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Characterization of the CDC6 as a Substrate of N-terminal Arginylation in the N-end Rule Pathway

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ABSTRACT

In eukaryotes, there are two machineries of protein degradation system composed of ubiquitin-proteasome system (UPS) and autophagy-lysosome system. The N-end rule pathway relates the regulation of the stability of a protein to the nature of its N-terminal amino acid. In mammalian N-end rule pathway, destabilizing N-terminal residues of proteins are recognized by N-recognins that mediate protein ubiquitination and degradation through 26S proteasome. Mammalian N-recognins are E3 ligases called UBR box proteins composed of UBR1, UBR2, UBR4 and UBR5.

Recently, our group was the first to validate that N-terminal Arg is an autophagic degron using endoplasmic reticulum (ER)-residing chaperone BiP. Through a genome-wide functional proteomic screening, CDC6 (cell division cycle 6) was found as a putative N-end rule substrate in our lab and used as a model substrate of cytosolic protein to generalize our study in this report. First, we raised a rabbit polyclonal anti-R-CDC6 antibody using N-terminally arginylated form of CDC6 peptide (R-CDC6 peptide). Immunoblotting and immunostaining analyses with this specific antibody show that caspase3-generated C-terminal fragment of CDC6 bearing N-terminal Asp is N-terminally arginylated by Arginyl-tRNA-protein transferase 1 (ATE1) in vivo. My results show that the CDC6 fragment is a new N-end rule substrate degraded by proteasome, and other C-terminal fragment of BRCA1 (breast cancer susceptibility type 1 gene product) is N-terminally arginylated and

degraded by N-end rule pathway. X-peptide pull-down assays demonstrate that these two arginylated proteins physically bind to autophagic adaptor p62/SQSTM1 through their N-terminal Arg. I also identify that a ZZ-type zinc finger domain (ZZ domain) of p62 is a binding site for N-terminal Arg using p62 point and deletion mutants. Moreover, I show that many kinds of protein fragments bearing arginylation-permissive N-terminal residues such as Asp and Glu also interact with p62 in an N-terminal arginylation-dependent manner.

In this study, my results propose that N-terminal Arg of the protein fragment has a dual function as an N-degron. First one is a degradation signal for UPS, which is well established already. The other is a new role that can act as an autophagic N-degron in autophagy. This report provides new insights into the connection between N-terminal arginylation and autophagy.

Keywords: N-end rule pathway, N-terminal arginylation, CDC6, Ubiquitin-proteasome system, Autophagy-lysosome system, p62/SQSTM1

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LIST OF ABBREVIATIONS

UPS: Ubiquitin-proteasome system

ER: Endoplasmic reticulum

CDC6: Cell division cycle 6

ATE1: Arginyl-tRNA-protein transferase 1

BRCA1: Breast cancer susceptibility type 1 gene product

ZZ domain: ZZ-type zinc finger domain

Ub: Ubiquitin

N-terminal degron: N-degron

PB1 domain: Phox and Bem1 domain

LC3: Microtubule-associated protein-1 light chain 3

UBA domain: Ubiquitin-associated domain

LIR: LC3-interacting region

KIR: Keap1-interacting region

MEFs: Mouse embryonic fibroblasts

X-CDC6^{f}: X-CDC6-flag

mATE1: Mouse ATE1

ELISA: Enzyme-linked immunosorbent assay

aa: Amino acid

INTRODUCTION

Protein degradation in cells has been studied for over 70 years and is a central protein quality control system that maintains cellular protein homeostasis (proteostasis) (1, 2). Proteolysis (targeted protein degradation) plays a role in eliminating misfolded or abnormal proteins, controlling the levels of regulatory proteins and producing protein fragments that function as antigens, hormones or other effectors (1, 3, 4). Failure to eliminate misfolded and aggregated proteins causes a variety of human disorders like diabetes, cancers and neurodegenerative diseases including Alzheimer's disease and Parkinson's disease (5, 6). The protein degradation pathways can be largely divided into two types in eukaryotes (7). One is the UPS involved in degradation of proteins including many abnormal, damaged, short-lived or regulated proteins (7). The other is the autophagy-lysosome system which is responsible for destruction of proteins, pathogens, and cellular organelles including mitochondria, peroxysomes and ribosomes (7). Autophagy-lysosome system is a catabolic degradative process in which cytoplasmic materials are degraded within the lysosome (8, 9).

Ubiquitin (Ub), a 76-amino acid protein, is marked to proteins via an enzymatic cascade in UPS. Three classes of enzymes called E1 (activation), E2 (conjugation) and E3 (ligation) achieve protein ubiquitination, and this covalent modification serves as a signal for degradation of the conjugated

proteins through the 26S proteasome, an ATP-dependent multi-catalytic protease (2, 10).

The N-end rule pathway states that identity of N-terminal amino acid of a protein determines its half-life (Figure. 1) (3, 4, 11, 12). This fundamental principle of the proteolytic pathway is well conserved from prokaryotes to eukaryotes, although there are some differences in proteolytic machineries (11). The N-end rule pathway plays a role in many biological processes including the signaling by G proteins, cardiovascular development, meiosis, fat metabolism, DNA repair, neurogenesis, spermatogenesis, peptide import, apoptosis, and the elimination of misfolded proteins (4, 11). Especially, in mammalian N-end rule pathway, N-terminal degradation signals of substrates termed N-terminal degrons (N-degrons) are recognized by E3 ligases termed N-recognins, leading to protein ubiquitination and proteasomal degradation (4, 12). N-degrons comprise destabilizing N-terminal residues of proteins, and N-terminal Arg, Lys, His, Phe, Trp, Tyr, Leu, Ile, Asp, Glu, Asn, Gln and Cys are destabilizing residues (4, 12). N-terminal Asn and Gln are tertiary destabilizing residues which are deamidated to the secondary destabilizing residues Asp and Glu, respectively, by the N-terminal amidases NTAN1 and NTAQ1 (4, 12). The secondary destabilizing residues Asp and Glu are conjugated with Arg by R-transferase ATE1, which produces a new primary destabilizing residue Arg (4, 12). Primary destabilizing residues can be classified into type 1, composed of Arg, Lys and His (basic residues), and type 2 including Phe, Trp, Tyr, Leu, Ile (bulky hydrophobic residues) (12). The

primary destabilizing residues are directly recognized by N-recognins without further modifications (4, 12). The N-terminal Cys also functions as a tertiary destabilizing residue via its oxidation by nitric oxide (NO), oxygen or its derivatives, followed by N-terminal arginylation by ATE1 (4, 12, 13). These N-degrons can be recognized by four N-recognins, namely UBR1, UBR2, UBR4 and UBR5 (14, 15). These N-recognins share a ~70-residue zinc finger domain called UBR box that has two zinc fingers and acts as a substrate recognition site for type 1 N-degrons (12, 14-17).

The autophagy-lysosome system is used for recycling cytoplasmic contents to produce macromolecular building blocks and energy under unfavorable conditions, and to protect the cell during various stress conditions (8). The autophagy occurs in three distinct types in eukaryotes, depending on the delivery pathway of the cargo proteins to the lysosomes or autophagic vaculoes: microautophagy, chaperone-mediated autophagy (CMA), and macroautophagy, hereafter referred to as autophagy (9, 18, 19). We mainly focus on macroautophagy in which the cytosolic cargoes and dysfunctional organelles are sequestered by an isolated double-membrane called phagophore, leading to maturation to a closed double-membrane structure termed autophagosome (7, 8, 20). The autophagosomes are subsequently fused with lysosomes, and the cargoes are degraded by lysosomal hydrolases (21). In autophagic process, LC3 (microtubule-associated protein-1 light chain 3) plays a role at an early stage of phagophore expansion and functions as a protein interaction platform which is linked to the adaptor protein and

phagophore (20).

p62, also known as SQSTM1 (Sequestosome 1) is the autophagic adaptor protein that contains multiple protein-protein interaction domains comprising a Phox and Bem1 domain (PB1 domain), a ubiquitin-associated domain (UBA domain), a LC3-interacting region (LIR), a ZZ-type zinc finger domain (ZZ domain) and a Keap1-interacting region (KIR) (22, 23). p62 directly interacts with ubiquitinated proteins via a its UBA domain and with LC3 through a LIR motif, participating in the targeting cargoes to the autophagosome (9, 18). p62 is the autophagic substrate itself and a cargo receptor during autophagic process (7, 8, 18, 20).

It is known that N-terminal Arg of the protein serves as a degradation signal in Ub-dependent N-end rule pathway. However, the role of N-terminal Arg involved in autophagy remains poorly studied and has been overlooked. A recent paper published by our group showed that N-terminal arginylation by R-transferase ATE1 targets ER-residing chaperone BiP for autophagy by binding to p62 (24). In our lab, we performed genome-wide functional proteomic screening and found out candidate proteins for N-end rule substrates. The RGS (regulators of G protein signaling) proteins including RGS4, RGS5 and RGS16, the G protein-specific GTPase-activating proteins, were characterized as N-end rule substrates among candidate proteins (25, 26). In this report, I characterize other putative N-end rule substrate CDC6 and mainly use CDC6 as a model substrate to study function of N-terminal Arg in both UPS and autophagy. We raised anti-R-CDC6 and anti-R-BRCA1

antibodies that exclusively detect R-CDC6 and R-BRCA1, respectively. Immunoblotting assays with these antibodies show that cytosolic protein fragments of CDC6 and BRCA1 bearing the secondary destabilizing residue Asp are practically arginylated by R-transferase ATE1 in cells. I confirm that these two protein fragments are substrates of N-end rule pathway and produce a rabbit polyclonal anti-mouse ATE1 antibody. Then, I determine that arginylated forms of CDC6 and BRCA1 physically interact with the autophagic adaptor p62 using X-peptide pull-down assays, and N-terminal Arg binds to p62 via the ZZ domain. Moreover, other kinds of proteolytic fragments containing arginylation-permissive N-terminal residues bind to p62 in an N-terminal arginylation-dependent manner. I also identify interactions between multiple peptides and N-recognins through X-peptide pull-down assays.

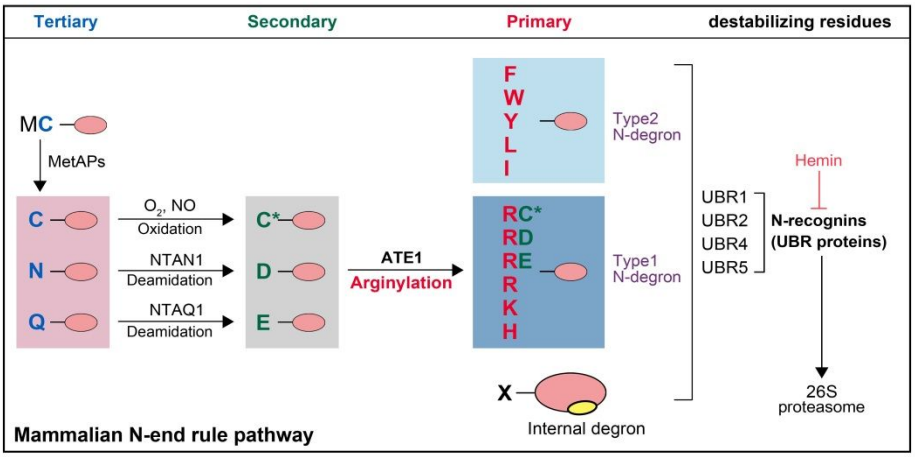


Figure 1. The N-end rule pathway in mammals. Single capital letter denotes N-terminal amino acid of a protein. Pink ovals denote substrates of N-end rule pathway. C* indicates oxidized N-terminal Cys by NO or oxygen. N-terminal Arg, Lys, His, Phe, Trp, Tyr, Leu, Ile, Asp, Glu, Asn, Gln and Cys of proteins are classified as destabilizing residues. MetAP, Met aminopeptidase

MATERIALS AND METHODS

1. Cell culture and immunoblotting

HeLa CCL2, HEK293 and MEF cells were cultured in DMEM (Gibco, 11995073) supplemented with 10% fetal bovine serum (FBS) at 37℃ in a 5% CO2 incubator.

For immunoblotting, cells were collected by centrifugation and lysed using 4X laemmli sample buffer (Bio-Rad, Hercules, California, USA, BR161-0747), followed by boiling for 5 min at 95℃. Whole cell lysates were separated by SDS-PAGE and transferred onto PVDF membrane (Millipore, Billerica, Massachusetts, USA, IPV00010) of which the pore size was 0.45μm, followed by immunoblotting. The membrane was incubated in 1% skim milk solution for 1 hr at room temperature, followed by incubation with primary antibody diluted in 1% skim milk for 1 hr at room temperature. Then the membrane was washed with PBST three times every 20 min and treated with secondary antibody for 1 hr at room temperature, followed by washing with PBST three times every 20 min.

2. Antibodies

Rabbit polyclonal anti-R-CDC6 and anti-R-BRCA1 antibodies against N-terminally arginylated peptides of CDC6 and BRCA1 were developed by AbFrontier (Seoul, Korea). A rabbit polyclonal anti-mouse ATE1 antibody

was also generated by AbFrontier.

Mouse monoclonal anti-p62 (Abcam, Cambridge, UK, ab56416, 1:15,000), mouse monoclonal anti-flag (Sigma-Aldrich, St. Louis, Missouri, USA F3165, 1:15,000), mouse monoclonal anti-actin (Sigma-Aldrich, A1978, 1:20,000), rabbit polyclonal anti-myc (Abcam, ab9106, 1:15,000), rabbit polyclonal anti-actin (Abclon, Seoul, Korea, AbC-2002, 1:5,000) and rabbit polyclonal anti-UBR4 (Abcam, ab86738, 1:2,500) antibodies were purchased from the indicated vendors. Goat anti-mouse IgG-HRP (Abclon, AbC5001, 1:10,000) and goat anti-rabbit IgG-HRP (Abclon, AbC5003, 1:10,000) antibodies were used.

3. X-peptide pull-down assay

C-terminally biotin-conjugated 12-mer peptides bearing different N-terminal amino acids (X) were synthesized. In case of X-CDC6 peptides, the N-terminal residues of the peptides were composed of three types including Arg-Asp (permanently arginylated), Asp (native) or Val (stable). These peptides were cross-linked to streptavidin-agarose beads (Thermo Scientific, Waltham, Massachusetts, USA, 20361) via their C-terminal biotin with a ratio of 0.5mg solubilized peptide per 1 ml beads. Protein extracts for pull-down assay were prepared from HEK293 or HeLa CCL2 cells. Cells were pelleted by centrifugation and lysed in a hypotonic solution (10mM KCl, 1.5mM $MgCl_2$ and 10mM HEPES at pH7.9) containing protease inhibitor (Sigma-Aldrich, P8340). After incubation for 30 min on ice, cell lysates were obtained through

cycles of freezing in liquid nitrogen and thawing in a 37℃ water bath about 5 to 10 times. Then cell lysates were diluted in a binding buffer (0.05% Tween20, 10% glycerol, 0.2M KCl and 20mM HEPES at pH 7.9) and mixed with 50µl (in packed volume) of X-peptide beads, followed by gentle rotation at 4℃ for 2 h. The X-peptide beads were washed with 1ml binding buffer five times and pelleted by centrifugation at 3000rpm for 1 min, resuspended in 25µl SDS sample buffer and heated at 95℃ for 5 min, followed by SDS-PAGE and immunoblotting.

4. Immunocytochemistry

For immunocytochemistry, cells were grown on coverslips coated by poly-L-lysine (Sigma-Aldrich, P8920) in 24well-plate. Cells were rinsed with PBS three times, fixed using 4% paraformaldehyde for 15 min at room temperature and washed with PBS three times. Then Cells were permeabilized with 0.5% triton diluted in PBS and rinsed with PBS three times, followed by blocking with 2% BSA solution. After blocking, the cells were incubated with primary antibody in 2% BSA solution for 1 hr and washed with PBS three times every 10 min. The cells were treated with Alexa fluor-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Invitrogen, Carlsbad, California, USA) for 30 min, followed by washing and mounting with hard-type mounting solution containing DAPI.

5. ELISA

Partial proteins of mouse ATE1 were coated on 96-well plate, followed by blocking with 2% skim milk at 37℃ for 1 hr. The antigen-coated wells were rinsed with TBST and incubated with anti-mATE1 antibody at 37℃ for 2 hr, followed by washing with TBST three times and incubation with HRP (horseradish peroxide)-labelled goat anti-rabbit IgG antibody at 37℃ for 1 hr. After washing with TBST five times, the TMB was added in each well. When color changes occurred, the stop solution (1N H_2SO_4) was added to stop the reaction and the amounts of anti-mATE1 antibody bound to partial mouse ATE1 proteins were determined by measuring the value of optical density at 495nm.

RESULTS

C-terminal proteolytic fragment of CDC6 is N-terminally arginylated by R-transferase ATE1 in vivo

CDC6 is an essential factor for initiation of DNA replication and an important component of the pre-replication complex (27). Deregulation of CDC6 causes genomic instability and rereplication (27, 28). CDC6 is cleaved by caspase-3 at an early stage of apoptosis (29-32). There are two or three cleavage sites of CDC6 and, one of them seems relevant to N-end rule pathway. The cleavage of CDC6 by caspase-3 results in generating a new C-terminal fragment D^{128}-CDC6 containing secondary destabilizing N-terminal residue Asp^{128} (P1' residue) in mice. The exposed N-terminal residues (P1' residues) resulting from the cleavage are destabilizing residues and conserved among mammals (Figure. 2A).

To verify whether this C-terminal proteolytic fragment of CDC6 is a real N-end rule substrate, I need to express CDC6 fragments bearing different N-terminal amino acids. We produced flag-DHFR-Ub-X-CDC6-flag (X= R-D^{128}, D^{128} or V^{128}) plasmid constructions using ubiquitin fusion technic (33), because methionine is the first amino acid in protein synthesis of eukaryotic systems (33, 34). In cells, the flag-DHFR-Ub-X-CDC6-flag was co-translationally cleaved by deubiquitinases into flag-DHFR-Ub and X-CDC6-flag, yielding a CDC6 protein with a desired N-terminal amino acid (X) (24,

33). D-CDC6 had an original N-terminal amino acid of the C-terminal fragment of CDC6, and R-CDC6 was a permanently arginylated form of CDC6, while V-CDC6 had a stabilizing N-terminal residue Val instead of Asp (Figure. 2B).

In the N-end rule pathway, the N-terminal Asp is a secondary destabilizing residue that must undergo post-translational modification like N-terminal arginylation by R-transferase ATE1 before degradation. Then to determine whether D-CDC6 is arginylated by ATE1 in vivo, we raised a rabbit polyclonal anti-R-CDC6 antibody could recognize N-terminally arginylated form of CDC6 (R-CDC6), not unarginylated form (D-CDC6). Rabbit was injected with 12-mer R-CDC6 peptide (R-DEPTFKASPPK) four times, followed by collection of immunized serum. This final anti-serum was carried out IgG purification and negatively purified using D-CDC6 peptide (DEPTFKASPPK). Then flowthrough obtained from the negative purification was positively purified with R-CDC6 peptide. I performed dot-blot assay to confirm the specificity of anti-R-CDC6 antibody. As a result, this antibody had high specificity for R-CDC6 peptide and there was no signal, even with the highest tested quantity of the D-CDC6 peptide (Figure. 2C). Using this specific antibody, immunoblotting and immunostaining showed that D-CDC6 was N-terminally arginylated in an ATE1-dependent manner (Figure. 2D, 2E). Wild-type and $ATE1^{-/-}$ mouse embryonic fibroblasts (MEFs) lacking their own ATE1 were transfected with flag-DHFR-Ub-X-CDC6-flag (X=RD, D or V). When wild-type MEFs compared with $ATE1^{-/-}$ MEFs, D-CDC6 bearing

arginylation-permissive N-terminal residue was only arginylated in wild-type MEFs, whereas not in ATE1$^{-/-}$ MEFs (Figure. 2D, 2E). Because R-CDC6 was permanently arginylated form, anti-R-CDC6 antibody could detect arginylated CDC6 in both wild-type and ATE1$^{-/-}$ MEFs. In contrast, N-terminal Val was not a substrate of ATE1. Thus V-CDC6 was not arginylated in both wild-type and ATE1$^{-/-}$ MEFs. Treatment with 10μM MG132, a proteasome inhibitor, caused accumulation of arginylated CDC6.

In conclusion, C-terminal fragment of CDC6 containing N-terminal Asp is a substrate of ATE1 and N-terminally conjugated with Arg in vivo. N-terminal arginylation of D-CDC6 is dependent on R-transferase ATE1.

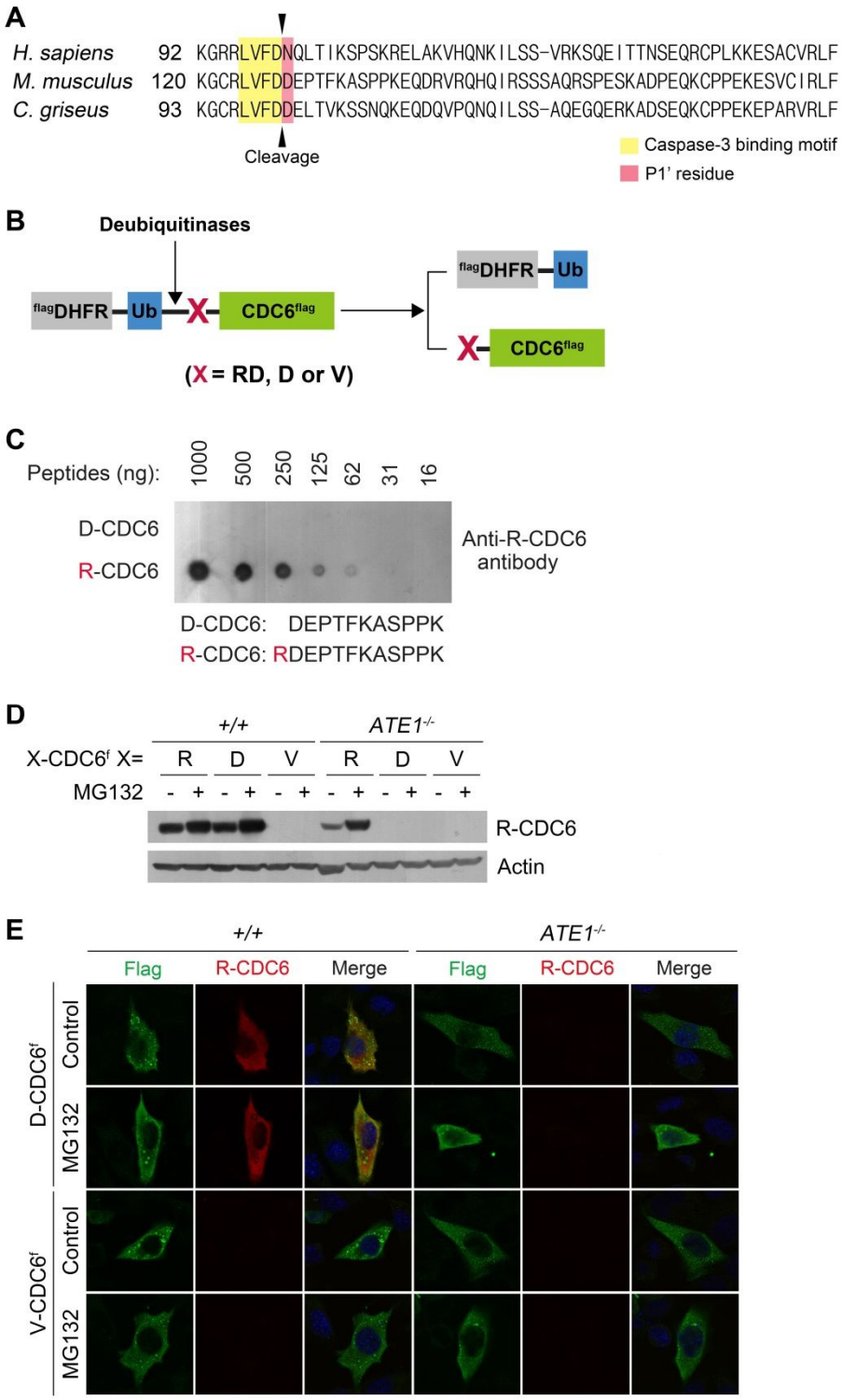

A
H. sapiens 92 KGRRLVFDNQLTIKSPSKRELAKVHQNKILSS-VRKSQEITTNSEQRCPLKKESACVRLF
M. musculus 120 KGCRLVFDDEPTFKASPPKEQDRVRQHQIRSSSAQRSPESKADPEQKCPPEKESVCIRLF
C. griseus 93 KGCRLVFDDELTVKSSNQKEQDQVPQNQILSS-AQEGQERKADSEQKCPPEKEPARVRLF
Cleavage
Caspase-3 binding motif
P1' residue
B
Deubiquitinases
flagDHFR
Ub
X
CDC6flag
flagDHFR
Ub
X
CDC6flag
(X = RD, D or V)
C
Peptides (ng):
1000
500
250
125
62
31
16
D-CDC6
R-CDC6
Anti-R-CDC6 antibody
D-CDC6: DEPTFKASPPK
R-CDC6: RDEPTFKASPPK
D
+/+
ATE1-/-
X-CDC6f X=
R
D
V
R
D
V
MG132
- + - + - + - + - + - +
R-CDC6
Actin
E
+/+
ATE1-/-
Flag
R-CDC6
Merge
Flag
R-CDC6
Merge
D-CDC6f
Control
MG132
V-CDC6f
Control
MG132

Figure 2. C-terminal proteolytic fragment of CDC6 is N-terminally arginylated by R-transferase ATE1 in vivo. (A) A sequence alignment of mammalian CDC6. The exposed N-terminal amino acids (P1' residues) resulting from cleavage by caspase-3 are destabilizing residues in mammals. **(B)** Schematic diagrams showing flag-DHFR-Ub-X-CDC6-flag plasmid construction (X=RD, D or V) produced by ubiquitin fusion technic. Deubiquitinases co-translationally cleave Ub, yielding flag-DHFR-Ub and X-CDC6-flag which has a desired N-terminal residue (X). **(C)** A dot-blot assay with anti-R-CDC6 antibody that we raised. This antibody has a high specificity for R-CDC6 peptide, not D-CDC6 peptide. **(D)** The comparison of N-terminal arginylation of CDC6 between wild-type and $ATE1^{-/-}$ MEFs. MEFs were transfected with flag-DHFR-Ub-X-CDC6-flag (X= RD, D or V) and incubated in the absence or presence of 10μM MG132 for 5 hr, followed by immunoblotting. D-CDC6 is arginylated by ATE1 in wild-type MEFs, not in $ATE1^{-/-}$ MEFs. $X\text{-}CDC6^{f}$, X-CDC6-flag. **(E)** Immunostaining of X-CDC6-flag (green) and arginylated CDC6 (red) in wild-type and $ATE1^{-/-}$ MEFs. D-CDC6, but not V-CDC6, is N-terminally arginylated by ATE1 in vivo.

The rabbit polyclonal anti-mouse ATE1 antibody can selectively detect mouse ATE1

To study about N-terminal arginylation which is involved in mouse ATE1, I required an anti-mouse ATE1 (mATE1) antibody. However, there were no commercial antibodies could detect mouse ATE1. Thus, I generated a rabbit polyclonal anti-mATE1 antibody.

R-transferase ATE1 mediates posttranslational modification that transfers Arg from tRNA to the N-terminal residue of a target protein (12, 35). N-terminal arginylation by ATE1 plays an important role in cell motility, angiogenesis, cardiovascular development, embryogenesis, and regulation of metabolic enzymes and cytoskeleton (35-39). Mammalian genomes have one ATE1 gene, and pre-mRNAs of mouse ATE1 are composed of 14 exons (4, 40). There are at least six isoforms of mouse ATE1 derived from alternative splicing of pre-mRNAs (40, 41). The alternative exons of these isoforms are two pairs of sequelogous exons, 1A/1B and 7A/7B (4, 40). The regions encoded by 1A/1B and 7A/7B exons have different amino acid sequences among isoforms. Thus, I raised anti-mATE1 antibody using partial mouse ATE1 proteins (25kDa) containing C-terminal sequences without variable regions among isoforms (Figure. 3A). These partial proteins with adjuvant were injected to rabbit four times doing enzyme-linked immunosorbent assay (ELISA) for checking immunization of rabbit. In ELISA using final anti-serum of rabbit, OD value that is from 1 to 1.2 at dilution factor 1:1000 of an antibody represents that the rabbit is well immunized. Therefore, ELISA showed that mATE1 antibody

specific for the partial mouse ATE1 protein was properly produced in rabbit (Figure. 3B). Then, the immunized serum was collected by heart puncture of rabbit and sequentially subjected to IgG purification and affinity purification. Immunoblotting showed that this antibody could selectively detect endogenous mouse ATE1 in wild-type MEFs and four constructions expressing different mouse ATE1 isoforms including ATE1-1 ($ATE1^{1B7A}$), ATE1-2 ($ATE1^{1B7B}$), ATE1-3 ($ATE1^{1A7A}$) and ATE1-4 ($ATE1^{1A7B}$) (Figure. 3C, 3D).

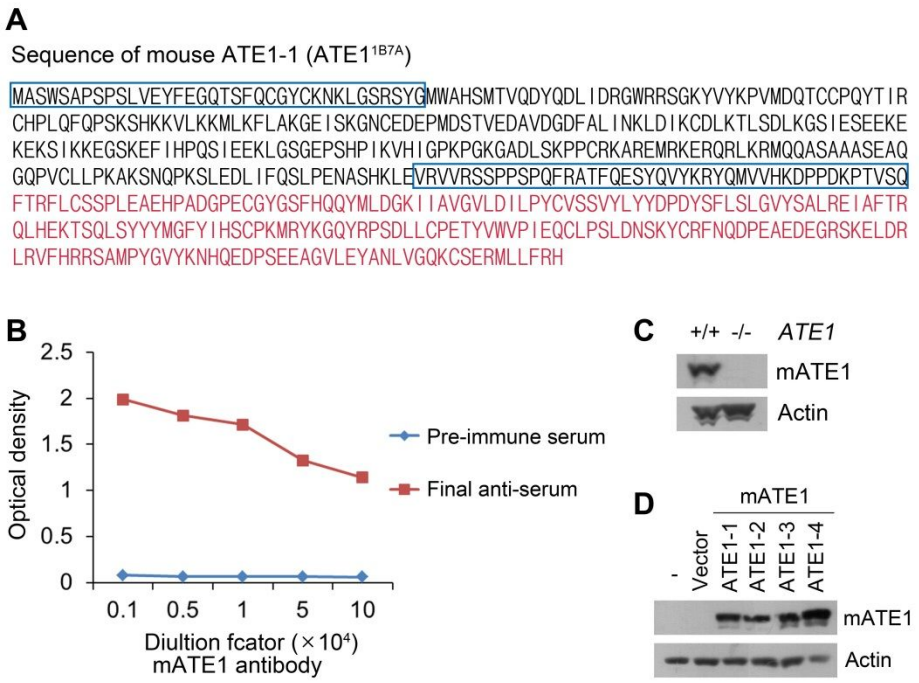


Figure 3. The rabbit polyclonal anti-mouse ATE1 antibody can selectively detect mouse ATE1. (A) Sequence of mouse ATE1-1 ($ATE1^{1B7A}$) containing variable regions encoded by 1B (first blue box) and 7A (second blue box) alternative exons. Red letters indicate sequence of a partial protein injected to rabbit for generation of anti-mATE1 antibody. **(B)** ELISA to confirm immunization of rabbit. This result shows that the rabbit is well immunized. **(C)** Immunoblotting to examine the specificity of anti-mATE1 antibody. This antibody can detect endogenous mouse ATE1 in wild-type MEFs, not in $ATE1^{-/-}$ MEFs. **(D)** HeLa CCL2 cells were transfected with four isoforms of mouse ATE1 (ATE1-1, ATE1-2, ATE1-3 or ATE1-4), followed by immunoblotting with anti-mATE1 antibody.

C-terminal fragment of CDC6 is an N-end rule substrate degraded by ubiquitin-proteasome system

To determine whether C-terminal fragment of CDC6 is a real N-end rule substrate, wild-type and ATE1$^{-/-}$ MEFs were transfected with flag-DHFR-Ub-X-CDC6-flag. In wild-type MEFs, R-CDC6 and D-CDC6 containing destabilizing N-terminal residues were rapidly degraded, whereas V-CDC6 bearing stabilizing N-terminal residue Val was not degraded and highly stable. When R-CDC6 compared with D-CDC6 in ATE1$^{-/-}$ MEFs, R-CDC6 was unstable but not D-CDC6. Because R-CDC6 containing primary destabilizing residue is not required ATE1 for degradation and, D-CDC6 must be preliminarily arginylated by ATE1 before degradation (Figure. 4A).

To test whether this degradation of CDC6 is mediated by UPS, a proteasome inhibitor MG132 was treated. In the presence of MG132, it was observed that accumulation of CDC6 proteins in comparison with the absence of MG132 in cells. Thus I demonstrate that proteolytic fragment of CDC6 is a new N-end rule substrate degraded by UPS (Figure. 4A).

To identify rescue of N-terminal arginylation and degradation of CDC6 by overexpression of ATE1 in ATE1$^{-/-}$ MEFs, cells were co-transfected with three isoforms of mouse ATE1 (ATE1-1, ATE1-2 or ATE1-3) and flag-DHFR-Ub-D-CDC6-flag. As a result, three isoforms of mouse ATE1 could arginylate D-CDC6 and confer metabolic instability (Figure. 4B). This result suggests that both N-terminal arginylation and degradation of D-CDC6 are dependent on ATE1.

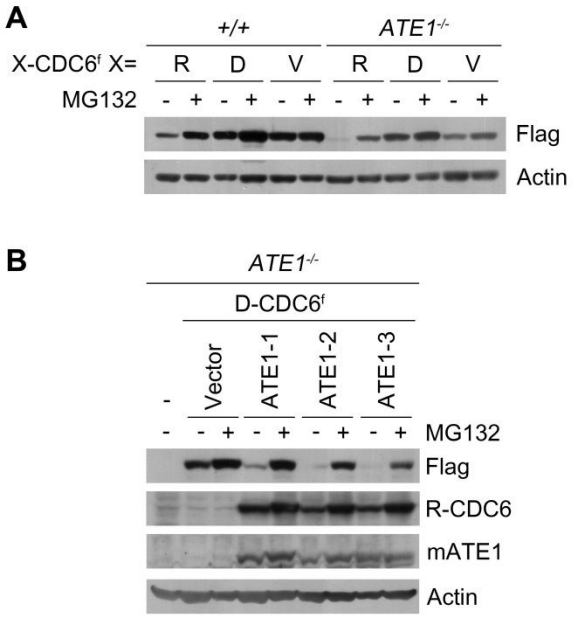


Figure 4. C-terminal fragment of CDC6 is an N-end rule substrate degraded by ubiquitin-proteasome system. (A) Wild-type and ATE1$^{-/-}$ MEFs were transfected with flag-DHFR-Ub-X-CDC6-flag in the absence or presence of MG132, followed by immunoblotting. Proteolytic fragment of CDC6 is degraded by Ub-dependent N-end rule pathway. **(B)** Three isoforms of mouse ATE1 were co-expressed with flag-DHFR-Ub-D-CDC6-flag in ATE1$^{-/-}$ MEFs. D-CDC6 is arginylated and degraded by overexpression of mouse ATE1 in ATE1$^{-/-}$ MEFs.

Other C-terminal fragment of BRCA1 is also N-terminally arginylated and degraded by N-end rule pathway

To expand our studies about N-terminal Arg, I used other cytosolic protein fragment of BRCA1. BRCA1 functions as a tumor suppressor, and germ-line mutations in BRCA1 gene confer susceptibility to ovarian and breast cancers (42, 43). BRCA1 mediates numerous physiological functions including cell cycle progression, DNA damage repair, mRNA transcription and chromatin remodeling (43). BRCA1 is also cleaved by caspase-dependent pathway upon the activation of apoptosis, releasing a C-terminal proteolytic fragment that has N-terminal Asp^{1156} (P1' residue) in human. It was reported that the fragment of BRCA1 is degraded by N-end rule pathway (44, 45). To confirm this, we generated flag-DHFR-Ub-X-BRCA1-flag (X= R-D^{1156}, D^{1156} or V^{1156}) plasmids using ubiquitin fusion technic like CDC6. HeLa CCL2 and HEK293 cells were transfected with flag-DHFR-Ub-X-BRCA1-flag and treated with MG132 for proteasomal inhibition (Figure. 5A, 5B). In the absence of MG132, R-BRCA1 and D-BRCA1 bearing destabilizing N-terminal residues were rapidly degraded in vivo, whereas V-BRCA1 containing stabilizing N-terminal residue was completely stable. The R-BRCA1 and D-BRCA1 proteins were accumulated by the treatment of MG132. There was no difference the level of V-BRCA1 protein in the absence and presence of MG132 because V-BRCA1 was not degraded by proteasome even in the absence of MG132. I identify that N-terminal arginylation of D-BRCA1 in

cells using anti-R-BRCA1 antibody that we raised (Figure. 5A, 5B). Thus, I confirm that C-terminal fragment of BRCA1 is also an N-end rule substrate arginylated by ATE1 and degraded by proteasome in vivo.

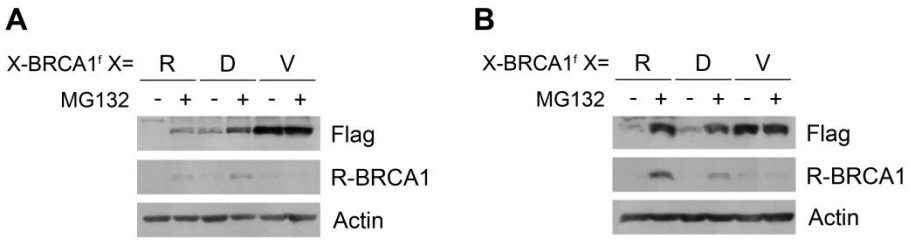


Figure 5. Other C-terminal fragment of BRCA1 is also N-terminally arginylated and degraded by N-end rule pathway. (A) HeLa CCL2 cells were transiently transfected with flag-DHFR-Ub-X-BRCA1-flag (X= RD, D or V) in the absence or presence of 10μM MG132 for 5 hr. N-terminal arginylation and degradation of D-BRCA1 are confirmed. **(B)** flag-DHFR-Ub-X-BRCA1-flag plasmids (X= RD, D or V) were expressed in HEK293 cells, followed by treatment of 10μM MG132 for 5 hr. R-BRCA1 and D-BRCA1, not V-BRCA1, were degraded by N-end rule pathway.

N-terminally arginylated forms of CDC6 and BRCA1 fragments physically bind to the autophagic adaptor p62

It was previously reported from our group that the autophagic adaptor p62 physically interacts with arginylated form of ER-chaperone BiP (R-BiP) using X-peptide pull-down assays (24). X-peptide pull-down assay is an in vitro method developed to confirm and identify protein-protein interactions. This method is very similar to immunoprecipitation except that the synthetic X-peptide is used instead of an antibody (14, 15). Thus, I synthesized C-terminally biotin-conjugated 12-mer peptides bearing different N-terminal amino acids. These synthetic X-peptides are cross-linked to streptavidin-agarose beads via their C-terminal biotin and then peptide-linked beads are incubated with cell lysates. The co-precipitated proteins with the peptide-linked beads are detected by immunoblotting (Figure. 6A).

To determine whether N-terminally arginylated form of CDC6 that I previously confirmed as a new N-end rule substrate also binds to p62, I carried out X-peptide pull-down assay using synthetic X-CDC6 peptides (X= RD, D or V) (Figure. 6B). I showed that endogenous p62 bound to only R-CDC6 peptide, but not D-CDC6 and V-CDC6 peptide (Figure. 6C). I next examined the binding of p62 with other cytosolic protein fragment of BRCA1. As expected, arginylated form of BRCA (R-BRCA1), but not D-BRCA1 and V-BRCA1 peptides, captured endogenous p62 (Figure. 6D). These data demonstrate that arginylated protein fragments bind to p62, and p62 is a recognition component of N-terminal Arg. Thus, these results imply that

arginylated proteins may have a chance to be targeted to autophagy by interacting autophagic adaptor p62 in some stress conditions inducing autophagy.

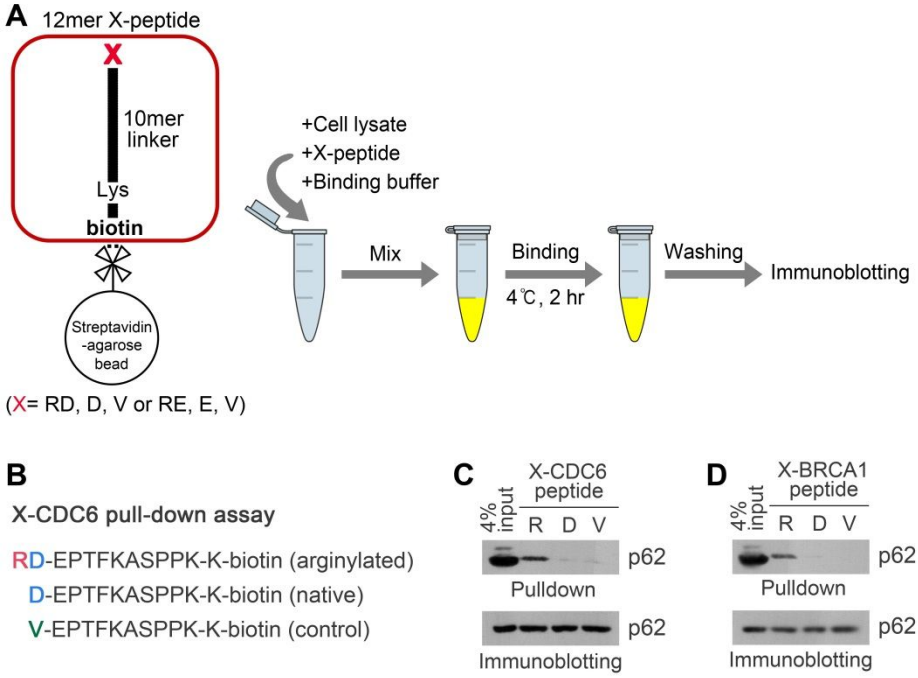


Figure 6. N-terminally arginylated forms of CDC6 and BRCA1 fragments physically bind to the autophagic adaptor p62. (A) Schematic diagrams to describe X-peptide pull-down assay. C-terminal biotin of 12-mer X-peptide is cross-linked to streptavidin-agarose beads. X-peptide conjugated beads are incubated with cell lysates diluted in a binding buffer for 2 hr at 4℃. Then proteins bound to X-peptide conjugated beads are detected by immunoblotting. **(B)** The sequences of three kinds of X-CDC6 peptides (X= RD, D or V) used to perform X-CDC6 pull-down assays. **(C)** X-peptide pull-down assay using X-CDC6 peptides. Cell lysates were prepared by freezing-thawing cycles of HEK293 cells. The R-CDC6 peptide only has affinity to endogenous p62. **(D)** X-peptide pull-down assay with X-BRCA1 peptides

(X= RD, D or V). HEK293 cells were used for X-peptide pull-down assay. The R-BRCA1 peptide only binds to endogenous p62.

Many protein fragments containing arginylation-permissive N-terminal residues also interact with p62 in an N-terminal arginylation-dependent manner

In cells, a large number of proteins can be cleaved by intracellular proteases, generating new C-terminal fragments that expose destabilizing N-terminal residues (4, 12). These proteins can be divided into cytosolic proteins and ER-residing proteins. To verify whether these cytosolic proteins and ER-residing proteins bearing arginylation-permissive N-terminal residues such as Asp and Glu bind to p62, I generated a series of C-terminally biotin-conjugated 12-mer peptides (Table. 1) and employed X-peptide pull-down assays. Earlier study showed that neurodegeneration-associated C-terminal fragment of Tau is an N-end rule substrate, and Aβ42 produced through cleavages of the amyloid precursor protein (APP) by secretases is an N-end rule substrate (46). ER-residing proteins BiP, PDI, CRT, ERdj5 and GRP94 are cleaved by signal peptidases, exposing arginylation-permissive residues on mature proteins. N-terminal arginylation of BiP, PDI and CRT was identified in vivo (24).

First, in the case of the cytosolic protein fragments, endogenous p62 bound to only arginylated forms of Aβ42 and Tau peptides (Figure. 7A). Arginylated forms of PDI, CRT and ERdj5 peptides that represent ER-residing proteins pulled down endogenous p62. However, exceptionally, binding of p62 with any GRP94 peptide was not detected in our condition (Figure. 7B). Thus, I determine that various proteins containing arginylation-permissive residues also bind to p62 through the interaction of their N-terminal Arg.

Table 1. The Sequences of synthetic X-peptides

Protein	Sequence of X-peptide	P1' residue (X)	Protease	Subcellular localization
X-CDC6	X-E-P-T-F-K-A-S-P-P-K-K-biotin	**Asp**^{128}	Caspase-3	Cytosol
X-Aβ42	X-A-E-F-R-H-D-S-G-Y-E-K-biotin	**Asp**^{597}	β-secretase	
X-BRCA1	X-G-E-I-K-E-D-T-S-F-A-K-biotin	**Asp**^{1156}	Caspase-3	
X-Tau	X-P-R-Q-E-F-E-V-M-E-D-K-biotin	**Glu**^{3}	Calpains	
X-BiP	X-E-E-D-K-K-E-D-V-G-T-K-biotin	**Glu**^{19}	Signal peptidase	Endoplasmic reticulum (ER)
X-PDI	X-A-P-E-E-E-D-H-V-L-V-K-biotin	**Asp**^{20}		
X-CRT	X-P-A-V-Y-F-K-E-Q-F-L-K-biotin	**Glu**^{19}		
X-GRP94	X-D-E-V-D-V-D-G-T-V-E-K-biotin	**Asp**^{22}		
X-ERdj5	X-Q-D-F-Y-S-L-L-G-V-S-K-biotin	**Asp**^{34}		

(X= RD, D, V or X= RE, E, V)

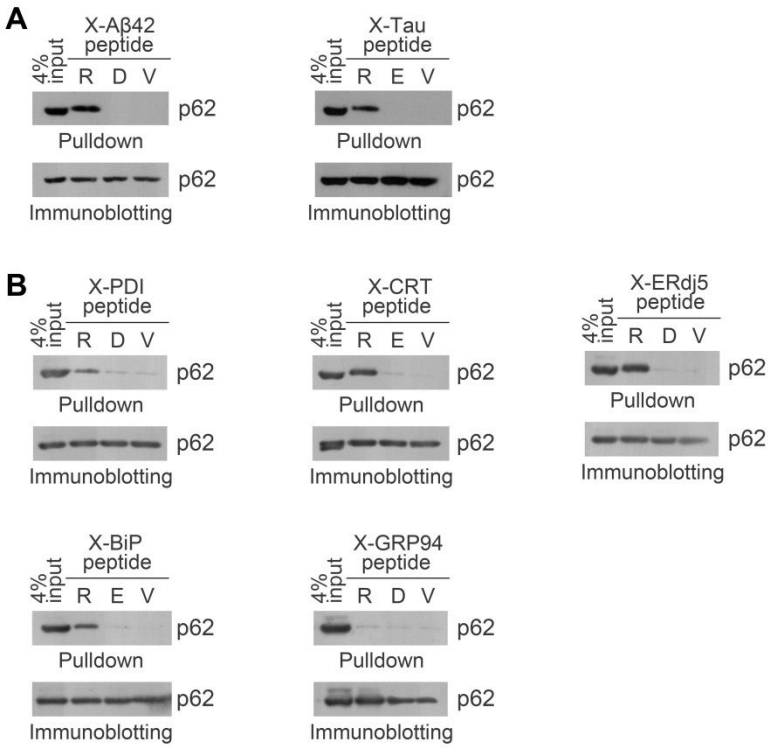


Figure 7. Many protein fragments containing arginylation-permissive N-terminal residues also interact with p62 in an N-terminal arginylation-dependent manner. (A) X-peptide pull-down assays with Aβ42 and Tau

peptides that indicate cytosolic protein fragments. R-Aβ42 and R-Tau peptides precipitate endogenous p62. **(B)** X-peptide pull-down assays using various X-peptides including PDI, CRT, ERdj5, BiP and GRP94 peptides that represent ER-residing proteins. Endogenous p62 binds to arginylated peptides of PDI, CRT, ERdj5 and BiP.

ZZ domain of p62 is a recognition site for N-terminal Arg

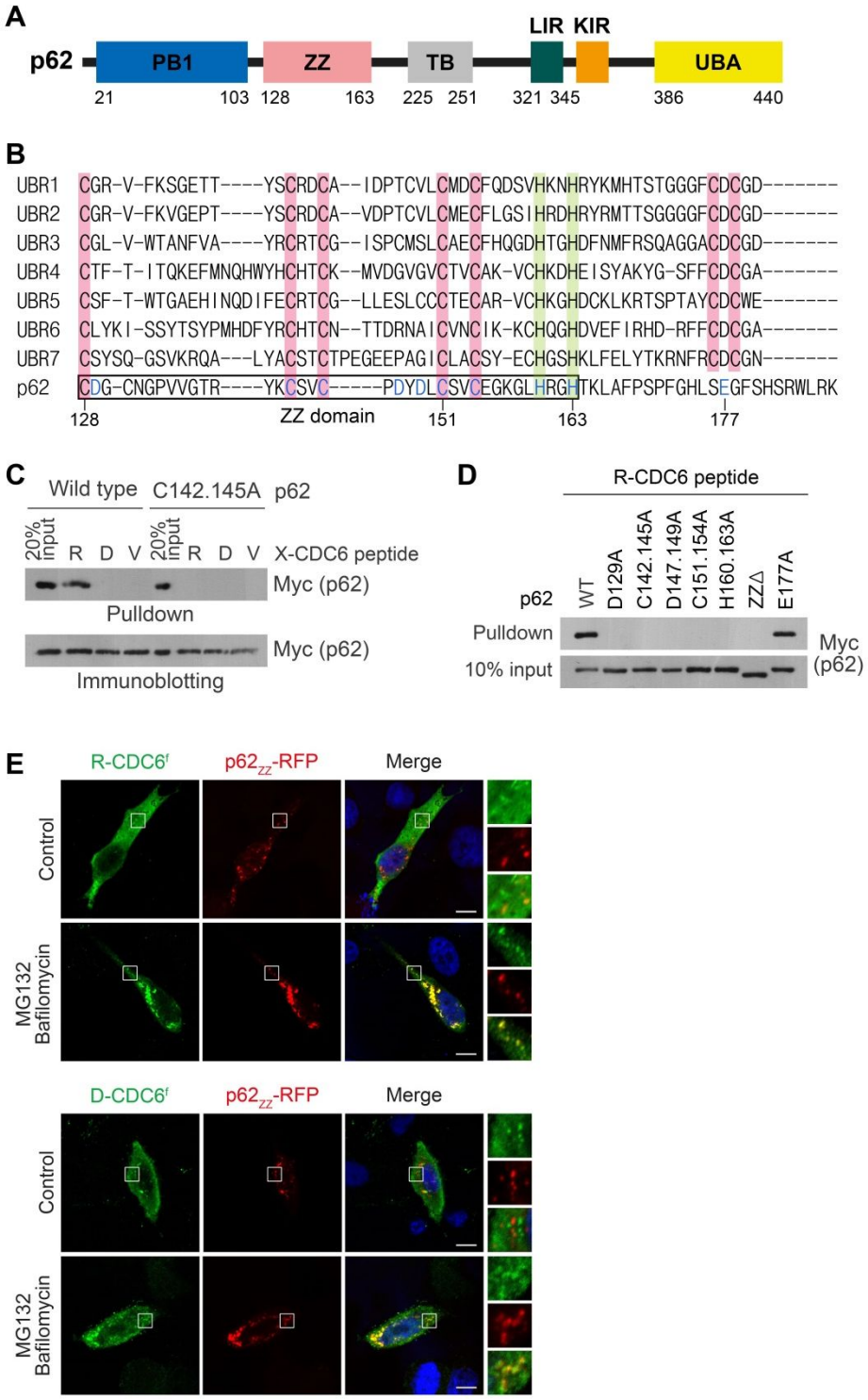
p62 has multiple domains containing PB1 (aa. 21-103), ZZ (aa. 128-163), LIR (aa. 321-345), KIR (aa. 346-359) and UBA (aa. 386-440) domains (Figure. 8A) (22, 23, 47). Among these domains of p62, I want to demonstrate which domain of p62 is a binding site for arginylated proteins. In Ub-dependent N-end rule pathway, UBR box protein family known as N-recognins interact with N-degrons through two distinct substrate recognition domains, the UBR box for type 1 N-degrons and N-domain for type 2 N-degrons (12, 48). The UBR box has two contiguous zinc fingers composed of three zinc ions (13, 16, 17). The first zinc finger is atypical ($CX_{24}CX_2CX_{21}C$ $XCX_{11}CX_2H$) and a novel binuclear zinc finger containing two zinc ions that are shared by a cysteine ligand for tetrahedral coordination (12, 17). The second zinc finger is typical ($CX_2CX_{20}HX_2H$) with one zinc ion (17). These two zinc fingers stabilize UBR box structure and form a binding site for type 1 N-degrons (16, 17, 48). Similarly, p62 has a zinc finger motif in its ZZ domain. Moreover, Asp residues in UBR box are very important for binding to N-terminal Arg by forming hydrogen bond and, p62 also has three Asp residues in ZZ domain (Figure. 8B) (12, 16, 17). Thus, we found that UBR box was structurally similar to ZZ domain and assumed that the ZZ domain of p62 is necessary to interact with N-terminal Arg of CDC6.

To demonstrate this hypothesis, we produced distinct mutants of human p62 tagged with myc-his (D129A, C142.145A, D147.149A, C151.154A, H160.163A, ZZΔ, E177A). I first carried out X-peptide pull-down assay with

ZZ domain mutant (C142.145A) expressed in HEK293 cells. As expected, R-CDC6 peptide precipitated wild-type p62 but not its C142.145A mutant (Figure. 8C). Next, HEK293 cells were transiently transfected with various p62 mutants, and I employed X-peptide pull-down assay. Wild-type p62 bound to R-CDC6 peptide, whereas the binding of p62 with R-CDC6 was completely abolished by overexpression of a ZZ-deletion mutant or ZZ-point mutants (ZZΔ, D129A, C142.145A, D147.149A, C151.154A or H160.163A). The p62 mutant (E177A), in which Glu 177 was mutated to Ala locating outside of ZZ domain, had no effect on binding to R-CDC6 peptide (Figure. 8D).

To further confirm the ZZ domain of p62 is responsible for binding to R-CDC6, I carried out immunostaining using a 93-residue ZZ-only fragment fused with RFP, $p62_{ZZ}$-RFP (aa. 83-175). HeLa CCL2 cells were co-transfected with flag-DHFR-Ub-X-CDC6-flag and $p62_{ZZ}$-RFP. In normal condition, CDC6 puncta were rarely formed and did not co-localize with $p62_{ZZ}$-RFP. In cells treated with both MG132 and bafilomycin, prolonged proteasomal inhibition and blocking autophagy flux, respectively, R-CDC6 and D-CDC6 formed cytosolic puncta and co-localized with $p62_{ZZ}$-RFP puncta, while V-CDC6 failed in forming puncta and could not co-localize with $p62_{ZZ}$-RFP puncta (Figure. 8E). These results suggest that ZZ-only fragment $p62_{ZZ}$-RFP is sufficient to interact with N-terminal Arg of CDC6 under stress condition. Moreover, both puncta formation of CDC6 and co-localization with $p62_{ZZ}$-RFP by prolonged proteasomal inhibition occur in an N-terminal

arginylation-dependent manner. In conclusion, ZZ domain of p62 is a recognition site for N-terminal Arg.


A
p62
PB1
ZZ
TB
LIR
KIR
UBA
21
103
128
163
225
251
321
345
386
440
B
UBR1 CGR-V-FKSGETT----YSCRDCA--IDPTCVLCMDCFQDSVHKNHRYKMHTSTGGGFCDCGD-------
UBR2 CGR-V-FKVGEPT----YSCRDCA--VDPTCVLCMECFLGSIHRDHRYRMTTSGGGGFCDCGD-------
UBR3 CGL-V-WTANFVA----YRCRTCG--ISPCMSLCAECFHQGDHTGHDFNMFRSQAGGACDCGD-------
UBR4 CTF-T-ITQKEFMNQHWYHCHTCK--MVDGVGVCTVCAK-VCHKDHEISYAKYG-SFFCDCGA-------
UBR5 CSF-T-WTGAEHINQDIFECRTCG--LLESLCCCTECAR-VCHKGHDCKLKRTSPTAYCDCWE-------
UBR6 CLYKI-SSYTSYPMHDFYRCHTCN--TTDRNAICVNCIK-KCHQGHDVEFIRHD-RFFCDCGA-------
UBR7 CSYSQ-GSVKRQA---LYACSTCTPEGEEPAGICLACSY-ECHGSHKLFELYTKRNFRCDCGN-------
p62 CDG-CNGPVVGTR----YKCSVC-----PDYDLCSVCEGKGLHRGHTKLAFPSPFGHLSEGFSHSRWLRK
128
ZZ domain
151
163
177
C
Wild type
C142.145A
p62
20% input
R
D
V
20% input
R
D
V
X-CDC6 peptide
Myc (p62)
Pulldown
Myc (p62)
Immunoblotting
D
R-CDC6 peptide
p62
WT
D129A
C142.145A
D147.149A
C151.154A
H160.163A
ZZΔ
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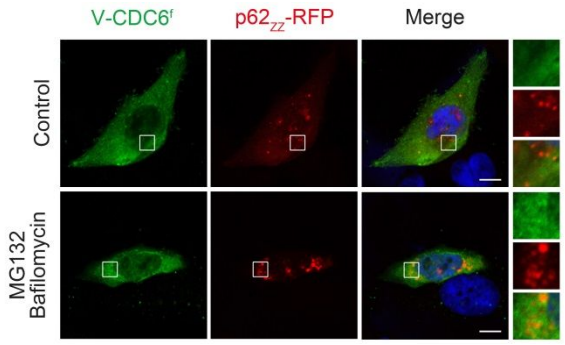

Figure 8. ZZ domain of p62 is a recognition site for N-terminal Arg. (A) Structure of human p62. **(B)** A sequence alignment of the UBR box of human UBR proteins and ZZ domain of human p62. Cysteine and histidine residues that are marked indicate zinc-coordinating residues. Blue letters of p62 denote mutation sites used to generate p62 mutants. **(C)** X-peptide pull-down assay using R-CDC6 and V-CDC6 peptides. HEK293 cells were transfected with p62 mutant, in which Cys 142 and 145 were mutated to Ala. **(D)** X-peptide pull-down assay with various p62 mutants tagged with myc-his. The p62 mutants were transiently expressed in HEK293 cells. **(E)** Puncta formation and co-localization analyses of X-CDC6^{f} (green) with p62$_{zz}$-RFP in HeLa CCL2 cells treated with both 0.3 μM MG132 for 18 hr and 0.2 μM bafilomycin for 5 hr. X-CDC6^{f} are visualized by anti-flag antibody. Enlarged views of the white box areas are shown. Scale bar, 10μM

Several protein fragments bearing arginylation-permissive N-terminal residues bind to UBR proteins in an N-terminal arginylation-dependent manner

The N-end rule substrates are recognized by N-recognins such as UBR1, UBR2, UBR4 and UBR5 for ubiquitination and delivery of target proteins to the proteasome (14, 15). UBR1 and UBR4 were previously validated as N-recognins that interact with both type 1 and type 2 N-degrons through X-peptide pull-down assays with X-nsP4 peptides (14, 15). nsP4, the RNA polymerase of the Sindbis virus, is a representative N-end rule substrate that exposes N-terminal Tyr (4). To confirm the interactions between X-nsP4 peptides (X= R, F or V) and N-recognins including UBR1 and UBR4, X-peptide pull-down assays were carried out. Consistently, R-nsP4 and F-nsP4 bearing type 1 and type 2 N-degrons, respectively, pulled down endogenous UBR1 and UBR4. V-nsP4 containing stabilizing N-terminal residue did not precipitate both UBR1 and UBR4 (Figure. 9A).

To identify the binding properties of the UBR proteins to putative and confirmed N-end rule substrates bearing arginylation-permissive residues, I performed X-peptide pull-down assays with various X-peptides. R-CDC6 and R-Aβ42 peptides precipitated endogenous UBR1, and R-CRT and R-ERdj5 peptides weakly bound to endogenous UBR1 (Figure. 9B). The interaction of endogenous UBR4 with all the X-peptides that I had was not detected in our experimental setting (Figure. 9C).

In conclusion, several arginylated forms of X-peptides bind to UBR proteins through their N-terminal Arg. The recognition of putative and confirmed N-end rule substrates by UBR proteins is identified in vivo and has certain specificity among different kinds of proteins.

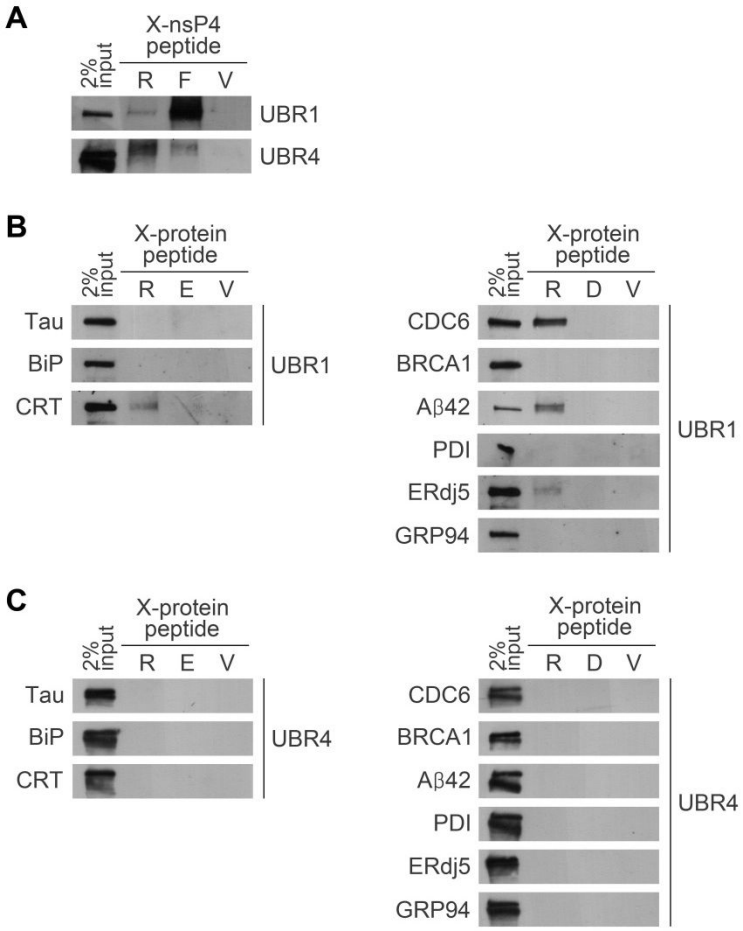

Figure 9. Several protein fragments bearing arginylation-permissive N-terminal residues bind to UBR proteins in an N-terminal arginylation-dependent manner. (A) X-peptide pull-down assays using X-nsP4 peptides (X= R, F or V) to confirm interactions with N-recognins including UBR1 and UBR4. **(B)** X-peptide pull-down assays with six X-peptides bearing N-terminal Asp and with three X-peptides containing N-terminal Glu to identify interaction with endogenous UBR1. Arginylated peptides of CRT, CDC6,

Aβ42 and ERdj5 pull down endogenous UBR1. **(C)** X-peptide pull-down assays using various X-peptides, followed by immunoblotting with anti-UBR4 antibody.

DISCUSSION

In the N-end rule pathway, it is known that proteins with N-degrons are recognized by E3 ligases called N-recognins such as UBR1, UBR2, UBR4 and UBR5 in mammals, and are subsequently targeted to UPS for proteolysis (4, 12, 15). Looking at the studies performed to date, N-degrons in the N-end rule pathway has been just studied in connection with UPS (3, 4, 11-13). Little is known about the function of N-degrons in autophagy. In previously published paper from our group, for the first time, it validated the relationship between N-degron, especially N-terminal Arg that can be generated by ATE1, and autophagy using ER chaperone BiP as a model substrate (24).

In this study, I focus on the characterization of the replication licensing factor CDC6 as a new N-end rule substrate and expansion of our previous paper using cytosolic protein fragments. Here, I demonstrate that natural C-terminal fragments of CDC6 and BRCA1 are N-end rule substrates arginylated by R-transferase ATE1 and degraded through the proteasome. Then, X-peptide pull-down assays show that a number of proteins which are known to have arginylation-permissive N-terminal residues including CDC6, BRCA1, Tau, Aß42, ERdj5, PDI, CRT and BiP physically interact with p62 in an N-terminal arginylation-dependent manner. Especially, N-terminal Arg binds to the ZZ domain of p62 that has a C_2H_2 zinc finger motif. Thus, I demonstrate

that the autophagic adaptor p62 plays a role as a recognition component of N-terminal Arg.

In this study, it proposes the possibility that N-terminal Arg of the cytosolic protein fragment can act as an autophagic N-degron by interacting with p62 like ER-residing chaperone BiP (24), although there may be a little differences in details of action mode. In summary, I suggest a model of a bimodal function of N-terminal Arg as a degron (Figure. 10). In addition to the above mentioned, there are various proteins can be cleaved by intracellular proteases such as caspases, separases, calpains and secretases, yielding new C-terminal proteolytic fragments (12). Especially, some of them expose arginylation-permissive residues and undergo posttranslational conjugation with Arg by ATE1. Under normal condition, these arginylated protein fragments are recognized by UBR box of UBR proteins, proteasomal N-recognins, and are subsequently degraded by UPS. In other words, N-terminal Arg acts as a proteasomal N-degron, a degradation signal for UPS. However, in stress conditions such as proteasomal inhibition or autophagy induction, N-terminal Arg that can function as an autophagic N-degron binds to the ZZ domain of p62. Then, arginylated proteins can be targeted to autophagy for degradation. Thus, I suggest the biomodal function of N-terminal Arg in two protein degradation system. Although there are many things to do to generalize and expand our studies using cytosolic protein fragments, this study provides some important evidences for further research.

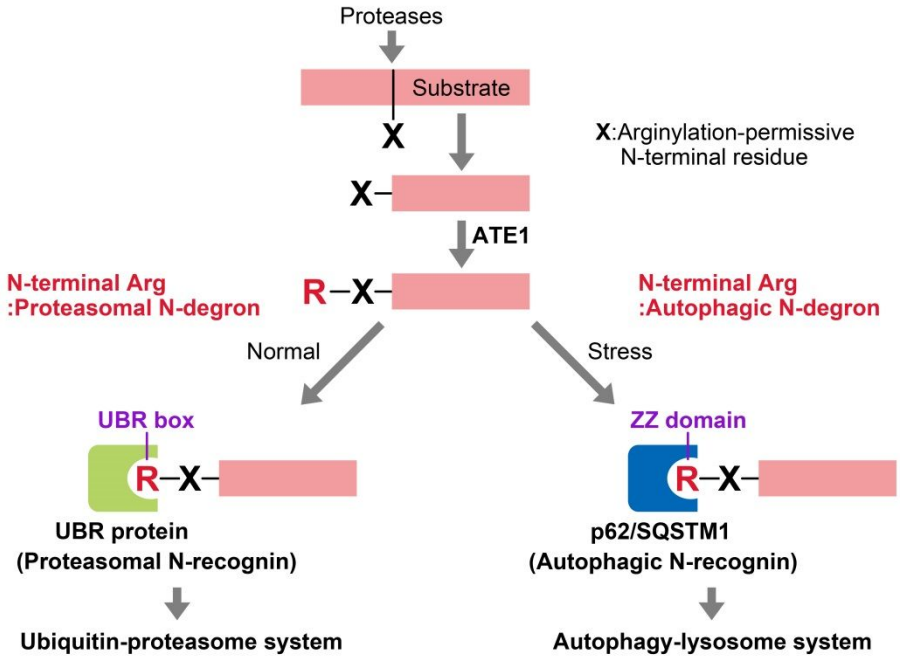

Figure 10. A model of the bimodal function of N-terminal Arg as a degron. Proteases-generated C-terminal fragments exposing arginylation-permissive N-terminal residues (X) are conjugated with Arg by ATE1. In normal condition, N-terminal Arg that functions as a proteasomal N-degron is recognized by UBR protein. Under stress conditions, N-terminal Arg acts as an autophagic N-degron by binding to p62, an autophagic N-recognin.

REFERENCES

1. Amm I, Sommer T, Wolf DH. Protein quality control and elimination of protein waste: the role of the ubiquitin-proteasome system. Biochimica et biophysica acta. 2014;1843(1):182-96.

2. Ravid T, Hochstrasser M. Diversity of degradation signals in the ubiquitin-proteasome system. Nature reviews Molecular cell biology. 2008;9(9):679-90.

3. Gibbs DJ, Bacardit J, Bachmair A, Holdsworth MJ. The eukaryotic N-end rule pathway: conserved mechanisms and diverse functions. Trends in cell biology. 2014;24(10):603-11.

4. Varshavsky A. The N-end rule pathway and regulation by proteolysis. Protein science : a publication of the Protein Society. 2011;20(8):1298-345.

5. Buchberger A, Bukau B, Sommer T. Protein quality control in the cytosol and the endoplasmic reticulum: brothers in arms. Molecular cell. 2010;40(2):238-52.

6. Tanaka K, Matsuda N. Proteostasis and neurodegeneration: the roles of proteasomal degradation and autophagy. Biochimica et biophysica acta. 2014;1843(1):197-204.

7. Lilienbaum A. Relationship between the proteasomal system and autophagy. International journal of biochemistry and molecular biology. 2013;4(1):1-26.

8. Feng Y, He D, Yao Z, Klionsky DJ. The machinery of macroautophagy. Cell research. 2014;24(1):24-41.

9. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. The Journal of biological chemistry. 2007;282(33):24131-45.

10. Shaid S, Brandts CH, Serve H, Dikic I. Ubiquitination and selective

autophagy. Cell death and differentiation. 2013;20(1):21-30.

11. Mogk A, Schmidt R, Bukau B. The N-end rule pathway for regulated proteolysis: prokaryotic and eukaryotic strategies. Trends in cell biology. 2007;17(4):165-72.

12. Sriram SM, Kim BY, Kwon YT. The N-end rule pathway: emerging functions and molecular principles of substrate recognition. Nature reviews Molecular cell biology. 2011;12(11):735-47.

13. Tasaki T, Sriram SM, Park KS, Kwon YT. The N-end rule pathway. Annual review of biochemistry. 2012;81:261-89.

14. Tasaki T, Mulder LC, Iwamatsu A, Lee MJ, Davydov IV, Varshavsky A, et al. A family of mammalian E3 ubiquitin ligases that contain the UBR box motif and recognize N-degrons. Molecular and cellular biology. 2005;25(16):7120-36.

15. Tasaki T, Zakrzewska A, Dudgeon DD, Jiang Y, Lazo JS, Kwon YT. The substrate recognition domains of the N-end rule pathway. The Journal of biological chemistry. 2009;284(3):1884-95.

16. Choi WS, Jeong BC, Joo YJ, Lee MR, Kim J, Eck MJ, et al. Structural basis for the recognition of N-end rule substrates by the UBR box of ubiquitin ligases. Nature structural & molecular biology. 2010;17(10):1175-81.

17. Matta-Camacho E, Kozlov G, Li FF, Gehring K. Structural basis of substrate recognition and specificity in the N-end rule pathway. Nature structural & molecular biology. 2010;17(10):1182-7.

18. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. Autophagy. 2011;7(3):279-96.

19. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451(7182):1069-75.

20. Klionsky DJ, Schulman BA. Dynamic regulation of macroautophagy by distinctive ubiquitin-like proteins. Nature structural & molecular biology.

2014;21(4):336-45.

21. Ryter SW, Cloonan SM, Choi AM. Autophagy: a critical regulator of cellular metabolism and homeostasis. Molecules and cells. 2013;36(1):7-16.

22. Lin X, Li S, Zhao Y, Ma X, Zhang K, He X, et al. Interaction domains of p62: a bridge between p62 and selective autophagy. DNA and cell biology. 2013;32(5):220-7.

23. Puissant A, Fenouille N, Auberger P. When autophagy meets cancer through p62/SQSTM1. American journal of cancer research. 2012;2(4):397-413.

24. Cha-Molstad H, Sung KS, Hwang J, Kim KA, Yu JE, Yoo YD, et al. Amino-terminal arginylation targets endoplasmic reticulum chaperone BiP for autophagy through p62 binding. Nature cell biology. 2015;17(7):917-29.

25. Davydov IV, Varshavsky A. RGS4 is arginylated and degraded by the N-end rule pathway in vitro. The Journal of biological chemistry. 2000;275(30):22931-41.

26. Lee MJ, Tasaki T, Moroi K, An JY, Kimura S, Davydov IV, et al. RGS4 and RGS5 are in vivo substrates of the N-end rule pathway. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(42):15030-5.

27. Tan CY, Hagen T. Destabilization of CDC6 upon DNA damage is dependent on neddylation but independent of Cullin E3 ligases. The international journal of biochemistry & cell biology. 2013;45(7):1489-98.

28. Archambault V, Ikui AE, Drapkin BJ, Cross FR. Disruption of mechanisms that prevent rereplication triggers a DNA damage response. Molecular and cellular biology. 2005;25(15):6707-21.

29. Pelizon C, d'Adda di Fagagna F, Farrace L, Laskey RA. Human replication protein Cdc6 is selectively cleaved by caspase 3 during apoptosis. EMBO reports. 2002;3(8):780-4.

30. Schories B, Engel K, Dorken B, Gossen M, Bommert K. Characterization of apoptosis-induced Mcm3 and Cdc6 cleavage reveals a

proapoptotic effect for one Mcm3 fragment. Cell death and differentiation. 2004;11(8):940-2.

31. Yim H, Hwang IS, Choi JS, Chun KH, Jin YH, Ham YM, et al. Cleavage of Cdc6 by caspase-3 promotes ATM/ATR kinase-mediated apoptosis of HeLa cells. The Journal of cell biology. 2006;174(1):77-88.

32. Yim H, Jin YH, Park BD, Choi HJ, Lee SK. Caspase-3-mediated cleavage of Cdc6 induces nuclear localization of p49-truncated Cdc6 and apoptosis. Molecular biology of the cell. 2003;14(10):4250-9.

33. Varshavsky A. Ubiquitin fusion technique and related methods. Methods in enzymology. 2005;399:777-99.

34. Drabkin HJ, RajBhandary UL. Initiation of protein synthesis in mammalian cells with codons other than AUG and amino acids other than methionine. Molecular and cellular biology. 1998;18(9):5140-7.

35. Wong CC, Xu T, Rai R, Bailey AO, Yates JR, 3rd, Wolf YI, et al. Global analysis of posttranslational protein arginylation. PLoS biology. 2007;5(10):e258.

36. Karakozova M, Kozak M, Wong CC, Bailey AO, Yates JR, 3rd, Mogilner A, et al. Arginylation of beta-actin regulates actin cytoskeleton and cell motility. Science. 2006;313(5784):192-6.

37. Kwon YT, Kashina AS, Davydov IV, Hu RG, An JY, Seo JW, et al. An essential role of N-terminal arginylation in cardiovascular development. Science. 2002;297(5578):96-9.

38. Rai R, Wong CC, Xu T, Leu NA, Dong DW, Guo C, et al. Arginyltransferase regulates alpha cardiac actin function, myofibril formation and contractility during heart development. Development. 2008;135(23):3881-9.

39. Wang J, Han X, Saha S, Xu T, Rai R, Zhang F, et al. Arginyltransferase is an ATP-independent self-regulating enzyme that forms distinct functional complexes in vivo. Chemistry & biology. 2011;18(1):121-30.

40. Hu RG, Brower CS, Wang H, Davydov IV, Sheng J, Zhou J, et al. Arginyltransferase, its specificity, putative substrates, bidirectional promoter, and splicing-derived isoforms. The Journal of biological chemistry. 2006;281(43):32559-73.

41. Kwon YT, Kashina AS, Varshavsky A. Alternative splicing results in differential expression, activity, and localization of the two forms of arginyl-tRNA-protein transferase, a component of the N-end rule pathway. Molecular and cellular biology. 1999;19(1):182-93.

42. Futreal PA, Liu Q, Shattuck-Eidens D, Cochran C, Harshman K, Tavtigian S, et al. BRCA1 mutations in primary breast and ovarian carcinomas. Science. 1994;266(5182):120-2.

43. Narod SA, Foulkes WD. BRCA1 and BRCA2: 1994 and beyond. Nature reviews Cancer. 2004;4(9):665-76.

44. Piatkov KI, Brower CS, Varshavsky A. The N-end rule pathway counteracts cell death by destroying proapoptotic protein fragments. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(27):E1839-47.

45. Xu Z, Payoe R, Fahlman RP. The C-terminal proteolytic fragment of the breast cancer susceptibility type 1 protein (BRCA1) is degraded by the N-end rule pathway. The Journal of biological chemistry. 2012;287(10):7495-502.

46. Brower CS, Piatkov KI, Varshavsky A. Neurodegeneration-associated protein fragments as short-lived substrates of the N-end rule pathway. Molecular cell. 2013;50(2):161-71.

47. Matsumoto G, Wada K, Okuno M, Kurosawa M, Nukina N. Serine 403 phosphorylation of p62/SQSTM1 regulates selective autophagic clearance of ubiquitinated proteins. Molecular cell. 2011;44(2):279-89.

48. Sriram SM, Kwon YT. The molecular principles of N-end rule recognition. Nature structural & molecular biology. 2010;17(10):1164-5.

국문 초록

진핵생물에서 단백질 분해 기작은 크게 유비퀴틴-프로테아좀 시스템과 오토파지-라이소좀 시스템 두 가지로 구성된다. N-end rule pathway는 단백질의 N-말단에 존재하는 아미노산 종류에 따른 단백질의 안정성 조절과 관련된다. 포유류의 N-end rule pathway에서 단백질의 불안정 N-말단 잔기는 그 단백질의 유비퀴틴화와 26S 프로테아좀에 의한 분해를 매개하는 N-recognins에 의해 인식된다. 포유류 N-recognins은 UBR box 단백질이라고 불리는 E3 ligases이며 이것은 UBR1, UBR2, UBR4, UBR5로 구성된다.

최근 본 그룹에서 처음으로 N-말단 아르기닌이 오토파지 분해신호(autophagic degron)라는 것을 소포체 샤페론인 BiP을 이용해서 증명하였다. 본 연구실에서는 genome-wide functional proteomic screening을 통해서 CDC6가 N-end rule 기질로 추정되는 단백질이라는 것을 발견하였으며 이 단백질을 우리의 연구를 일반화하기 위한 세포질 단백질의 모델 기질로서 사용하였다. 먼저 N-말단 아르기닌화 CDC6 펩타이드 (R-CDC6 펩타이드)를 이용해 토끼에서 다클론 R-CDC6 항체를 제작하였다. 이 특이적 항체를 이용한 웨스턴 블롯과 면역염색 방법을 통해 in vivo에서 N-말단 아스파르트산을 가지는 caspase-3에 의해 생성되는 C-말단 단백질 조각이

ATE1에 의해 N-말단 아르기닌화 되는 것을 규명하였다. 본 연구는 CDC6 조각이 프로테아좀에 의해 분해되는 새로운 N-end rule 기질이라는 것을 증명하였고, BRCA1의 C-말단 조각 또한 N-말단 아르기닌화 되고 N-end rule pathway에 의해 분해되는 것을 관찰하였다. X-peptide pull-down assays는 이 두 가지 아르기닌화 된 단백질들이 그들의 N-말단 아르기닌을 통해 오토파지 어댑터인 p62/SQSTM1과 물리적으로 결합하는 것을 보여준다. 또한 p62의 ZZ 도메인이 N-말단 아르기닌의 결합위치라는 것을 p62 점돌연변이와 결실 돌연변이를 이용하여 규명하였다. 아르기닌화 허용 N-말단 잔기들인 아스파르트산과 글루탐산을 가지는 다른 많은 종류의 단백질들도 N-말단 아르기닌화-의존적 방식으로 p62와 상호작용하는 것을 관찰하였다.

본 연구에서는 단백질 조각의 N-말단 아르기닌의 N-degron으로서의 두 가지 기능을 제시한다. 이미 잘 정립되어있는 첫 번째 기능은 유비퀴틴-프로테아좀 시스템에서 분해신호로 작용하는 것이다. 두 번째는 오토파지에서 오토파지 분해신호로 기능할 수 있는 새로운 역할이다. 이를 통해 본 연구는 N-말단 아르기닌화와 오토파지 사이의 관계에 대한 새로운 이해를 제공한다.

주요어 : N-end rule pathway, N-말단 아르기닌화, CDC6, 유비퀴틴-프로테아좀 시스템, 오토파지-라이소좀 시스템, p62/SQSTM1,
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