The lysosome pathway degrades CD81 on the cell surface by poly-ubiquitination and clathrin-mediated endocytosis

Kohei Hosokawa,\textsuperscript{a} Hanako Ishimaru,\textsuperscript{a} Tadashi Watanabe,\textsuperscript{a} Masahiro Fujimuro\textsuperscript{* a}

\textsuperscript{a}Department of Cell Biology, Kyoto Pharmaceutical University, Kyoto 607-8412, Japan

*To whom correspondence should be addressed:
Masahiro Fujimuro: Department of Cell Biology, Kyoto Pharmaceutical University, Kyoto 607-8412, Japan;

fuji2@mb.kyoto-phu.ac.jp; TEL:+81-75-595-4717; FAX:+81-75-595-4793.
SUMMARY

CD81 is a highly conserved four-transmembrane protein in mammals and widely expressed on many tissues. It belongs to the tetraspanin family and forms complexes with various cell surface membrane proteins. It also functions in cell migration and B-cell activation, which is induced by CD81 complexing with CD19, CD21 and the B-cell receptor. Thus, CD81 is thought to play a key role in regulating cell function and fate. However, little is known about the degradation mechanism of CD81. Here we found that CD81 on the plasma membrane is degraded by the lysosome pathway via endocytosis. The expression levels of CD81 in HEK293T cells treated with a proteasome inhibitor (lactacystin) and lysosome inhibitors (chloroquine and bafilomycin A1) were analyzed by flow cytometry. The expression of CD81 on the cell surface was increased by the lysosome inhibitors, but not lactacystin. A pulldown assay revealed that CD81 was conjugated with a K63- and K29-linked poly-ubiquitin chain before its degradation, and the poly-ubiquitination site was Lys8 at the N-terminal intracellular domain of CD81. Furthermore, mutant CD81, in which Lys8 was substituted with Ala, extended the CD81 half-life compared with wildtype. CD81 was mainly localized on the plasma membrane in normal cells, but also co-localized with lysosomal LAMP1 and early endosomal EEA1 in chloroquine-treated cells. Furthermore, a
clathrin-mediated endocytosis inhibitor, chlorpromazine, stabilized CD81 expression on the cell surface. Hence, we demonstrated that CD81 is internalized by clathrin-mediated endocytosis and subsequently degraded via a lysosome pathway requiring the K63- and K29-linked poly-ubiquitination of CD81.

Keywords: CD81, tetraspanin family, ubiquitin, lysosome, poly-ubiquitination, endocytosis
INTRODUCTION

CD81 is a four-transmembrane protein that is composed of two extracellular loops and three short intracellular domains.\(^1\sim3\) CD81 belongs to the tetraspanin family, which modulates biological processes such as cell adhesion, motility, activation and proliferation. Tetraspanin molecules can laterally associate with one another and cluster with multiple cell surface receptors.\(^4\) On B cells, CD81 is required for CD19 maturation and surface expression.\(^5\) Additionally, CD81 interacts with CD19, CD21 and B-cell receptor (BCR) to form a complex that induces B cell activation by amplifying receptor signaling. CD81 expression also is heavily involved in a number of other cell functions including cell motility and signal transduction.\(^2\sim3\) Although CD81 turnover is thought to depend on the proteasome and lysosome pathways, little is known about the CD81 degradation mechanism.\(^6\sim7\)

In eukaryotes, the two major organelles for proteolysis, the proteasome and lysosome, play a key role in the manifestation and/or maintenance of physiological functions, e.g., cell growth, signal transduction, immune response, protein quality control, cellular homeostasis, and so on. The proteasome pathway is regulated by poly-ubiquitination of the substrate. Poly-ubiquitination is catalyzed by the sequential actions of ubiquitin (Ub)-activating enzyme (E1), Ub-conjugating enzyme (E2) and E3 Ub ligase, and the
poly-ubiquitin chain is classified as degradation and non-degradation signals.\(^8, 9\) Ub has seven internal Lys residues at positions 6, 11, 27, 29, 33, 48, and 63. The poly-ubiquitination linked through Lys48 of Ub and C-terminus of Ub (K48-linked poly-ubiquitination) functions as a signal for degradation by the 26S proteasome, whereas poly-ubiquitination linked through the other Lys is involved in protein-protein interactions, endocytosis, and lysosomal degradation. The type of poly-Ub chain (e.g., K63- or K48-linked) is mainly defined by acting E2 and E3 Ub ligase.\(^8, 9\) In the cases of membrane proteins such as notch receptor, interferon receptor, and MHC class-I, Lys29- and Lys63-linked poly-Ub chains have been implicated for protein transport to the late endosome and lysosome.\(^10-12\) The poly-ubiquitinated membrane proteins are then internalized by endocytosis and subsequently degraded by acidic hydrolases including cathepsins in the lysosomes.\(^13\)

Only two studies have investigated how CD81 is degraded.\(^6, 7\) The gene related to anergy in lymphocytes (GRAIL) induces Lys48-linked poly-ubiquitination of CD81, causing CD81 instability.\(^6\) On the other hand, membrane-associated RING-CH (MARCH) induces the downregulation of CD81 from the cell surface and its localization to the lysosome.\(^7\) MARCH is also the E3 Ub ligase that mediates Lys63-linked poly-ubiquitination for MHC class-I. In the current study, we looked deeper into the effects of the proteasome, lysosome,
and ubiquitination on CD81 degradation and found that CD81 on the cell surface is conjugated by K29- and K63-linked poly-ubiquitin for degradation by the lysosome pathway via clathrin-mediated endocytosis.

MATERIALS AND METHODS

Cells, compounds, plasmids, and antibodies

293T, MCF7, and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Lactacystin, MG132, (PEPTIDE INSTITUTE, Osaka, Japan), chloroquine (TOCRIS, Bristol, UK), bafilomycin A1 (AdipoGen, San Diego, CA, USA), and chlorpromazine (Nacalai Tesque Inc., Kyoto, Japan) were dissolved in DMSO. Methyl-β-cyclodextrin (Tokyo Chemical Industry, Tokyo, Japan) and sucrose (Nacalai Tesque Inc., Kyoto, Japan) were dissolved in water.

To construct N-terminal 2xS-tagged CD81 wildtype (2xS-CD81-WT) and C-terminal 2xHA-tagged CD81 wildtype (CD81-2xHA) plasmids, CD81 cDNA was obtained by PCR from the cDNA library of HeLa cells and cloned into pCIneo-2xS and pCIneo-2xHA (C-terminal tag) vectors, which were generated by inserting oligonucleotides encoding two repeats of S-tag peptides and HA-tag peptides, respectively, into pCIneo.
pCIneo-2xHA (C-terminal tag) has cloning sites upstream of the 2xHA-tag sequences. CD81-mutant plasmids (2xS-CD81-K8A, 2xS-CD81-K11A, 2xS-CD81-KK8,11AA, CD81-K8A-2xHA, and CD81-K11A-2xHA) were amplified from the 2xS-CD81-WT or CD81-2xHA-WT plasmid by site-directed mutagenesis using PCR. HA tagged Ub-WT (#17608), Ub-K48 (#17605), and Ub-K63 (#17606) plasmids were purchased from Addgene (MA, USA). Ub-K48 and Ub-K63 are mutants that have a single Lys residue at amino acids 48 and 63, respectively, in the Ub gene, and all other lysines substituted to arginines. The Myc-tagged Ub-K29R, -K48R, and -K63R plasmids were generated by site-directed mutagenesis from the Myc-tagged Ub-wildtype plasmid (pCIneo-Myc-Ub) we previously constructed.14)

Anti-S-tag, anti-HA-tag, anti-Myc-tag (MBL, Nagoya, Japan), anti-CD81, anti-LAMP1, anti-p62, anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), and EEA1 (BD Biosciences, Franklin Lakes, NJ, USA) were used as primary antibodies. FK2, which we established previously,15) was used to detect the poly-Ub chain.

**Pulldown assay and western blot**

For pulldown assays, transfected 293T cells were lysed with RIPA buffer (50 mM Tris-HCl...
(pH 7.4), 0.15 M NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and protease inhibitors and sonicated. The cell extracts were subjected to affinity purification using S-protein-immobilized beads (Novagen, MA, USA), and the purified proteins (containing 2xS-tagged CD81 or mutants) were subjected to western blotting. Western blotting was performed as described previously.15)

Flow cytometry

Trypsinized 293T cells were washed with FACS buffer (PBS containing 3% FBS). For staining cell-surface CD81, 293T cells were fixed with paraformaldehyde (Wako, Osaka, Japan) for 20 min. After washing with FACS buffer, resuspended cells were incubated with mouse anti-CD81 antibody and then with secondary antibodies conjugated to Alexa fluor 647 for 60 min at 4 °C. The samples were analyzed by using a LSRFortessa Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FACSDiva™ software (BD Biosciences, Franklin Lakes, NJ, USA).

Immunofluorescence assay (IFA)

MCF7 cells treated with proteasome inhibitors, lysosome inhibitors, endocytosis inhibitors or
vehicle for 24 h were fixed on glass slides in 4% paraformaldehyde and permeabilized with 0.1% saponin/PBS. After blocking with 3% BSA in PBS, cells were treated with each primary antibody (except anti-CD81) and subsequently a secondary antibody, then the cells were co-stained with anti-CD81 conjugated to Alexa fluor 647 (Santa Cruz Biotechnology, Dallas, TX, USA). DAPI was stained using Fluoro-KEEPER Antifade Reagent, Non-Hardening Type with DAPI (Nacalai). Immunofluorescence signals were obtained using a LSM800 laser scanning confocal microscope (Carl Zeiss, Jena, Germany), and staining images were visualized using ZEN system imaging software (Carl Zeiss).

Densitometry and statistical analyses

Densitometric analysis of the blotting data was performed using ImageJ software (NIH, Bethesda, MD, USA). The statistical significance between each group and the control (DMSO-treated cells) was analyzed by one-way ANOVA followed by Dunnett's or Tukey’s test for multiple comparisons. Statistically significant data were analyzed with GraphPad prism7 (GraphPad Software, San Diego, CA, USA). ***$p<0.001$ indicates statistical significance, and “ns” indicates not significant.
RESULTS

CD81 is degraded by a lysosome pathway involving K63- and K29-linked poly-ubiquitination.

To reveal which degradation pathway (proteasome or lysosome) is involved in the turnover of CD81, we analyzed the CD81 expression level on the cell surface in the presence of proteasome or lysosome inhibitors by flow cytometry using an anti-CD81 antibody that recognizes an extracellular loop of CD81 (Fig. 1A). The fluorescence intensity of FACS was quantified (Fig. 1B). Lactacystin, bafilomycin A1, and chloroquine were used as inhibitors of the proteasome, lysosome, and lysosome, respectively. MG132 was used as an inhibitor of both the proteasome and lysosome. Although MG132 is a well-known proteasome inhibitor, it also inhibits cathepsin K, a member of the lysosomal cathepsin family.16) When 293T cells were exposed to these inhibitors, the amount of CD81 on the cell surface was comparable between DMSO- and lactacystin-treated cells. Bafilomycin A1, chloroquine, and MG132 increased CD81 expression compared to DMSO- and lactacystin-treatment. In addition, we evaluated CD81 turnover in MCF7 whole cells. MCF7 cells were treated with cycloheximide (CHX) in the presence or absence of MG132 or chloroquine for the indicated time, and CD81 was detected by western blotting (Fig. 1C). Supplemental Figures-S1 shows the densitometric
analysis of CD81 expression in blotting of Fig. 1C. The half-life of CD81 upon DMSO-, MG132-, and chloroquine-treatment was approximately 24, over 36, and 36 hours, respectively, meaning that lysosome inhibition induced CD81 stability. Hence, we disclosed that CD81 on the cell membrane is degraded by the lysosome pathway, not proteasome pathway. We then examined whether the poly-ubiquitination of CD81 correlated with the lysosomal degradation of CD81. 293T cells transfected with 2xS-tagged CD81 or co-transfected with 2xS-tagged CD81 and HA-tagged Ub plasmids were treated with lactacystin, MG132 or chloroquine, and 2xS-CD81 was precipitated from cell extracts by S-protein-immobilized beads. To detect the ubiquitination of CD81, precipitates were subjected to western blotting with FK2 (Fig. 1D) and anti-HA antibody (Supplemental Figure-S2). As a result, the ubiquitination of CD81 was increased in MG132- and chloroquine-treated cells compared with DMSO- and lactacystin-treated cells. Furthermore, to determine which poly-Ub chain (K48-linked or K63-linked) was conjugated to CD81, we analyzed the poly-ubiquitination of CD81 in cells expressing mutant-Ub (Ub-K48 and Ub-K63), in which all Lys residues (except for K48 or K63, respectively) of Ub were changed to Arg residues. Cells transfected with 2xS-CD81 and HA-tagged Ub (wildtype, Ub-K48, or Ub-K63) were treated with MG132, and cell extracts was analyzed with a pulldown assay. The expression of Ub-K63 reduced ubiquitinated CD81,
but the expression of Ub-K48 completely eliminated it (Fig. 1E). In addition, we assayed whether CD81 was poly-ubiquitinated by the expression of Ub-K29R, -K48R, and -K63R mutants, in which the Lys29, Lys48 and Lys63 were respectively substituted with Arg. Interestingly, the poly-ubiquitination of CD81 was decreased not only by Ub-K63R but also Ub-K29R(Fig. 1F). These results indicated that CD81 conjugated with K63- or K29-linked poly-Ub chains before degradation.

The endocytic pathway is involved in CD81 degradation.

Normally, CD81 is localized on the plasma membrane. To further explore the lysosomal degradation pathway of CD81, we observed the localization of CD81 and marker proteins of the lysosome, early endosome, and autophagosome in MCF7 cells cultured with or without chloroquine. CD81 showed plasma membrane localization and partial cytoplasmic distribution in normal cells, whereas cytosolic CD81 was colocalized with LAMP1, a lysosomal membrane protein, and EEA1, an early endosome marker, in chloroquine-treated cells (Fig. 2). It is known that p62, an autophagosome marker, promotes autophagosome maturation and the degradation of ubiquitinated proteins by autophagy. However, CD81 and p62 were not colocalized, suggesting that CD81 is not degraded via autophagy. Supplemental Figures-S3, -S4 and –S5
show high-magnification images of the localization of CD81 with LAMP1, EEA1 and p62, respectively, in chloroquine-treated cells. These data provide further evidence for the lysosomal degradation of CD81 and indicate a strong relationship between CD81 degradation and endocytosis.

CD81 is internalized by clathrin-mediated endocytosis.

We found that CD81 is degraded by the lysosome pathway through endocytosis. To gain further insight into the CD81-degradation machinery, we analyzed CD81 endocytosis in detail. 293T cells were treated with three types of endocytosis inhibitors for 24 h, and CD81 expression on the cell surface and in the whole cell was evaluated by FACS and western blotting (Fig. 3A-C). Chlorpromazine (CPMZ) and methyl-β-cyclodextrin (MBCD) were used as inhibitors of clathrin- and caveolae-mediated endocytosis, respectively, and sucrose as an inhibitor of both clathrin- and caveolae-independent endocytosis. Neither MBCD nor sucrose affected CD81 expression on the cell surface, but CPMZ caused a remarkable increase in CD81 cell-surface expression (Fig. 3A and B). No treatment had an effect on CD81 expression in the whole cell (Fig. 3C). These results indicate that the inhibition of clathrin-mediated endocytosis induced CD81 stabilization on the cell surface, i.e., CD81 was
Biological and Pharmaceutical Bulletin Advance Publication

internalized by clathrin-mediated endocytosis. In addition, we analyzed the localization of CD81 in CPMZ-, MBCD-, and sucrose-treated cells by IFA (Fig. 3D). Interestingly, CD81 was localized on the plasma membrane in all observed conditions, but only CPMZ induced the cytoplasmic accumulation of CD81. Thus, we propose that CPMZ might influence not only clathrin-mediated endocytosis but also clathrin-dependent vesicular trafficking, e.g., vesicular transport between the endosome and Golgi (or lysosome).

The poly-ubiquitin chain binds to Lys8 at the N-terminal intracellular domain of CD81.

Above we show that poly-ubiquitination and clathrin-mediated endocytosis are involved in the degradation of CD81. CD81 is composed of four transmembrane domains, three intracellular domains, and two extracellular domains. The three intracellular domains harbor two Lys residues, K8 and K11, which are located at the N-terminal intracellular domain of CD81 (Fig. 4A). To disclose which Lys (K8 or K11) of CD81 conjugates with the poly-Ub chain, we elucidated the ubiquitination of mutant CD81, in which the ubiquitinated Lys is substituted with Ala. 293T cells were transiently transfected with HA-tagged Ub and 2xS-tagged CD81-WT, -K8A, -K11A, or -KK8,11AA plasmids, and cell extracts were subjected to pulldown assays. The precipitates were probed with anti-HA antibody to detect
the binding of Ub and CD81 (Fig. 4B). As a result, CD81-K8A and CD81-KK8,11AA expression showed remarkably decreased poly-ubiquitination on CD81 compared with CD81-WT and CD81-K11A expression, meaning that the poly-Ub chain binds to Lys8 at the intracellular N-terminal domain of CD81. In addition, we evaluated whether defects in the poly-ubiquitination for CD81 affected the stability of CD81. HeLa cells were transfected with HA-tagged CD81-WT, -K8A, or -K11A plasmids and harvested for 0-24 h. Blotting data showed that CD81-K8A extended the CD81 half-life (Fig. 4C). These results indicate that the degradation of CD81 depends on the poly-ubiquitination of Lys8 at the N-terminal intracellular domain of CD81. However, CD81-K8A was still efficiently degraded (Fig. 4C). Therefore, it could be speculated that ubiquitination-independent pathway also participates in CD81 degradation (e.g., transmembrane protease and ubiquitination-independent endocytosis).

DISCUSSION

CD81 is a well-known surface receptor that plays a crucial role in B-cell activation and differentiation.4, 5) CD81 expression is upregulated at the hematopoietic stem cell (HSC) stage, and its high expression persists among HSC, pro-B, pre-B and immature B cell stages. However, CD81 expression is rapidly downregulated upon B cell maturation.11) Despite its
importance in B cell function, little is known about the CD81 degradation machinery. Here we demonstrated that poly-ubiquitinated CD81 is degraded by the lysosome pathway via endocytosis. We also revealed that Lys8 at the N-terminal intracellular region of CD81 was conjugated to the K63- and K29-linked poly-Ub chain and that mutant CD81-K8A, in which Lys8 was substituted with Ala, extended the half-life of CD81. To our knowledge, this is the first report showing that the lysosomal degradation of CD81 requires K29- and K63-linked poly-ubiquitination and clathrin-mediated endocytosis.

Two transmembrane proteins harboring the RING-finger domain, which is necessary for binding to E2, have been reported as candidates of the E3 Ub ligase for CD81. Lineberry et al. identified GRAIL, which has an intracellular RING-finger domain. They found that GRAIL induced Lys48-linked poly-ubiquitination of CD81 and CD81 instability. In addition, they showed that poly-ubiquitinated CD81 accumulated upon treatment with MG132, an inhibitor of both the proteasome and lysosome, or MG101, an inhibitor of lysosomal cathepsins. They also found poly-ubiquitination at Lys8 of CD81, which agrees with our data. Bartee et al. reported that membrane-associated RING-CH (MARCH) downregulated the cell-surface expression of CD81 and sequestered CD81 to endo-lysosomal vesicles. In addition, they found that internalization and lysosomal
localization of CD81 was MARCH-dependent. The MARCH family is known to induce the K63-linked poly-ubiquitination and internalization of cell surface receptors such as MHC class I, B7, ICAM-1, and CD4. Notably, the RING-CH domain of MARCH interacts with Ubc13 as an E2 enzyme, which contributes to K63-linked poly-ubiquitination. We therefore speculate that the K63-linked poly-ubiquitination at Lys8 of CD81 is catalyzed by MARCH (or GRAIL) with Ubc13.

The K48-linked poly-Ub chain functions as a signal for proteasomal degradation, while the K63- and K29-linked poly-Ub chain is involved in proteasome-independent events such as endocytosis and protein trafficking. Many cell surface receptors are known to be conjugated with K63-linked poly-Ub chains, leading to their internalization and lysosomal degradation. On the other hand, notch receptors are conjugated with mono-Ub and K29-linked poly-Ub chain for their internalization and degradation. K29-linked poly-ubiquitination of notch receptors is mediated by Itch (HECT-type E3 Ub ligase), which harbors the HECT domain for binding to E2. Finding K63- and K29-linked poly-ubiquitinated CD81 suggests that multiple E3 Ub ligases (i.e., MARCH, GRAIL and Itch) might mediate the poly-ubiquitination of CD81. More study is needed to identify the precise effects of K63- and K29-linked poly-ubiquitination on the endocytosis or intracellular
trafficking of CD81.

In sum, we report a molecular mechanism for the degradation of CD81 expressed on the cell membrane. K63- and K29-linked poly-ubiquitination conjugate to the N-terminal Lys8 of CD81, leading to the internalization of CD81 by clathrin-mediated endocytosis, upon which it is degraded by the lysosome.

Acknowledgements

This work was supported by the JSPS Grant-in-Aid for Scientific Research (18K06642 and 18K14910). We thank Dr. Peter Karagiannis for scientific advice and critical proofreading of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
REFERENCES

1) Levy S, Todd SC, Maecker HT. CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. *Annu. Rev. Immunol.*, **16**, 89–109 (1998).

2) Hong IK, Byun HJ, Lee J, Jin YJ, Wang SJ, Jeoung DI, Kim YM, Lee H. The tetraspanin CD81 protein increases melanoma cell motility by up-regulating metalloproteinase MT1-MMP expression through the pro-oncogenic Akt-dependent Sp1 activation signaling pathways. *J. Biol. Chem.*, **289**, 15691–15704 (2014).

3) Quast T, Eppler F, Semmling V, Schild C, Homsi Y, Levy S, Lang T, Kurts C, Kolanus W. CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration. *Blood*, **118**, 1818–1827 (2011).

4) Levy S, Shoham T. The tetraspanin web modulates immune-signalling complexes. *Nat. Rev. Immunol.*, **5**, 136–148 (2005).

5) Shoham T, Rajapaksa R, Boucheix C, Rubinstein E, Poe JC, Tedder TF, Levy S. The tetraspanin CD81 regulates the expression of CD19 during B cell development in a postendoplasmic reticulum compartment. *J. Immunol.*, **171**, 4062–4072 (2003).

6) Lineberry N, Su L, Soares L, Fathman CG. The single subunit transmembrane E3 ligase gene related to anergy in lymphocytes (GRAIL) captures and then
ubiquitinates transmembrane proteins across the cell membrane. *J. Biol. Chem.*, **283**, 28497–28505 (2008).

7) Bartee E, Eyster CA, Viswanathan K, Mansouri M, Donaldson JG, Fruh K. Membrane-Associated RING-CH proteins associate with Bap31 and target CD81 and CD44 to lysosomes. *PLoS One*, **5**, e15132 (2010).

8) Kwon YT, Ciechanover A. The Ubiquitin Code in the Ubiquitin-Proteasome System and Autophagy. *Trends Biochem. Sci.*, **42**, 873–886 (2017).

9) Pickart CM. Mechanisms underlying ubiquitination. *Annu. Rev. Biochem.*, **70**, 503–533 (2001).

10) Moretti J, Brou C. Ubiquitinations in the notch signaling pathway. *Int. J. Mol. Sci.*, **14**, 6359–6381 (2013).

11) Bagwell CB, Hill BL, Wood BL, Wallace PK, Alrazzak M, Kelliher AS, Preffer FI. Human B-cell and progenitor stages as determined by probability state modeling of multidimensional cytometry data. *Cytometry B. Clin. Cytom.*, **88**, 214–226 (2015).

12) Duncan LM, Piper S, Dodd RB, Saville MK, Sanderson CM, Luzio JP, Lehner PJ. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J.*, **25**, 1635–1645 (2006).
13) Verbovsek U, Van Noorden CJF, Lah TT. Complexity of cancer protease biology: Cathepsin K expression and function in cancer progression. *Semin. Cancer Biol.*, **35**, 71–84 (2015).

14) Shigemi Z, Furukawa Y, Hosokawa K, Minami S, Matsuhiro J, Nakata S, Watanabe T, Kagawa H, Nakagawa K, Takeda H, Fujimuro M. Diallyl trisulfide induces apoptosis by suppressing NF-kappaB signaling through destabilization of TRAF6 in primary effusion lymphoma. *Int. J. Oncol.*, **48**, 293–304 (2016).

15) Fujimuro M, Sawada H, Yokosawa H. Production and characterization of monoclonal antibodies specific to multi-ubiquitin chains of polyubiquitinated proteins. *FEBS Lett.*, **349**, 173–180 (1994).

16) Votta BJ, Levy MA, Badger A, Bradbeer J, Dodds RA, James IE, Thompson S, Bossard MJ, Carr T, Connor JR, Tomaszek TA, Szewczuk L, Drake FH, Veber DF, Gowen M. Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both in vitro and in vivo. *J. Bone Miner. Res.*, **12**, 1396–1406 (1997).

17) Danieli A, Martens S. p62-mediated phase separation at the intersection of the ubiquitin-proteasome system and autophagy. *J. Cell Sci.*, **131**, (2018).

18) Ohmura-Hoshino M, Goto E, Matsuki Y, Aoki M, Mito M, Uematsu M, Hotta H, Ishido S. A novel family of membrane-bound E3 ubiquitin ligases. *J. Biochem.*, **140**, 147–154 (2006).
19) Hoer S, Smith L, Lehner PJ. MARCH-IX mediates ubiquitination and downregulation of ICAM-1. *FEBS Lett.*, **581**, 45–51 (2007).
Figure 1. CD81 was ubiquitinated and subsequently degraded by the lysosome pathway.

(A) Inhibitory effects of proteasome and lysosome inhibitors on the cell-surface expression of CD81. 293T cells were treated with lactacystin (proteasome inhibitor), bafilomycin A1 (lysosome inhibitor), MG132 (proteasome and lysosome inhibitor), and chloroquine (lysosome inhibitor) for 24 h, and the amount of cell-surface CD81 was measured by FACS using an anti-CD81 antibody that recognizes the extracellular loop of CD81. The FACS data of CD81-positive cells are shown (inhibitor: red, DMSO: dark grey). The light grey histograms present cells stained with control IgG (control). (B) Averages of the FACS data normalized to vehicle (DMSO)-treated cells. (C) The effects of proteasome and lysosome inhibitors on the stability of CD81. MCF7 cells were cultured in DMEM containing 100 µg/mL cycloheximide (CHX) with vehicle (DMSO), 100 nM MG132, or 75 mM chloroquine for 12, 24, and 36 h, and cell extracts were subjected to western blotting using anti-CD81 antibody. The band intensities of CD81 were calculated using ImageJ software and normalized to those of total β-actin. The values of CD81/β-actin are presented at the bottom of the picture, and the value of 0 h-treated cells is presented as 1.0. (D) An analysis of the ubiquitination of CD81 in the presence of proteasome or lysosome inhibitors. 293T cells were transfected with 2xS-CD81-WT and then treated with 1 µM lactacystin, 100 nM MG132, or 75 mM chloroquine for 6 h. 2xS-CD81 was precipitated from cell lysates by

Biological and Pharmaceutical Bulletin Advance Publication
S-protein-immobilized beads and subjected to immunoblotting with FK2 antibody to detect ubiquitination. (E) The poly-ubiquitination of CD81 in cells expressing Ub-K48 or Ub-K63 mutants. 293T cells were cotransfected with 2xS-tagged CD81-WT and HA-tagged Ub-WT, -K48, or -K63 plasmids. Ub-K48 and Ub-K63 are mutants that have a single Lys residue at amino acids 48 and 63, respectively, in the Ub gene, and all other lysines substituted to arginines. The cotransfected cells were treated with 100 nM MG132 for 6 h, and the cell lysates were applied to pulldown assays to detect the poly-ubiquitination of 2xS-CD81. (F) The poly-ubiquitination of CD81 in cells expressing Ub-K29R, Ub-K48R, or Ub-K63R mutants. 293T cells were cotransfected with 2xS-tagged CD81-WT and Myc-tagged Ub-WT, -K29R, -K48R, or -K63R, in which the Lys29, Lys48, and Lys63 were respectively substituted with Arg. Cell lysates were subjected to pulldown assays using S-protein-immobilized beads. The precipitates were immunoblotted with FK2 or anti-Myc antibody to detect the poly-ubiquitination of 2xS-CD81. (Color figure can be accessed in the online version.)
Figure 2. Endocytosis was involved in the degradation of CD81.

(A) CD81 colocalized with endosomes and lysosomes but not autophagosomes in cells. MCF7 cells were incubated with or without 50 µM chloroquine for 6 h, and the fixed cells were stained with anti-CD81 antibody and either anti-LAMP1 (lysosome marker), EEA1 (early endosome marker), or p62 (autophagosome marker) antibody. CD81 (red) and DNA (blue) are shown. LAMP1, EEA1, and p62 are shown as green. Scale bars represent 20 nm.

(B) The colocalization ratios of CD81 and LAMP1, EEA1 or p62. Pearson’s correlation coefficient (y-axis) was calculated using Zen system imaging software. (Color figure can be accessed in the online version.)
Figure 3. CD81 was downregulated by clathrin-mediated endocytosis.

(A) The expression level of cell-surface CD81 in the presence of endocytosis inhibitors. 293T cells were treated with 10 µM CPMZ, 400 µM MBCD, or 10 mM sucrose for 24 h in order to inhibit clathrin-mediated, caveolae-mediated, or clathrin- and caveolae-independent endocytosis. The expression of CD81 on the cell surface was analyzed by FACS. 

(B) Relative expression of CD81 in different conditions based on the FACS data. Results are normalized to 0.1% DMSO-treated cells.

(C) The amount of CD81 in whole cell extracts in the presence of endocytosis inhibitors. CD81 was detected by western blotting.

(D) The localization of CD81 in MCF7 cells treated with endocytosis inhibitors. MCF7 cells were treated with 10 µM CPMZ, 400 µM MBCD, or 10 mM sucrose for 24 h and stained with anti-CD81 antibody (green) and DAIP (blue). The fluorescence signal was detected by laser scanning confocal microscopy (LSM 800). (Color figure can be accessed in the online version.)
Figure 4. Poly-ubiquitination at Lys8 in the N-terminus of CD81 is related to CD81 degradation.

(A) A cartoon of CD81 on the plasma membrane. Three intracellular domains harbor two Lys residues, K8 and K11, which are located at the N-terminal intracellular domain of CD81. (B) Identification of the poly-ubiquitination site in CD81. 293T cells were cotransfected with HA-tagged Ub and 2xS-tagged CD81-WT, CD81-K8A, CD81-K11A, or CD81-KK8,11AA plasmids. CD81-K8A, -K11A, and -KK8,11AA are point-mutated CD81, in which Lys8 or/and Lys11 was/were substituted to Ala. S-tagged CD81 was pull-downed with S-protein beads from cell extracts and applied to immunoblotting with anti-S and anti-HA. (C) Missing poly-ubiquitination sites prolonged the stability of CD81. HeLa cells transfected with 2xS-CD81-WT or KA mutant CD81 were treated with 100 µg/ml cycloheximide for the indicated times and harvested. Cell lysates were subjected to immunoblotting using anti-CD81 antibody. The values of CD81/β-actin are presented at the bottom of the picture, and the value of 0 h cells is presented as 1.0.