Complex of Fas-associated Factor 1 (FAF1) with Valosin-containing Protein (VCP)-Npl4-Ufd1 and Polyubiquitinated Proteins Promotes Endoplasmic Reticulum-associated Degradation (ERAD)*5

Jae-Jin Lee1,2, Joon Kyu Park1, Jaeho Jeong1, Hyesung Jeon5, Jong-Bok Yoon6, Eunice EunKyeong Kim3, and Jong-Joo Lee4

From the 4Center for Cell Signaling and Drug Discovery Research, College of Pharmacy, Division of Life and Pharmaceutical Sciences, Department of Bioinspired Science, Ewha Womans University, Seoul 120-750, Korea, 5Biomedical Research Institute, Korea Institute of Science and Technology, Seoul 136-791, Korea, and 6Department of Biochemistry and Translational Research Center for Protein Function Control, Yonsei University, Seoul 120-749, Korea

Background: FAF1, which has multiple ubiquitin-like domains, interacts with various proteins (VCP, Hsp70, and polyubiquitinated proteins).

Results: Association of FAF1 UBX with VCP-Npl4-Ufd1 complex regulates ubiquitin binding to FAF1 UBA domain and promotes CD36 degradation in ERAD.

Conclusion: FAF1 is a ubiquitin receptor that promotes ERAD by delivering polyubiquitinated proteins from UBX domain to UBA domain.

Significance: FAF1 plays a role in ERAD by modulating domain-domain interaction.

Fas-associated factor 1 (FAF1) is a ubiquitin receptor containing multiple ubiquitin-related domains including ubiquitin-associated (UBA), ubiquitin-like (UBL) 1, UBL2, and ubiquitin regulatory X (UBX). We previously showed that N-terminal UBA domain without direct interaction between UBA and UBX domains. These interactions are well characterized by structural and biochemical analysis. VCP-Npl4-Ufd1 complex is known as the machinery required for endoplasmic reticulum-associated degradation. We demonstrate here that FAF1 binds to VCP-Npl4-Ufd1 complex via UBX domain and polyubiquitinated proteins via UBA domain to promote endoplasmic reticulum-associated degradation.

FAF15 is a ubiquitin receptor containing a ubiquitin-associated (UBA) domain and three domains with ubiquitin-like folds, UBL1, UBL2, and ubiquitin regulatory X (UBX) domains. N-terminal UBA domain (47 amino acids long) recruits polyubiquitinated proteins crucial for FAF1-mediated apoptosis and stress response (1, 2). UBL1 binds to Hsp70 and regulates its chaperone activity by promoting Hsp70 degradation (3, 4). C-terminal UBX domain of FAF1 binds to valosin-containing protein (VCP), a molecular chaperone in the ubiquitin-proteasome system. VCP, the mammalian homologue of the multifunctional Cdc48p in yeast and p97 in Xenopus, is a member of the AAA (ATPase associated with a variety of cellular activities) family and acts as a molecular chaperone in diverse functions such as cell cycle regulation, apoptosis, transcription activation, organelle biogenesis, vesicular transport, and endoplasmic reticulum-associated degradation (ERAD) (5–8). In the performance of these functions, VCP uses two classes of cofactors, substrate-recruiting cofactors and substrate-processing cofactors. FAF1 is a substrate-recruiting cofactor, whereas E3 ligases and deubiquitinating enzymes are substrate-processing cofactors (9).

The ER is the organelle that produces secretory proteins to be properly folded before they are delivered to their functional

6998 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 288 • NUMBER 10 • MARCH 8, 2013

5 The abbreviations used are: FAF1, Fas-associated factor 1; VCP, valosin-containing protein; ERAD, endoplasmic reticulum-associated degradation; UBA, ubiquitin-associated; UBL, ubiquitin-like; UBX, ubiquitin regulatory X; ER, endoplasmic reticulum; aa, amino acids; Ni-NTA, nickel-nitrilotriacetic acid; UPLC-ESI-q-TOF, ultraperformance LC-electrospray ionization-quadrupole time of flight; ITC, isothermal titration calorimetry; Npl4, nuclear protein localization protein 4.
FLAG as described before (15). Because pYR-CD3
ment containing human CD3
constructed by introducing a PCR-generated BglII-XbaI frag-
serum (FBS) at 37 °C in 5% CO2. Transfections were carried out
imal essential medium) supplemented with 10% fetal bovine
HeLa cells were grown and maintained in DMEM (Eagle’s min-
EXPERIMENTAL PROCEDURES
strate CD3
Ufd1 complex is known to play a key role in ERAD, we investi-
VCP-Npl4-Ufd1 complex. Furthermore, because VCP-Npl4-
misfolded proteins and prevents their accumulation. The
destination. ERAD as a quality control for proteins eliminates
bodies from Abcam (Cambridge, UK); anti-ubiquitin antibody from Sigma; anti-VCP, anti-Npl4, and anti-Ufd1 anti-
Korea); and anti-Hsp70 antibody from Stressgene (British
Healthcare). Purified FAF1 proteins were dissolved in 50 mM
histidine-tagged proteins were purified using an Ni-NTA col-
and gel filtration on a Superdex-200 26/60 column (GE
preparation on a Superdex-200 26/60 column (GE
Healthcare). Purified FAF1 proteins were dissolved in 50 mM
HEPES, pH 7.4, 1.5 mM MgCl2, and 1mM DTT.
preparation to .pkl files using ProteinLynx Global ServerTM 2.3 data pro-
Raw data obtained from the mass spectrometer were converted
protease inhibitors (10 mM NaF, 10 mM Na3VO4) for 30
min on ice and centrifuged at 16,000 x g for 15 min. The super-
natant was incubated for 3 h at 4 °C with anti-FLAG-agarose
affinity beads. The beads were washed five times with 1 ml of
lysis buffer containing 0.5% Nonidet P-40 to remove nonspe-
cific binding, and the immune complex was solubilized in SDS
gel sample buffer, separated by 10% SDS-PAGE, and detected
FAF1(82–650) and
Human FAF1 was cloned into pFLAG-CMV2 vector to gener-
and HeLa cells were analyzed 72 h after siRNA transfection.
expression of FAF1 in HEK293T
instructions. Changes in the expression of FAF1 in HEK293T
0.1-mm inner diameter; NanoEaseTM dC18, Waters) cartridge. Peptides were separated using a C18
reversed-phase 75-
Full-length FAF1 (aa 1–650), UBL1-UBL2 domain (aa 100–
UAS-UBX domain (aa 308–650), UBX domain (aa 571–
UBX domain mutant TFPR → AG of FAF1 and
pNpl4 and FAF1, respectively, at a final concentration of
FAF1(TFPR → AG) were cloned into pET-22b (Novagen). The dissolved samples were desalted on line prior to separation
using a trap column (5-µm particle size; NanoEaseTM dC18,
preparation to .pkl files using ProteinLynx Global ServerTM 2.3 data pro-
Raw data obtained from the mass spectrometer were converted
to .pkl files using ProteinLynx Global ServerTM 2.3 data pro-
MS/MS spectra were matched
FAF1 Regulates ERAD via VCP-Npl4-Ufd1 Complex
FAF1(TFPR → AG), which shortened 618TFPR to
AG; FAF1(82–650) (aa 82–650); FAF1ΔUBX (aa 1–568); FAF1ΔUBL1 (aa Δ100–174); FAF1ΔUBL1−2 (aa Δ100–270); FAF1(TFPR → AG)ΔUBL1, FAF1(TFPR → AG) (aa Δ100–174); and FAF1(TFPR → AG)ΔUBL1−2, FAF1(TFPR → AG) (aa Δ100–270) constructs were generated based on structural
studies (14). CD36 expression plasmid, pYR-CD36-FLAG, was
constructed by introducing a PCR-generated BglII-Xbal frag-
ment containing human CD36 ORF into the same sites in pYR-
cFLAG as described before (15). Because pYR-CD36-FLAG
carries a tetracycline-regulated promoter, cells were co-trans-
fected with pYR-CD36-FLAG and the plasmid encoding tTS (pTet-Off, Clontech) to express CD36-FLAG.
Cell Extracts and Immunoprecipitation—HEK293T and
HeLa cells were grown and maintained in DMEM (Eagle’s min-
imal essential medium) supplemented with 10% fetal bovine
serum (FBS) at 37 °C in 5% CO2. Transfections were carried out
with Effectene (Qiagen) transfection reagent in a 1:10 ratio as
instructed by the manufacturer. For immunoprecipitation, cells
(2–10 × 10^6) were lysed with a hypotonic lysis buffer (10 mM
HEPES, pH 7.4, 1.5 mM MgCl2, 0.5% Nonidet P-40) containing protease inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1
µg/ml pepstatin, 100 µg/ml phenylmethylsulfonyl fluoride) and phosphatase inhibitors (10 mM NaF, 10 mM Na3VO4) for 30
min on ice and centrifuged at 16,000 x g for 15 min. The supernatant was incubated for 3 h at 4 °C with anti-FLAG-agarose
affinity beads. The beads were washed five times with 1 ml of
lysis buffer containing 0.5% Nonidet P-40 to remove nonspe-
cific binding, and the immune complex was solubilized in SDS
gel sample buffer, separated by 10% SDS-PAGE, and detected
with silver staining or Western analysis.
Preparation of FAF1, VCP, and Npl4-Ufd1 Heterodimer—
Full-length FAF1 (aa 1–650), UBL1-UBL2 domain (aa 100–
283), UAS-UBX domain (aa 308–650), UBX domain (aa 571–
650), and UBX domain mutant TFPR → AG of FAF1 and
full-length VCP (aa 2–806), VCP N-domain (aa 2–458), and
VCP N-domain (aa 2–208) were cloned into pET-28a (Novagen).
Ubiquitin fold domain of Npl4 (aa 1–80) was cloned into
pET-15b (Novagen). Full-length Ufd1 (aa 1–307) and Npl4 (aa
1–608) were cloned into pET-28a and pET-22b (Novagen).
GST-fused FAF1 UBA domain (aa 1–81), full-length VCP, and
Ufd1-Npl4 complex were purified as described previously (2, 33). The full-length FAF1 was overexpressed in Escherichia coli
Rosetta-gami (DE3) cells (Novagen), whereas the others were
overexpressed in E. coli Rosetta (DE3) cells. The N-terminal histidine-tagged proteins were purified using an Ni-NTA col-
umn (GE Healthcare) followed by thrombin treatment (Sigma)
and gel filtration on a Superdex-200 26/60 column (GE
Healthcare). Purified FAF1 proteins were dissolved in 50 mM
HEPES buffer, pH 7.5 containing 150 mM NaCl, whereas VCP
and Npl4-Ufd1 were dissolved in 50 mM HEPES buffer, pH 7.5
containing 150 mM NaCl, 5 mM MgCl2, and 1 mM DTT.
Silencing RNAs—ON-TARGETplus SMARTpool siRNAs and siControl duplex siRNA (Dharmacon) were used to knock
down Npl4 and FAF1, respectively, at a final concentration of
100 nM. Silencing was achieved using Dharma FECT1 transfe-
sion reagent (Dharmacon) according to the manufacturer’s
instructions. Changes in the expression of FAF1 in HEK293T
and HeLa cells were analyzed 72 h after siRNA transfection.
Protein Identification Using UPLC-ESI-q-TOF Tandem MS—
To identify the proteins and modifications, the gel bands were
destained and digested with trypsin, and the resulting peptides
were extracted as described previously (16). The peptide
extracts were evaporated to dryness in a SpeedVac and dis-
solved in 10% acetonitrile solution containing 1.0% formic acid.
The dissolved samples were desalted on line prior to separation
using a trap column (5-µm particle size; NanoEaseTM dC18,
Waters) cartridge. Peptides were separated using a C18
reversed-phase 75-µm-inner diameter × 150-mm analytical
column (3-µm particle size; AtlantisTM dC18, Waters) with an
integrated electrospary ionization SilicaTipTM (10-µm inner
diameter; New Objective). Chromatography was performed on
line to a mass spectrometer (Q-Tof UltimaTM Global, Waters).
Raw data obtained from the mass spectrometer were converted
to .pkf files using ProteinLynx Global ServerTM 2.3 data pro-
MARCH 8, 2013 • VOLUME 288 • NUMBER 10
JOURNAL OF BIOLOGICAL CHEMISTRY 6999
against amino acid sequences in Swiss-Prot. Large numbers and types of potential post-translational modifications were considered. All reported assignments were verified by automatic and manual interpretation of spectra from Mascot and MODi (17) in a blind mode.

**Isothermal Titration Calorimetry (ITC)**—ITC experiments were performed using VP-ITC and ITC200 instruments (MicroCal, Northampton, MA) at 298 K, and the data were analyzed using the program Origin 7.0. All samples (in 50 mM HEPES, pH 7.5, 150 mM NaCl) were centrifuged and degassed prior to the measurements at 298 K. The injectants were added at 150-s intervals to the sample solution in the cell.

**Electron Microscopy (EM)**—For EM, purified N-terminal His-tagged full-length FAF1 at 50 μg/ml in 50 mM HEPES buffer, pH 7.5 containing 150 mM NaCl was incubated with a 10-fold molar excess of 1.5-nm Ni-NTA-Nanogold (Nanoprobes Inc.) for 30 min, and the excess Ni-NTA-Nanogold was removed using a Superdex-200GL column (GE Healthcare). For generating FAF1 and VCP-Ufd1-Npl4 complex, gold-labeled FAF1 was incubated with purified VCP-Ufd1-Npl4 complex at a 1:1 molar ratio for 1 h at 4 °C, and the product was further purified on a Superdex-200S column to ensure removal of unbound FAF1 in 50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM AMP-PNP. The purified complex was diluted to 0.3 mg/ml concentration, then applied to glow-discharged carbon-coated grids, rinsed, and stained with 2% uranyl acetate. Images were recorded on a 2000 × 2000 charge-coupled device camera using a Tecnai F20 field emission gun electron microscope operated at 200 kV (FEI Co.). For imaging Nanogold (~1.5 nm in diameter) on the complex, a Tecnai Titan microscope was used at 300 kV without staining the sample (FEI Co.). To observe both gold particles and protein complex, we used a

---

**FIGURE 1. Constructs of FAF1 and VCP mutants studied.**

A, FAF1 WT; FAF1(TFPR→AG), VCP-interacting loop (618TFPR621) of FAF1 UBX domain was shortened to AG; FAF1(82–650), FAF1 UBA domain (1–47) was deleted; FAF1 ΔUBX, FAF1 UBX domain (569–650) was deleted; FAF1 ΔUBL1, FAF1 UBL1 domain (100–174) was deleted; FAF1 ΔUBL1–2, FAF1 UBL1 domain (100–174) and UBL2 domain (195–270) were deleted (Δ100–270); FAF1(TFPR→AG) ΔUBL1, UBL1 domain was deleted in FAF1(TFPR→AG) construct; FAF1(TFPR→AG) ΔUBL1–2, UBL1,2 domains were deleted in FAF1(TFPR→AG) construct; FAF1 I41N, point mutation of UBA domain at Ile⁴¹, which is crucial for ubiquitin interaction with N-terminal UBA domain (2). B, constructs of FAF1, VCP, and Npl4-Ufd1 complex used for structural and biochemical studies. Constructs used for the crystal structure are marked with a star.
cryotransmission EM method. For cryoexperiments, we diluted purified samples to a concentration of ~0.02–0.1 mg/ml for VCP-Npl4-Ufd1 with Nanogold-labeled FAF1 complex and loaded samples onto holey carbon film-supported grids and plunged froze them. We recorded images on a charge-coupled device camera (2000 × 2000 charge-coupled device camera, Gatan) using a Tecnai F20 field emission gun electron microscope operated at 200 kV. Particles were selected from the individual digital micrographs.

X-ray Crystallography of FAF1 UBX-VCP N-domain Complex—Diffraction quality crystals of FAF1 UBX domain and VCP N-terminal domain complex (see supplemental Fig. S1) were obtained from 100 mM sodium acetate, 1 mM LiCl, 30% PEG 4000. FAF1 UBX-VCP N-domain complex was methylated and crystallized following the procedure described earlier (18). The crystal structure was determined by the molecular replacement method (MOLREP from the CCP4 suite) (19) using the N-domain of the VCP ND1 crystal structure (Protein Data Bank code 1E32) (20) as a search model. The resulting electron density was good enough to orient the FAF1 UBX using CAPRA. Manual building using Coot (21) and refinement using CNS (22) gave the final models, which were assessed with PROCHECK (23). Crystallographic data collected and the refinement statistics are summarized in supplemental Table 1. The atomic coordinates and structure factors of FAF1 UBX-VCP N-domain complex have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ under accession code 3QWZ.

RESULTS

Binding of VCP to C-terminal UBX Domain of FAF1 Is Necessary for the Recruitment of Polyubiquitinated Proteins to N-terminal UBA Domain—To investigate the effect of VCP binding on the biological function of FAF1, we designed a mutant of FAF1 defective in VCP binding. A previous structural study showed that UBX has a well conserved FPR motif in the S3/S4 loop that is a VCP binding motif (14, 24) and that FAF1 also has an S3/S4 loop in UBX domain, 618TFPR621, that is a possible site for VCP binding of FAF1 UBX domain. We produced a mutant of UBX domain, FAF1(TFPR→AG), that has a shorter S3/S4 loop (618TFPR621 mutated to AG). HEK293T cells were transfected with control FLAG, FLAG-FAF1, and FLAG-FAF1(TFPR→AG), and the cell lysates were immunoprecipitated with anti-FLAG antibody, respectively. Immune complexes were detected with Western analysis using anti-FLAG antibody. As shown in Fig. 2A, FLAG-FAF1(TFPR→AG) with the deletion in the S3/S4 loop of UBX abrogated the interaction of FAF1 with VCP. Using the mutant defective in VCP binding, we investigated whether VCP binding to UBX domain affects the scaffolding ability of FAF1 to interact with Hsp70 through UBL1 domain and to recruit polyubiquitinated proteins through UBA domain. HEK293T cells overexpressing FLAG-FAF1 wild type (WT) and FLAG-FAF1(TFPR→AG) were lysed, and the cell lysates were immunoprecipitated with anti-FLAG antibody. The immune complexes were separated by 10% SDS-PAGE and detected with anti-Hsp70 and anti-ubiquitin antibodies. As shown in Fig. 2A, there were no discernible interaction changes of Hsp70 between FAF1 WT and FAF1(TFPR→AG). However, FAF1(TFPR→AG) could not recruit polyubiquitinated substrates to N-terminal UBA domain of FAF1. These results indicate that VCP binding to FAF1 is required to recruit polyubiquitinated proteins to UBA domain of FAF1. We therefore hypothesize that VCP binding to FAF1-UBX domain is necessary for the biological function of FAF1.

UBX Domain Regulates UBA Domain through VCP Binding without Direct Interaction—It was previously reported that ubiquitin-like folds mimic ubiquitin to interact with ubiquitin binding motifs such as UBA, ubiquitin-interacting motif (UIM), and proteasome subunits (25–27). To elucidate how UBX domain regulates UBA domain in recruiting polyubiquitinated proteins, we examined whether there is a direct interaction between UBLs and UBX with UBA domain, similar to ubiquitin-UBA domain interaction, because they both contain ubiquitin-like folds. We constructed various deletion mutants of UBX, UBL1, and UBL2 domains of FLAG-FAF1 WT and FLAG-FAF1(TFPR→AG) (Fig. 1) and examined whether ubiquitin-like folds inactivate UBA domain by direct interaction.

Because FLAG-FAF1(TFPR→AG) failed to recruit polyubiquitinated proteins, we examined whether ubiquitin-like folds of UBX domain directly inactivate UBA domain by assessing whether the FLAG-FAF1 UBX domain deletion mutant (FAF1ΔUBX) possesses ubiquitin recruiting function of UBA domain. HEK293T cells were transfected with FLAG-FAF1 WT, FLAG-FAF1(TFPR→AG), or FLAG-FAF1ΔUBX; cell lysates were immunoprecipitated with anti-FLAG antibody; and the immune complexes were detected by Western analysis using anti-ubiquitin, anti-FLAG, and anti-VCP antibodies. As shown in Fig. 2B, the recruitment of polyubiquitinated substrates through UBA domain disappeared in cells overexpressing FLAG-FAF1ΔUBX as well as FLAG-FAF1(TFPR→AG). This suggests that UBA domain is regulated by UBX domain through VCP binding rather than via direct UBX-UBA interaction mediated by ubiquitin-like folds of UBX domain.

FAF1 has two UBL domains (UBL1 (aa 100–176) and UBL2 (aa 195–270)) located next to UBA domain (aa 1–47). We examined whether these UBL domains directly interact with UBA domain. Such an interaction is possible based on structural considerations, similar to Rad23 and Dsk2 (26, 28). We constructed UBL domain deletion mutants of FAF1 WT and FAF1(TFPR→AG) (Fig. 1A) and examined the interaction of UBA domain with polyubiquitinated proteins. HEK293 cells were transiently transfected with FLAG-FAF1 WT, FLAG-FAF1(TFPR→AG), and UBL domain deletion constructs ∆UBL1 and ∆UBL1–2. The cell lysates were immunoprecipitated with anti-FLAG antibody, and the immune complexes were separated by 10% SDS-PAGE and detected with Western analysis using anti-FLAG, anti-VCP, and anti-ubiquitin antibodies. As shown in Fig. 2C, recruitment of polyubiquitinated proteins to FAF1 WT and FAF1(TFPR→AG) was not affected by deletion of UBL domains. This indicates that UBL domains do not play any role in this interaction. We also confirmed domain-domain interaction using various domain proteins: FAF1 WT, FAF1(82–650) as a UBA deletion mutant, FAF1 I41N as a mutant defective in UBA domain, and FAF1(TFPR→AG).
FIGURE 2. FAF1 UBX interaction with VCP regulates the recruitment of polyubiquitinated substrates to UBA domain without direct interaction with UBX or UBL domain(s). A, HEK293T cells were transfected with FLAG, FLAG-FAF1 WT, or FLAG-FAF1(TFPR→AG). At 24 h after transfection, lysates were immunoprecipitated (IP) with anti-FLAG antibody. Immune complexes were separated by 10% SDS-PAGE and detected by Western analysis using anti-FLAG, anti-VCP, anti-Hsp70, and anti-ubiquitin antibodies. Whole cell lysates were probed as input. B, immune complexes purified with anti-FLAG tag antibodies in HEK293T cells overexpressing FLAG, FLAG-FAF1 WT, FLAG-FAF1(TFPR→AG), or FLAG-FAF1∆UBX constructs were separated by SDS-PAGE, and proteins were detected by Western analysis using anti-FLAG, anti-VCP, and anti-ubiquitin antibodies. Whole cell lysates were probed as input. C, immune complexes purified with anti-FLAG antibodies in HEK293T cells overexpressing FLAG, FLAG-FAF1 WT, or the indicated constructs were separated by SDS-PAGE, and proteins were detected by Western analysis using anti-FLAG, anti-VCP, and anti-ubiquitin antibodies. D, HEK293T cells were transfected with various mutants including FLAG, FLAG-FAF1, FLAG-FAF1(82–650), FLAG-FAF1(TFPR→AG), and FLAG-FAF1 I41N. At 24 h after transfection, cell lysates were immunoprecipitated with anti-FLAG antibodies. Immune complexes were separated by 10% SDS-PAGE, and proteins were detected by Western analysis using anti-FLAG, anti-VCP, anti-Hsp70, and anti-ubiquitin antibodies.
AG) as a mutant defective in UBX domain (Fig. 2D). These results indicate that the mutant FAF1(TFPR → AG), which does not interact with VCP, also failed to recruit polyubiquitinated proteins like UBA deletion mutants.

Next, we determined the dissociation constants (K_{D}) between FAF1 UBA (aa 1–81) and UBX (aa 571–650) and between UBA and UBL1–2 (aa 100–280) using ITC to examine direct domain interactions (Table 1). FAF1 UBA domain did not interact with UBX domain or UBL1–2 domain, whereas UBA interacted with Lys^{48}-linked diubiquitin with a K_{D} value of 3.5 μM as we reported previously (2). Thus, it appears that UBX domain regulates UBA domain through VCP binding without direct domain interaction mediated by ubiquitin-like folds of UBX or UBL domains.

**FAF1 UBX Domain Interacts with VCP N-domain at a 1:1 Molar Ratio Using the S3/S4 Loop**—The crystal structure of FAF1 UBX domain complexed to N-terminal domain of VCP at 2.0-Å resolution shows that FAF1 UBX domain binds at the interface between the two barrels of VCP N-domain (Fig. 3A). The β1 and β5 as well as the loop connecting β3 and β4 of FAF1 UBX domain (S3/S4 loop) are involved in both hydrophobic and hydrophilic interactions (Fig. 3B). Hydrophobic interactions involve Phe^{585}, Phe^{619}, Pro^{620}, Pro^{640}, and Phe^{645} of FAF1 UBX domain and Val^{29}, Phe^{52}, Ile^{70}, Leu^{72}, Pro^{106}, Tyr^{110}, and Tyr^{143} of p97 N-domain. Hydrogen bonds are formed between Gln^{641} of FAF1 UBX domain and Gln^{43} and Asp^{47} of p97 N-domain.

**ITC data**

| Cell | Injectant | n | K_{D} (μM) | ΔH (kcal/mol) |
|------|-----------|---|------------|---------------|
| Lys-{diubiquitin} | FAF1 UBA | 0.79 ± 0.01 | 3.5 ± 0.2 | -7.7 ± 0.64 |
| UBL1-UBL2 | FAF1 UBA | ND | ND | ND |
| UBX | FAF1 UBA | ND | ND | ND |
| VCP N | FAF1 UBX | 1.13 ± 0.03 | 25.6 ± 2.1 | -4.3 ± 0.2 |
| VCP N | FAF1(TFPR → AG) | ND | ND | ND |
| VCP ND1 | Npl4 UBD | 1.04 ± 0.02 | 17.8 ± 1.4 | -5.1 ± 0.20 |
| VCP ND1 + ATP | FAF1 UBX | 0.72 ± 0.05 | 22.7 ± 1.1 | -5.0 ± 0.51 |
| VCP ND1D2 | FAF1 UBX | 0.61 ± 0.02 | 20.1 ± 1.7 | -5.1 ± 0.33 |
| VCP ND1D2 | FAF1 UAS-UBX | ND | ND | ND |
| VCP ND1D2 + Ufd1-Npl4 | FAF1 UBX | 0.23 ± 0.11 | 0.84 ± 0.1 | -23.4 ± 13.83 |
| VCP ND1D2 + Ufd1-Npl4 + ATP | FAF1 UBX | 0.27 ± 0.08 | 0.96 ± 0.2 | -18.3 ± 07.03 |
| VCP ND1D2 + Ufd1-Npl4 + ATP | FAF1 UAS-UBX | 0.25 ± 0.09 | 19.5 ± 4.2 | -28.0 ± 16.47 |

show that the highly conserved motif 618-TPFR621 in the S3/S4 loop of FAF1 UBX domain is crucial for binding to the N-domain of VCP.

**FAF1 Interacts with the Substrate-recruiting Cofactor Npl4-Ufd1 Heterodimer via VCP**—To characterize further the proteins that interact with FAF1, we performed immunoprecipitation on a large scale using anti-FLAG affinity beads from lysates of HEK293T cells overexpressing FLAG or FLAG-FAF1. Immune complexes were separated by 10% SDS-PAGE, and the FAF1-interacting protein was detected after silver staining (Fig. 4A). Using peptide sequencing analysis with UPLC-ESI-q-TOF tandem MS, we identified the FAF1-interacting protein as Npl4 (Fig. 4B). Because it is well known that Npl4 forms heterodimers with Ufd1 and binds to VCP through the ubiquitin fold domain (31), we confirmed the binding of Npl4-Ufd1 complex to FAF1 by Western analysis of FLAG-FAF1 immune complex with anti-Npl4, anti-Ufd1, and anti-VCP antibodies as reported previously (32). Interaction of FAF1 with Npl4-Ufd1 heterocomplex is shown in Fig. 4C. To further investigate whether FAF1 interacts directly with Npl4-Ufd1 heterodimer or through VCP, we performed immunoprecipitation of HEK293T cells overexpressing FLAG-FAF1 WT and FLAG-FAF1(TFPR → AG) with anti-FLAG antibody. As shown in Fig. 4D, FAF1(TFPR → AG), a VCP binding-deficient mutant did not interact with Npl4-Ufd1 heterodimer, suggesting that Npl4-Ufd1 heterodimer interacts with FAF1 through VCP.

We further examined the Ufd1-Npl4 dependence. The binding between full-length as well as various fragments of VCP (see Fig. 1B) and FAF1 was examined using ITC, and the results are summarized in Table 1. When VCP was presented as ND1, UBX domain binds with an apparent K_{D} of 20.1 μM in the presence of ATP and 22.7 μM in the absence of ATP, but the stoichiometry was no longer 1:1 (supplemental Fig. S2, B and C). These values are in good agreement with value of 30 μM reported previously (9). On the other hand, when the full-length VCP (denoted as VCP ND1D2) was titrated with FAF1 UBX domain, there was no detectable binding whether ATP was present or not (Fig. 4E). However, if VCP ND1D2 was complexed with Npl4-Ufd1 heterodimer, FAF1 UBX domain bound with a slightly increased binding affinity with a K_{D} value of 9.6 μM (Fig. 4F) with one or two FAF1 UBX domains bound to one VCP-Npl4-Ufd1 complex. We tested this further by extending...
the C-terminal end of FAF1 to include the UAS domain (i.e., FAF1 UAS-UBX) and found that its binding pattern with VCP ND1D2 was quite similar. That is, FAF1 UAS-UBX did not show any binding to full-length VCP alone but did bind to the VCP-Npl4-Ufd1 complex with a corresponding $K_D$ value of 19.5 M (supplemental Fig. S2, D and E). These results clearly suggest that prior binding of Npl4-Ufd1 to VCP is necessary for the interaction of FAF1 UBX domain with VCP.

Visualization of FAF1 Binding to VCP-Npl4-Ufd1 Complex by EM—The complex formation of VCP-Npl4-Ufd1 and FAF1 was initially established by size exclusion chromatography and SDS-PAGE studies (supplemental Fig. S3). To confirm the stoichiometry of the VCP-Npl4-Ufd1-FAF1 complex observed in ITC data, we tried EM imaging after gold labeling on the purified FAF1. Examination of VCP alone revealed a closed form with a hole in the center, consistent with a top view of the hexameric ring (33). In contrast to the relatively uniform fields observed for VCP, the VCP-Npl4-Ufd1-FAF1 complex revealed more irregular particles and additional density at the periphery of the VCP ring (Fig. 5A). The irregularity of the complex likely stems from the flexible, elongated structure of FAF1, which may also promote a more variable orientation of the complex on the grid (Fig. 5B). For stoichiometry, we labeled FAF1 with Nanogold and examined the reconstituted complex by high resolution and cryoelectron microscopy. Nanogold particles were readily visualized in association with the complex at 300 kV, revealing a single gold particle and thus a single molecule of FAF1 per complex (Fig. 5C). We also used the cryo-method to confirm both densities from the gold particle and protein complex because the sample used for high resolution EM was not stained to show enough protein density (Fig. 5D). 1.4–1.8-nm Nanogold particles were difficult to recognize in the conventional negatively stained EM but could be seen in the unstained complex with a ratio of one particle per complex when we used high resolution EM and cryotransmission EM. Arrows in Fig. 5 indicate the Nanogold-labeled FAF1 on the VCP-Npl4-Ufd1 complex. We tried the labeling with larger gold particles of 5-nm diameter, but the complex was not stable enough to go through the gel filtration step to get rid of excess gold particles.
Npl4-Ufd1 Heterodimer Is Required for VCP-FAF1 Interaction—VCP functions as a molecular chaperone by interacting with diverse cofactors (9). We showed that VCP binding is crucial for the scaffolding ability of FAF1 and that prior binding of Npl4-Ufd1 heterodimer to VCP is crucial for FAF1 binding to VCP. To confirm this, we examined FAF1-VCP interaction in HeLa cells in which Npl4 was knocked down with its specific siRNA. These cells were transfected with FLAG or FLAG-FAF1, the cell lysates were immunoprecipitated with anti-FLAG antibody, and the immune complexes were analyzed by Western analysis using anti-FLAG, anti-VCP, anti-Npl4, and anti-Ufd1 antibodies. We found that VCP could not bind to FAF1 in HeLa cells in which Npl4 was knocked down and that polyubiquitin binding to UBA domain was abolished as happened with mutant FAF1(TFPR→AG) (Fig. 6). This suggests that Npl4-Ufd1 heterodimer is crucial for the biological function of FAF1 conjugated to VCP.

FAF1 Promotes ER-associated Degradation via Ubiquitin Receptor Function—It is known that VCP-Npl4-Ufd1 complex plays a key role in ERAD. When ubiquitinated misfolded proteins are translocated through the ER membrane, VCP-Npl4-Ufd1 complex delivers them to the proteasome (12, 13, 34). Because FAF1 strongly binds to VCP and this binding is regulated by various stresses including heat shock (1) and by complex formation with Npl4-Ufd1, we examined the role of FAF1 in the ERAD pathway. Using the CD3δ Tet-Off system (pYR-CD3δ-FLAG co-transfected with pTet-Off) as a classical ERAD substrate, we monitored the degradation rates of CD3δ in HeLa
cells overexpressing FLAG or FLAG-FAF1. We blocked the synthesis of CD3\(\beta\) by treating the cells with 50 \(\mu\)g/ml doxycycline and then measured the degradation rates of CD3\(\beta\) through ERAD. To confirm that the Tet-Off system could be used to monitor CD3\(\beta\) degradation, we assessed CD3\(\beta\) degradation by treating the cells with 50 \(\mu\)g/ml doxycycline alone or together with 10 \(\mu\)g/ml cycloheximide. Supplemental Fig. S4 shows that the pattern of CD3\(\beta\) degradation promoted by FAF1 was the same whether the cells were treated with 50 \(\mu\)g/ml doxycycline alone or with doxycycline plus 10 \(\mu\)g/ml cycloheximide. Fig. 7A shows that FAF1 promoted CD3\(\beta\) degradation and is thus a component of the ERAD machinery. To further validate the function of FAF1 in ERAD, we examined ERAD in cells in which FAF1 was knocked down. Supplemental Fig. 5A shows that knocking down FAF1 did not affect protein levels of Npl4 and Ufd1. When HeLa cells in which FAF1 was knocked down or control cells treated with non-targeting siRNA were co-transfected with pYR-CD3\(\beta\)-FLAG and pTet-Off and treated with doxycycline 24 h after transfection, the degradation of CD3\(\beta\) was attenuated in cells in which FAF1 was knocked down (Fig. 7B). Degradation of CD3\(\beta\), which was reduced when FAF1 was knocked down, was restored in cells overexpressing FLAG-FAF1, confirming that FAF1 plays a role in ERAD (Fig. 7C). We further investigated the role of FAF1 in ERAD as a ubiquitin receptor by examining the degradation rates of CD3\(\beta\) in HeLa cells respectively overexpressing the following mutants of FAF1: FLAG-FAF1 WT, FLAG-FAF1(82–650) (UBA-deleted mutant), or FLAG-FAF1(TFPR\(^3\)AG) (VCP binding-defective mutant). As shown in Fig. 7D, neither FLAG-FAF1(82–650) nor FLAG-FAF1(TFPR\(^3\)AG) promoted CD3\(\beta\) degradation like FAF1 WT. FLAG-FAF1(82–650), the UBA deletion mutant having intact UBX to bind to VCP, and FAF1(TFPR\(^3\)AG), the mutant without the ability to bind to VCP and consequently its UBA function, did not affect the rate of ERAD. This suggests that FAF1 as a ubiquitin receptor plays a role in ERAD and that both UBA and UBX domains are required for promoting ERAD.

**FAF1 Promotes ER-associated Degradation in a VCP-Npl4-Ufd1-dependent Manner**—We further investigated the VCP-Npl4-Ufd1-related function of FAF1 in the ERAD pathway by examining the connection of CD3\(\beta\) degradation to VCP-Npl4-Ufd1 complex. We monitored CD3\(\beta\) degradation in HeLa cells in which Npl4 was knocked down with siRNA. Knocking down Npl4 did not affect the expression level of FAF1 but did decrease the level of Ufd1 (supplemental Fig. 5A) as reported previously (35). HeLa cells treated with non-Npl4-targeting siRNA (control) or Npl4 siRNA were co-transfected with pYR-CD3\(\beta\)-FLAG, pTet-Off, and FLAG-FAF1 or FLAG-FAF1(82–650) complex.
We demonstrated here that binding of FAF1 to VCP-Npl4-Ufd1 complex via UB domain is a prerequisite to the binding of polyubiquitinated proteins to UBA domain of FAF1 and that only intact FAF1 complex plays a role in ERAD. Our study shows that VCP N-domain interacts with C-terminal UB domain of FAF1, thereby facilitating the recruitment of polyubiquitinated proteins to N-terminal UB domain of FAF1. Fig. 3A, which depicts the crystal structure of the FAF1 UB-VCP N-domain complex, shows that the major interaction between the two involves the S3/S4 loop of the FAF1 UB domain. ITC results also confirmed that 618TFPR621 is critical for VCP binding because the 618TFPR621→AG mutation showed no detectable binding between the two. Furthermore, the crystal structure studies revealed that the cis-Pro620-centered β-turn of 618TFPR621 is important in the interaction.

We also showed that the Npl4-Ufd1 heterodimer is essential for VCP-FAF1 interaction. No direct binding of FAF1 UB domain to full-length VCP was observed in ITC studies. This was true even when UB domain was extended further to include a UAS domain. However, when VCP and Npl4-Ufd1 complex were used, the FAF1 UB domain titration showed binding at a molar ratio of about 1:6 with $K_d$ values of 9.6 and 19.5 μM, respectively. The EM study (Fig. 5) also clearly showed a single gold bead per VCP-Npl4-Ufd1-FAF1 complex, suggesting one FAF1 bound to the VCP-Npl4-Ufd1 complex. These results demonstrate that full-length FAF1 binds at a 1:6 molar ratio with full-length VCP but not at a 1:1 molar ratio, i.e., one FAF1 binding to a hexameric unit of VCP. Furthermore, FAF1 binding to VCP required prior binding of Npl4-Ufd1 to VCP.

Intriguingly, FAF1(TFPR→AG), a mutant deficient in VCP binding via C-terminal UB, could not recruit polyubiquitinated proteins to N-terminal UB domain (Fig. 2). We also showed by monitoring direct domain-domain interactions of various FAF1 mutants such as UBL and UB deletion mutants by ITC that FAF1 UBA domain does not directly interact with UBL1 and UBL2 or UB domain. Together with our previous findings that UBA domain is crucial for FAF1-mediated apoptosis and proteasomal inhibition (1) and that UBA domain facilitates Lys48-linked polyubiquitinated protein recruitment (2), we can conclude that UBA is the main functional domain of FAF1 and that UBA is regulated by UB binding to VCP already bound to Npl4-Ufd1 heterodimer. However, there is no direct interaction between UBA and UB domains.

Because UB domain is module that binds to VCP, many UB-containing proteins can promote VCP-mediated processes by changing the cofactors. In yeast, membrane-bound UBA-UB protein, Ub2 membrane-bound UBA-UB protein, recruits Cdc48-Npl4-Ufd1 to the ER membrane to perform ERAD (35–37). p47, another UB protein, acts as a cofactor for p97-mediated membrane fusion by forming a p97-p47 complex.
A recent study showed that various UBA-UBX proteins bind to ubiquitin ligases and that UBXD7 promotes binding of HIF1α to p97 by selectively interacting with von Hippel-Lindau tumor suppressor (pVHL) (32). How VCP performs its specific functions together with these various cofactors should be further studied. This concept of cooperative regulation between domains (UBX-UBA) in a ubiquitin receptor has not been reported before.

We examined the role of FAF1 in ERAD because FAF1 interacts with VCP-Npl4-Ufd1 complex, which is known to be involved in ERAD. ERAD was measured in cells that either overexpressed FAF1 or in which FAF1 was knocked down by monitoring degradation of CD3δ, an ERAD substrate. Overexpression of FAF1 promoted ERAD, whereas depletion of FAF1 retarded it (Fig. 7, A and B). These effects were examined again in cells in which FAF1 was knocked down. Wild type FAF1, FAF1(82–650) (UBA domain deletion mutant), and FAF1(TFPR3AG) (a mutant deficient in the interaction with VCP and polyubiquitinated proteins) were reintroduced in these cells. Although FAF1 WT accelerated CD3δ degradation,
FAF1 Regulates ERAD via VCP-Npl4-Ufd1 Complex

Our studies by examining the interaction between UBX and VCP-Npl4-Ufd1 complex. The FAF1 UBX-(VCP-Npl4-Ufd1) interaction is required for the substrate specificity of UBA domain. We propose that FAF1 promotes ERAD in a VCP-Npl4-Ufd1-dependent manner. This was confirmed in cells in which Npl4 was knocked down (Fig. 8). Because most cellular Npl4 interacted with FAF1 in immunoprecipitation with anti-FAF1 antibody (data not shown), Npl4 appears to be a major regulator of FAF1. We examined the effect of FAF1 on ERAD in cells in which Npl4 was knocked down. Effects of FAF1 on ERAD were completely abolished in these cells (Fig. 8). This finding implies that FAF1 exerts its biological function in ERAD by first interacting with VCP-Npl4-Ufd1 complex and then recruiting polyubiquitinated proteins to UBA domain. Specific scaffolding characteristics of FAF1, which are necessary for its biological role in ERAD, are finely regulated via changes in protein-protein interactions. The molecular mechanisms underlying this regulation should be studied further.

So far, UBL-UBA proteins Rad23 and Dsk2 have been proposed as proteasome-targeting factors that mediate ERAD (39, 40). It has been shown that UBA domain recruits polyubiquitinated ERAD substrates and that UBL domain interacts with proteasome subunits to deliver substrates. FAF1 also interacts with 26 S proteasome subunits including 20 S core particles (proteasome subunit α-3,4,5,6) and parts of 19 S regulatory particles (Rpn1, -2, -5, -12, and Rpt2,3) (data not shown). The present study proposes that FAF1 is a scaffolding ubiquitin receptor involved in ERAD by regulating protein-protein interaction through UBA and UBX domains and delivering ERAD substrates to the proteasome. However, the proteasome-interacting motif has yet to be identified.

FAF1 was reported to suppress NF-κB activity by interfering with nuclear translocation of the RelA subunit of NF-κB and to inhibit IkB kinase activation by interacting with p65 and IkB kinase (41). A Drosophila homolog of FAF1 called Caspar was also found to inhibit NF-κB. Caspar negatively regulates immune deficiency (Imd) responses by blocking nuclear translocation of NF-κB (42). However, no role for FAF1 as a ubiquitin receptor in NF-κB inactivation has been described, although such a role for FAF1 was demonstrated in other signaling pathways through interaction with various binding partners. Using the artificial ubiquitin-proteasome system substrate ubiquitin-X-GFP, we previously found that FAF1 inhibits proteasomal degradation through its UBA domain (1). In this study, we demonstrated that FAF1 UBA domain specificity is ensured by UBX interaction with VCP-Npl4-Ufd1 complexation. FAF1 seems to promote ERAD, whereas nonspecific ubiquitin-proteasome system substrates retard degradation. Nonetheless, the substrate specificity of FAF1 UBA domain is hard to explain because many types of polyubiquitinated proteins including Hsp70 and β-catenin have been shown to interact with FAF1 UBA, and their degradation is regulated by FAF1 (4). One possibility is that the specific UBX-interacting complex provides the substrate specificity of UBA domain by delivering specific polyubiquitinated proteins to FAF1.

In summary, both in vivo and in vitro data suggest that VCP-Npl4-Ufd1 complex selectively interacts with UBX domain of FAF1, which in turn regulates the recruitment of polyubiquitinated substrates to FAF1 UBA domain. We propose that FAF1-UBA domain is a ubiquitin receptor for ERAD regulated by the interaction between UBX and VCP-Npl4-Ufd1 complex. The multiple functions of FAF1 should be investigated in further studies by examining the interaction between UBX and VCP-
Npl4-Ufd1 complex and characterizing the various complexes of FAF1 formed inside cells in response to various stresses.

Acknowledgments—We thank Dr. Kyung Eun Lee at Advanced Analysis Center at Korea Institute of Science and Technology for help with electron microscope and the Advanced Analysis Center at Korea Institute of Science and Technology for use of the transmission electron microscope.

REFERENCES

1. Song, E. J., Yim, S. H., Kim, E., Kim, N. S., and Lee, K. J. (2005) Human Fas-associated factor 1, interacting with ubiquitinated proteins and valosin-containing protein, is involved in the ubiquitin-proteasome pathway. *Mol. Cell. Biol.* **25**, 2511–2524

2. Song, J., Park, J. K., Lee, J. J., Choi, Y. S., Ryu, K. S., Kim, J. H., Kim, E., Lee, K. I., Jeon, Y. H., and Kim, E. E. (2009) Structure and interaction of ubiquitin-associated domain of human Fas-associated factor 1. *Protein Sci.* **18**, 2265–2276

3. Kim, H. J., Song, E. J., Lee, Y. S., Kim, E., and Lee, K. J. (2005) Human Fas-associated factor 1 interacts with heat shock protein 70 and negatively regulates chaperone activity. *J. Biol. Chem.* **280**, 8125–8133

4. Lee, J. J., Kim, Y. M., Jeong, J., Bae, D. S., and Lee, K. J. (2012) Ubiquitin-associated (UBA) domain in human Fas associated factor 1 inhibits tumor formation by promoting hsp70 degradation. *PLoS One* **7**, e40361

5. Rape, M., Hoppe, T., Gorl, I., Kalocay, M., Richly, H., and Jentsch, S. (2001) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48 (UFD1/NPL4), a ubiquitin-selective chaperone. *Cell* **107**, 667–677

6. Wang, Q., Song, C., and Li, C. C. (2004) Molecular perspectives on p97-VCP: progress in understanding its structure and diverse biological functions. *J. Struct. Biol.* **146**, 44–57

7. Woodman, P. G. (2003) p97, a protein coping with multiple identities. *J. Cell Sci.* **116**, 4283–4290

8. Ye, Y. (2006) Diverse functions with a common regulator: ubiquitin takes command of an AAA ATPase. *J. Struct. Biol.* **156**, 29–40

9. Schuberth, C., and Buchberger, A. (2008) UBX domain proteins: major regulators of the AAA ATPase Cdc48/p97. *Cell. Mol. Life. Sci.* **65**, 2360–2371

FIGURE 9. A model depicting selective interaction of VCP-Npl4-Ufd1 complex with FAF1 to promote ERAD. A, FAF1 selectively interacts with VCP-Npl4-Ufd1 complex through UBX domain, and this interaction regulates the recruitment of polyubiquitinated substrate proteins to UBA domain. B, FAF1 promotes ERAD by interacting with VCP-Npl4-Ufd1 complex and polyubiquitinated proteins and delivering ERAD substrates to the proteasome. FAF1 is a ubiquitin scaffolding receptor that promotes ERAD by regulating domain interactions through UBX and UBA domains. N, Npl1; U, Ufd1; Ub, ubiquitin.
SUPPLEMENTARY TABLE 1

Crystallographic data and refinement statistics

| FAF1 UBX-VCP N-domain complex |
|--------------------------------|
| **Data collection**            |
| Wavelength                     | 1.0000 Å (PLS, BL4A MXW) |
| Resolution range, Å            | 20.0-2.0 (2.07-2.00)a     |
| Space group                    | C2                        |
| Unit cell parameters           |                           |
| $a = 67.490$                   |                           |
| $b = 59.938$                   |                           |
| $c = 75.541$                   |                           |
| $\alpha = 90$                  |                           |
| $\beta = 114.83$               |                           |
| $\gamma = 90$                  |                           |
| Total/unique reflections       |                           |
| Completeness, %                |                           |
| Mean $I/\sigma(I)$, %          |                           |
| $R_{merge}$, %                 |                           |
| $R / R_{free}$                 |                           |
| No. of protein atoms           |                           |

| Resolution range, Å            | 20-2.00                   |
| $R / R_{free}$                 | 22.5/24.9                 |
| No. of protein atoms           | 2088                      |
|                                |          |
|--------------------------------|----------|
| No. of water molecules         | 163      |
| R.m.s.d. from ideality         |          |
| Bond lengths, Å                | 0.0053   |
| Bond angles, °                 | 1.3009   |
| Ramachandran analysis          |          |
| Allowed region, %              | 88.5     |
| Additional allowed, %          | 11.5     |
| Generously allowed, %          | 0        |
| Disallowed region, %           | 0        |

*Values in parentheses are for the outer most resolution shell.

\[ R_{merge} = \frac{\sum_h \sum_i |I(h,i) - <I(h)>|}{\sum_h \sum_i I(h,i)}, \] where \( I(h,i) \) is the intensity of the \( i^{th} \) measurement of reflection \( h \) and \( <I(h)> \) is the mean value of \( I(h,i) \) for all \( i \) measurements.

\[^c]R_{free} \text{ was calculated from randomly selected 10\% set of reflections not included in the calculation of the } R \text{ value.}\]
SUPPLEMENTARY FIGURE LEGENDS

FIGURE S1. In vitro lysine methylation in FAF1 UBX-VCP N-domain complex. Electron density shows clear methylation at positions Lys614 and Lys649 of FAF1 UBX domain and Lys62 and Lys164 of VCP N-domain. Three of them are located at the crystallographic interfaces contributing further molecular contact. The chemical modification certainly facilitated the crystallization of otherwise intractable.

FIGURE S2. ITC studies of Npl4 and FAF1 binding to VCP. ITC results of (A) VCP N-domain + Npl4 UBD, (B) VCP ND1 domain + FAF1 UBX, (C) VCP ND1 domain in the presence of ATP + FAF1 UBX domain (D) VCP ND1D2 in the presence of ATP + FAF1 UAS-UBX domain, (E) VCP ND1D2 complexed with Ufd1-Npl4 in the presence of ATP + FAF1 UAS-UBX domain.

FIGURE S3. Purification of VCP-Ufd1-Npl4-FAF1 quaternary complex. (A) Profile of size exclusion chromatography. (B) SDS-PAGE of purified Ufd1-Npl4 alone, gold labeled FAF1 alone, and VCP-Ufd1-Npl4-FAF1 quaternary complex.

FIGURE S4. Effects of treatment with cycloheximide and doxycycline on degradation of CD3δ. We monitored CD3δ degradation by Tet-off system by treating doxycycline, alone, or with cycloheximide and with doxycycline. HeLa cells were co-transfected with Flag vector or Flag-FAF1 and tet-off–able CD3δ (pYR-CD3δ-Flag and pTet-off). At 24 h after transfection, cells were treated with 50 µg/mL doxycycline, alone, or with 50 µg/mL doxycycline and, with 10 µg/mL cycloheximide for indicated time points (0, 1 and 2 h). Proteins were analyzed by Western analysis using anti-Flag, anti-GAPDH antibodies. GAPDH served as a loading control. The amounts of CD3δ remaining after Dox treatment were determined by MultiGauge V3.0 (Fuji Film).

FIGURE S5. Protein expression changes in cells in which FAF1 (A) or Npl4 (B) is knocked down. Knocking down of FAF1 does not affect other proteins (A), and knocking down of Npl4 (B) doesn’t affect cellular protein expression level of FAF1, but lowers the expression of Ufd1.
Supplementary Figure S3

A

VCP-Ufd1-Npl4-goldFAF1

Void

goldFAF1

B

marker  Ufd1-Npl4  goldFAF1  Complex

97 kDa

66 kDa

36 kDa
Supplementary Figure S4

### Western Blot Analysis

**Flag**

- **Dox+CHX**
  - 0, 1, 2
  - Anti-Flag (FAF1)
  - Anti-Flag (CD3δ)
  - Anti-GAPDH

- **Dox**
  - 0, 1, 2
  - Anti-Flag (FAF1)
  - Anti-Flag (CD3δ)
  - Anti-GAPDH

**Flag-FAF1 WT**

- **Dox+CHX**
  - 0, 1, 2
  - Anti-Flag (FAF1)
  - Anti-Flag (CD3δ)
  - Anti-GAPDH

- **Dox**
  - 0, 1, 2
  - Anti-Flag (FAF1)
  - Anti-Flag (CD3δ)
  - Anti-GAPDH

### Graphical Representation

**Remaining of CD3δ (%)**

- **Flag (Dox+CHX)**
- **Flag-FAF1 (Dox+CHX)**
- **Flag (Dox)**
- **Flag-FAF1 (Dox)**

**Time (h)**: 0, 0.5, 1, 1.5, 2, 2.5

**Remaining of CD3δ (%)**: 0, 100, 200
Supplementary Figure S5

A

| siRNA | Control | FAF1 |
|-------|---------|------|
|       |         |      |

Anti-FAF1
Anti-GAPDH
Anti-Ufd1
Anti-Npl4

B

| siRNA | Control | Npl4 |
|-------|---------|------|
|       |         |      |

Anti-Npl4
Anti-Ufd1
Anti-GAPDH
Anti-FAF1
Complex of Fas-associated Factor 1 (FAF1) with Valosin-containing Protein (VCP)-Npl4-Ufd1 and Polyubiquitinated Proteins Promotes Endoplasmic Reticulum-associated Degradation (ERAD)
Jae-Jin Lee, Joon Kyu Park, Jaeho Jeong, Hyesung Jeon, Jong-Bok Yoon, Eunice EunKyeong Kim and Kong-Joo Lee

J. Biol. Chem. 2013, 288:6998-7011.
doi: 10.1074/jbc.M112.417576 originally published online January 4, 2013

Access the most updated version of this article at doi: 10.1074/jbc.M112.417576

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2013/01/04/M112.417576.DC1.html

This article cites 42 references, 13 of which can be accessed free at
http://www.jbc.org/content/288/10/6998.full.html#ref-list-1