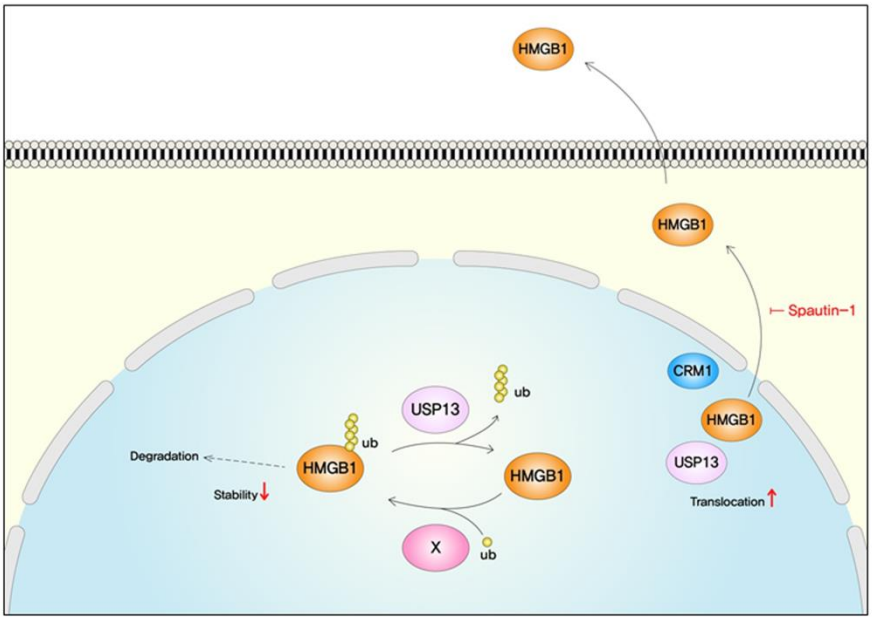



**Figure 7. USP13 regulates stability and localization of HMGB1.**

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**ABSTRACT (In Korean)**

## USP13에 의한 HMGB1 안정성과 위치이동 조절

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신 재 민

High Mobility Group Box 1(HMGB1)은 손상 관련 분자적 패턴으로서 선천면역에 있어서 핵심적인 역할을 담당한다. HMGB1은 염증성 자극에 능동적으로 분비되어 염증반응의 후기 매개자로서 작용한다. 아세틸화, 인산화, 그리고 산화 같은 몇몇 번역 후 변형이 HMGB1 분비를 조절한다고 알려져 있으나, HMGB1이 탈유비퀴틴화에 의해서 어떻게 조절되는지는 밝혀진 바 없다. 이 연구에서는 ubiquitin-specific-protease (USP13)가 HMGB1의 새로운 결합 파트너이며, 탈유비퀴틴화를 통하여 그 안정성을 조절함을 밝혔다. 또한 과발현된 USP13은 HMGB1의 세포질로의 이동과 세포 밖 분비도 유도했는데, 탈유비퀴틴화 효소 활성이 제거된 돌연변이 형의 USP13도 HMGB1 분비를 촉진시킴을 관찰할 수 있었다. 이는 USP13이 HMGB1의 세포 내 위치와 이동을 탈유비퀴틴화 효소 활성과는 무관하게 조절한다는 것을 의미한다. 결론적으로, USP13은 HMGB1의 안정성과 그 분비로 이어지는 위치 이동

을 조절하는 두 가지 역할을 한다는 것을 보고하는 바이다.

핵심되는 말: HMGB1, 탈유비퀴틴화, USP13, 분비