이학석사 학위논문

The Autophagic Adaptor p62 Binds to Destabilizing N-terminal Residues of the N-end rule pathway.

아미노 말단 법칙의 불안정한 아미노 말단 잔기에 붙는 자가포식 어댑터 p62 단백질에 관한 연구

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Abstract

The Autophagic Adaptor p62 Binds to Destabilizing N-terminal Residues of the N-end Rule Pathway.

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The N-end rule pathway is a proteolytic system in which a destabilizing N-terminal residue {type 1 (Arg, Lys, His) and type 2 (Phe, Trp, Tyr, Lue, Ile) in mammals} acts as a degradation signal (N-degron). Known functions of the pathway include the regulated proteolysis of short-lived proteins through the ubiquitin-proteasome system. So far, known N-recognins include UBR1, UBR2, UBR4 and UBR5, which possess an evolutionally conserved 72-residue UBR box that is required for recognizing N-degrons. We used synthetic peptides carrying N-

terminal destabilizing residues to purify recognition components (N-recognins) of the pathway. Our proteomic screen for N-degron binding proteins identified p62/SQSTM1 known to mediate autophagic degradation of aberrant proteins as a new recognition component of the N-end rule pathway. In this study, we characterized the binding specificity and other biochemical properties of p62 as a potential N-recognin of the N-end rule pathway. Pull-down assays with synthetic N-end rule peptides show that its ZZ domain whose function had remained unclear is responsible for the binding to N-end rule N-termini. p62 binds N-terminal Arg and other type-1 peptides (Lys and His) as well as a subset of type-2 peptides (Phe, Trp and Tyr) but not Leu and Ile. Site-directed mutagenesis revealed specific residues critical for the interaction with N-end rule peptides and conserved in p62 and known N-recognins. And the surface plasmon resonance biacore assay again demonstrated that the interaction between p62 and destabilizing N-end rule N-termini. Our results suggest that p62 may be a new recognition component of the N-end rule pathway and hints that to be an effective autophagy inducer, a ligand should have high affinity to the ZZ domain.

Key words : N-end rule pathway, UBR, autophagy, p62(sequestosome-1)

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1.Introduction

The N-end rule pathway is a ubiquitin-dependent proteolytic system in which N-terminal residues of short-lived protein act as an essential degradation signal (called N-degrons) that is recognized by E3 ligases (called N-recognins) leading to polyubiquitination and proteasomal degradation of a protein [1-15]. This system is well conserved throughout evolution and is involved in many biological processes such as cardiac development and signaling, angiogenesis, DNA repair, apoptosis, meiosis, neurogenetis, pancreatic functions, learning and memory, female development, muscle atrophy, and olfaction. N-degrons are generated either by the removal of N-terminal methionine or by the endoproteolytic cleavage of an otherwise stable protein. An N-degron can be created from a pre-N-degron through specific N-terminal modifications (Fig 1A). Specifically, in mammals, N-terminal Asn and Gln are tertiary destabilizing residues which function through their deamidation by N-terminal amidohydrolases into the secondary destabilizing N-terminal residues Asp and Glu [6] . Exposed secondary pro-N-degrons, Asp, Glu and Cys undergo ATE1-dependent post-translational arginylation which results in the principle N-degron Arg. N-terminal Cys can also function as a tertiary destabilizing residue through its oxidation in a manner depending on nitric oxide and oxygen (O2). Oxidized Cys is subsequently arginylated by ATE1. Primary N-degrons include basic residues such as Arg, Lys and His (Type 1) and bulky

hydrophobic residues such as Phe, Leu, Trp, Tyr and Ile (Type 2). In addition to a destabilizing N-terminal residue, a functional N-degron requires at least one internal Lys residue and a conformational feature required for optimal ubiquitylation. The primary destabilizing N-terminal residues directly bind specific UBR family E3 ligase and go through proteasomal degradation. A UBR protein contains two binding pockets and internal N-degron binding site (Fig 1B).

The mammalian genome encodes at least seven UBR box-containing proteins, termed UBR1 through UBR7 [10]. UBR1, UBR2 and UBR4 contain both a UBR box (Type 1 binding site) and an N-domain (Type 2 binding site) and bind both type 1 and type 2 N-degrons while UBR5 only has a UBR box and prefers to bind to type1 N-degrons [16,17]. Other recognition components (N-recognins) in this pathway include ClpS in bacteria [18-20] and PRT1 and PRT6 in plants [21], (Fig 2). PRT1 mediates the degradation of model substrates bearing aromatic hydrophobic residues (Phe, Trp and Tyr) but not aliphatic hydrophobic and basic residues (Leu and Ile) presumably through its ZZ domain [22,23]. On the other hand, PRT6 contains only a UBR box and regulates the stability of model substrates bearing type 1 N-degrons but not type 2 [24]. ClpS prefers to bind type 2 N-degrons, including Leu, Phe, Trp and Tyr. The crystal structure of the yeast UBR box has recently been solved [25]. This UBR box contains three zinc finger motifs; a typical and two atypical binuclear motifs, which stabilize the substrate binding pocket. Substrate contacting residues with negative charges are

positioned near the binding pocket on the surface of the UBR box domain to interact with basic type 1 N-degrons.

Macroautophagy of the autophagy-lysosome system is responsible for the degradation of long-lived proteins, misfolded and aggregated proteins, damaged organelles, and intracellular microorganisms [26-31]. Autophagy provides metabolic building blocks to maintain the synthesis of macromolecules and ATP production, which is important for the preservation of cellular bioenergetics and promotes cell survival. Autophagy activation leads to the formation of autophagosomes which involves the covalent conjugation of Atg12 to Atg5 [26,28]. The Atg5-Atg12 conjugates facilitate the conjugation of phosphatidylethanolamine (PE) to LC3. LC3 is used as a marker of autophagy because its lipidation reflects the formation of autophagosomes. Matured autophagosomes fuse with lysosomes to generate autolysosomes where the enclosed contents as well as the inner membrane are degraded by lysosomal enzymes.

p62 is a selective autophagy substrate comprised of multi-domains such as a PB1 oligomerization domain, a ZZ type zinc finger domain, a LC3 interacting region (LIR) and a UBA domain [32-34]. p62 functions to target itself and polyubiquitinated protein aggregates to the autophagosome for degradation. The ablation of autophagy leads to the marked accumulation of p62 as well as p62 containing protein inclusions [34]. It is believed that the targeting of p62 to the autophagosome depends on its interaction with LC3 associated on the autophagosomal membrane through its LIR domain. In addition, mutations in the

PB1 domain of p62 also resulted in the accumulation of p62, indicating that p62 oligomerization is also required for its autophagic degradation. A recent report demonstrated that p62 oligomerization but not its interaction with LC3 is required for the targeting of p62 to the autophagosomal initiation site [33].

In this study, we investigated the similarity between the UBR box of UBR family E3 ligase and the ZZ domain of p62 and characterized substrate binding specificities and recognition domains of p62 using X-peptide pull down assays. In our binding assays, p62 was able to pulled down by type-1, specially R-11, and type-2 N-terminal amino acid residues, but not by V-11 a stable type N-terminal amino acid residue. We also report that in contrast to other E3 systems which usually recognize substrates through protein-protein interface, p62 has a general substrate recognition domain termed the ZZ domain.

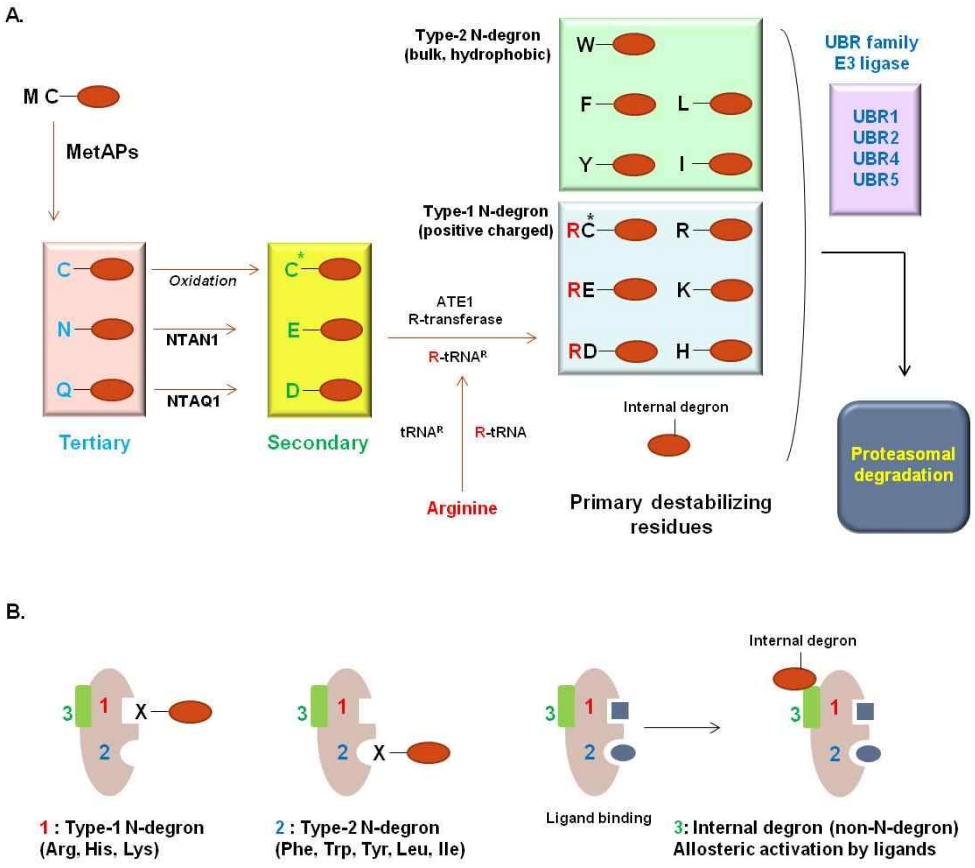


Figure 1. The N-end rule pathway. A, The N-terminal residues are indicated by single letter (Tertiary structure : blue, Secondary structure : green. And Primary structure : black). C* indicates oxidized N-terminal Cys which requires nitric oxide and oxygen. **B,** UBR family E3 ligase has two binding pocket. The UBR box recognizes type-1 N-end rule peptides and the N-domain recognizes type-2 N-end rule peptides. An internal N-degron is also recognized by UBR E3 ligase when ligands bind.

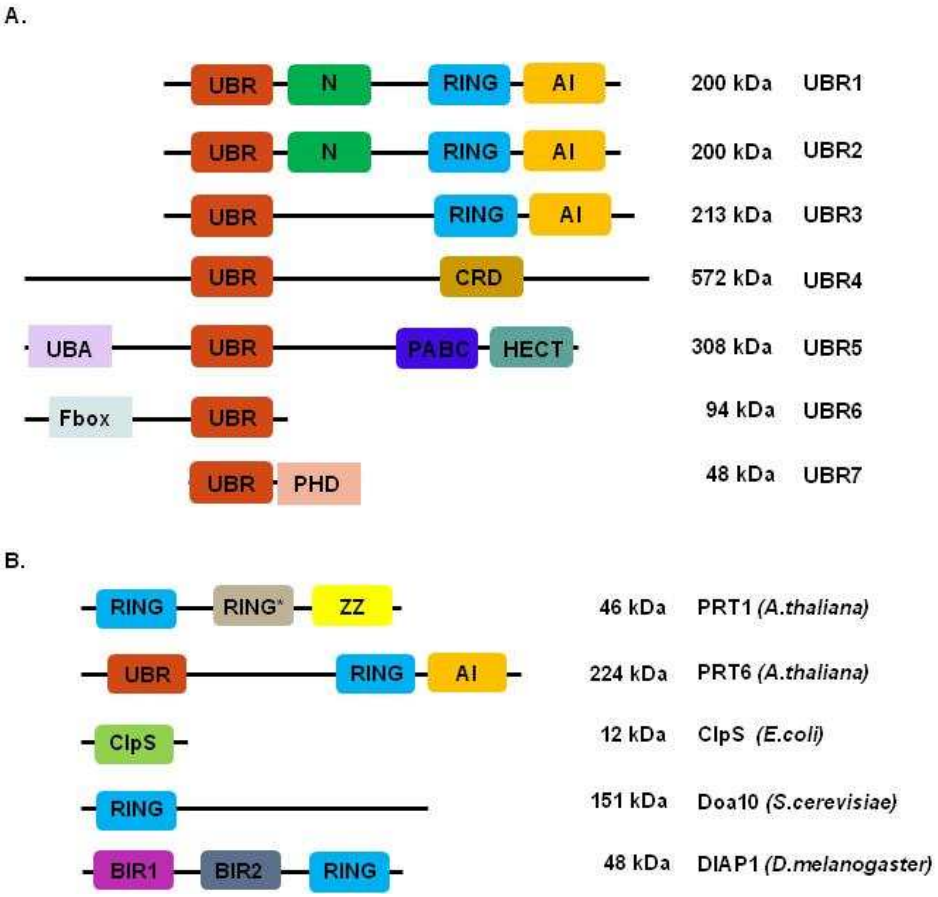


Figure 2. The domain structures of mammalian UBR family E3 ligase. A, All UBR family proteins have UBR box(red) which recognizes type-1 N-terminal residues. In addition UBR1 and UBR2 have N-domain(green) which recognizes type-2 N-terminal residues. **B,** Known and putative N-recognins of other species.

2. Materials and Methods

2.1 Plasmids and antibodies.

Human p62/sqstm1 cDNA fragment (1320 bp) amplified by PCR from hMU012675 clone (21C Frontier Human Gene Bank) was subcloned into pcDNA 3.1/*myc*-His (invitrogen) plasmid using EcoRI and XhoI sites. Using same enzyme sites, series of domain deletion mutants of p62/sqstm1 amplified by PCR were subcloned into pcDNA.3.1/*myc*-His. We employed site-directed mutagenesis to examine the effects of amino acid substitutions (X to Ala) around p62/sqstm1-ZZ domain on binding to type-1 and type-2 N-end rule N-termini. Six mutant constructs were generated using overlap extension PCR.

Rabbit polyclonal p62 antibody (1:1000, sc-25575, Santa Cruz Biotechnology) raised against recombinant full length protein, corresponding to amino acids 151-440 of human SQSTM1 / p62, were used for immunostaining of human p62/sqstm1 proteins in cultured cells. Other primary antibodies used are rabbit polyclonal anti-his tag (1: 1000, pm032, MBL International), rabbit polyclonal anti-myc (1:1000, ab9106, Abcam, Cambridge, UK), mouse monoclonal anti-myc (9E10), monoclonal anti-actin (1:2000, A1978, Sigma), and goat polyclonal anti-UBR2 (1:1000, NBP1-45243, Novus Biologicals). Donkey anti-mouse IgG-HRP (1:10000, sc-2314, Santa Cruz Biotechnology) and goat anti-rabbit IgG-HRP (1:10000, sc-2030, Santa Cruz Biotechnology) secondary antibodies were used.

2.2 Cell culture and immunoblotting.

HEK293 cell lines were incubated at 37℃ in a humidified 5% CO_2 incubator in Dulbecco's Modified Eagle Medium (DMEM)-high glucose (Invitrogen, Gibco) supplemented with 10% fetal bovine serum (FBS) until approaching confluency(`90%).

Whole cell lysates and pull down samples were separated by SDS-PAGE and overnight transferred onto 0.45 ㎛ PVDF membrane (Millipore, IPVH00010). We performed 1hr Incubation for primary, secondary antibody and washing (20 min, 3 times).

2.3 Overexpression of p62 protein in HEK293 and pull down preparation

p62 mutants which were inserted to pcDNA3.1 myc-his tag by T7 promoter/priming site and BHG reverse priming site were transfected into HEK293 cells transiently using lipofectamine 2000 (Invitrogen). Constructs came from WCI. After 24hr incubation, cells were collected by Trypsin-EDTA and resuspended to 5X volume using hypotonic solution (10mM HCl, 1.5mM $MgCl_2$, 10mM HEPES pH=7.9) and incubated on ice for 30 minutes. The mixture were freezed at liquid nitrogen and thawed in a 37℃ water bath five times quickly or Dounce homogenized (No detergent environment, physical lysis) and centrifuged at 12000rpm, 4℃ for 10 minutes.

2.4 X-peptide pull down assay

In the X-peptide pull down assay, a set of 12-mer peptides (X-I-F-S-T-I-E-G-R-T-Y-K-biotin) bearing N-terminal Arg, His, Lys(Type-1), Phe, Trp, Tyr, Leu(Type-2), Val, Asp or Gly(stabilizing control) residues were cross-linked through C-terminal biotin to streptavidin agarose resin(Thermo) [16]. The ratio of biotinylated peptide to streptavidin agarose beads was 0.5 ㎎ solubilize peptide per 1 ㎖ settled resin. The mixtures were diluted in 5X volume PBS and incubated overnight at 4℃. The beads were centrifuged at 2000rpm for 3 minutes and washed by equal volume of PBS three times and then used in the pull down assay. A aliquoted soluble extract (30 ㎕), containing 150-200 ㎍ of total protein, was diluted in 300 ㎕ binding buffer (0.05% Tween 20, 10% Glycerol, 0.2M KCl, 20mM HEPES pH=7.9) and mixed with X-peptide beads (50 ㎕ packed vol). The mixtures were incubated at 4℃ for 2hrs with gentle rotation. The beads were pelleted by centrifugation at 2000rpm for 30 sec, washed five times with 1 ㎖ of binding buffer quickly or three times at 4℃ for 20 minutes, resuspended in 2X SDS-PAGE 20 ㎕ and heated at 100℃ for 5 minutes. A analysis was performed by SDS-PAGE and immunoblotting.

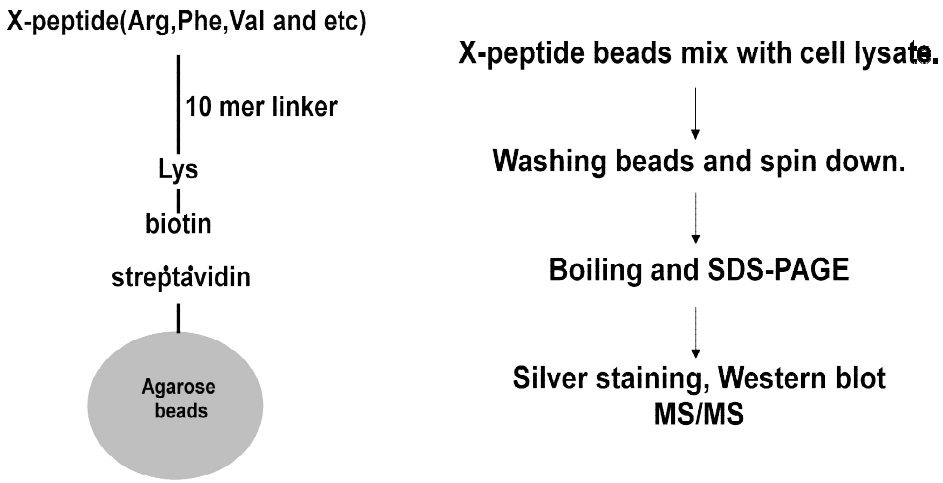


Figure 3. The X-peptide pull down assay. A 10-mer peptide bearing an N-terminal X-peptide (Type-1, Type-2 and stabilizing control) residue was cross-linked through biotin to streptividin- Sepharose beads. After mixing with cell lysates, bounded proteins which were captured the peptide beads were boiled and analysed by SDS-PAGE, Silver staining and Immunoblot.

2.5 Surface Plasmon Resonance (Biacore) assay

The Sensor chip SA with pre-immobilized streptavidin (GE Healthcare, Piscataway, NJ, USA) in one flow cell was first saturated with biotinylated-R11/V11/F11 peptides. 140ul of biotinylated R11/V11/F11-peptide (10 uM) was injected to a flow cell for 10 min at a 10 ul/min rate to allow saturation of the streptavidin chip by R11/V11/F11-peptide via the tight biotin–streptavidin binding. To analyze the binding kinetics, we used purified p62-D3-GST (from KRIBB) which was inserted in pGEX4T1 and expressed in E.coli. Various concentrations of p62-D3-GST were diluted in HBS-EP buffer (consisting of 0.01M HEPES, pH7.4 / 0.15M NaCl / 3mM EDTA / 0.005% Surfactant P20) and injected onto the sensor chip for 150 sec at 30 ul/min, then the response unit (RU) was recorded. After injection of the analyte was stopped, HBS-EP buffer was poured over the chip for 420 sec at 30 ul/min to allow the bound analytes to dissociate from the immobilized R11/V11/F11-peptide and dissociation curves were obtained. The RU elicited by injecting 1% elution buffer (20mM HEPES pH=7.0, 0.2M NaCl, 10% Glycerol, 2mM DTT) included HBS-EP buffer and was used as the vehicle control. Biacore X-100 control software was used to measure the changes in RU and to plot the binding curve. The curves obtained from the SPR experiments were analyzed and the dissociation equilibrium constant (KD) of p62-D3-GST to immobilized biotinylated peptides were calculated using kinetic evaluation software. The dissociation constant KD (M) was derived from the equation, KD = kd/ka, where kd and ka are dissociation- and association-rate constants, respectively [16].

3.Results

3.1 p62 is a selective autophagy substrate with high affinity for N-terminal Arg and may be a new component of the N-end rule pathway.

To obtain a comprehensive list of N-degron interacting proteins, we performed X-peptide pull down assays using rat testis extracts and biotinylated X-peptide R-11, F-11 and V-11 covalently immobilized on streptavidin beads as an affinity ligand. The 10-mer linker following the X-peptides was derived from the Sindbis virus polymerase nsP4, known N-end rule substrate. As a result, we identified 200 proteins. Among these proteins were the expected UBR box E3 family members of the N-end rule pathway. UBR1, UBR2, UBR4 and UBR5 as judged by analysis of silver-stained gels and immunoblotting. Specifically, UBR1 was identified in both R-11 and F-11 peptide bead pull down samples, UBR2 was identified in F-11 only and UBR4 and UBR5 were identified in R-11 (Fig. 4A). No UBR proteins were identified in V-11 peptide bead pull down samples. We confirmed these results with immunoblotting.

In addition to the UBR family proteins, p62 (squestosome-1) was identified in the R-11 pull down sample by mass spectrometry (Fig. 4B). Our silver-stained gel showed a strong band signal appearing around the molecular weight of 62 kDa in the R11-pull down sample but not in the V11-pull down sample. p62 is a known autophagy marker protein as is LC3 and is a selective autophagy substrate that

targets ubiquitinated proteins to autophagosomes for degradation. We verified this results employing X-peptide pull-down assays followed by immunoblotting by using HEK293 total cell lysates. The results showed that p62 strongly bound to R-11 peptide beads at an efficiency of greater than 40%, while weakly bound to F-11 peptide beads at an efficiency of 5% and didn't have binding affinity for V-11 (Fig. 4C). To confirm this result, we employed a dipeptide competition assay which showed that the RA peptide most effectively competed with R-11 peptide for p62 binding. Among type 2 peptides, WA seemed to show a small effect, but FA showed no effect. This means that p62, like UBR proteins(E3 ligases), may function as an N-recognin in the N-end rule pathway and may be able to bind a broad range of type 1 and type 2 N-end rule peptides.

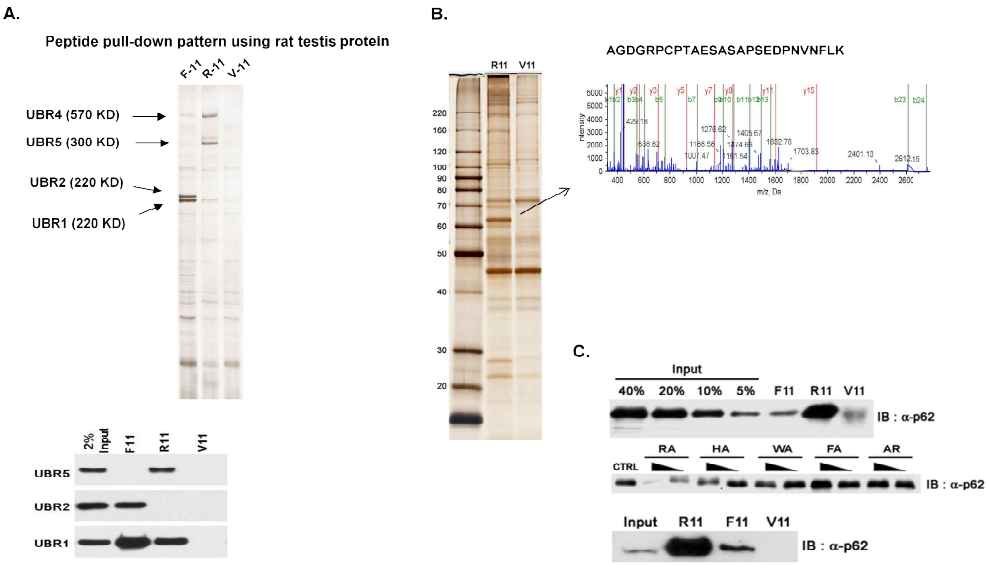


Figure 4. p62 may be a new component of the N-end rule pathway. **A,** This panel shows the results of the x-peptide pull down assay using R-11, F-11 and V-11 peptides and rat testis protein. UBR1, UBR2, UBR4 and UBR5 were identified in both the R-11 and F-11 peptide beads samples and confirmed by immunoblotting. **B**, p62 protein was detected in the R-11 peptide bead samples using mass spectrometry. Our silver stained gel showed a strong band signal around the molecular weight of 62 kDa in the R-11 pull down sample. But no signal was detected in the V-11. **C**, Immunoblot analysis of the x-peptide pull down assay. Input indicates 40%, 20%, 10% and 5% of total lysate for binding.

3.2 p62 can bind a broad range of type-1 and type-2 N-end rule N-termini.

In the N-end rule pathway, an N-degron is classified as a type-1, or type-2 destabilizing residue according to the R-group of the amino acid. Type-1 residues have a positive charge R- group, and type-2 residues have a bulky and hydrophobic R-group. To determine the binding affinity of p62 for both type-1 and type-2 destabilizing residues, we performed an X-peptide pull down assay. We used endogenous p62 protein from total protein lysates of HEK293 and also p62 deletion mutant protein (D3) from transient transfected HEK293 cells. R-11, H-11, K-11 (Type-1), F-11, W-11, L-11, I-11 (Type-2) and V-11, D-11, G-11 (stabilizing control) X-peptide beads were used and analyzed by SDS-PAGE and immunoblotting. Endogenous p62 exhibited binding affinity to all type-1 and type-2, except Leu, N-end rule substrate but not the stabilizing controls (Fig 5). p62 strongly bound to the R-11 peptide, moderately to the K-11 peptide, and weakly to the H-11 peptide. p62 exhibited the strongest binding affinity for R-11, followed by W-11 and F-11 (type-2) N-end rule peptides. Arg (R), His (H) and Lys (K) have positively charged side chain and Phe (F), Typ (W) and Tyr (Y) have aromatic side chain. The properties of the amino acids binding to p62 indicate that p62 prefers to bind to amino acids with positively charged and aromatic side chains.

We checked the binding affinity of UBR2 by stripping the same blot. UBR2 exhibited stronger binding affinity to type-2 N-end rule peptides than type-1. Compared to endogenous p62, overexpressed p62 deletion mutant (D3) exhibited

similar binding affinity for type-1 and type-2 N-end rule peptides. These results suggest that p62 can binds broad range of type 1 and type 2 N-end rule N-termini, which strongly supports our hypothesis that p62 is a new component of the N-end rule pathway.

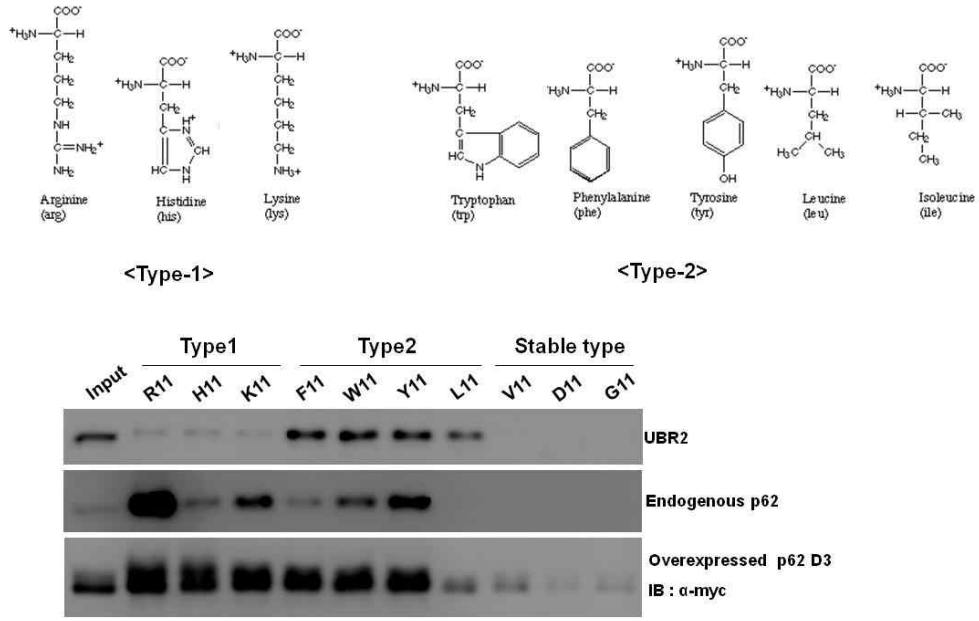


Figure 5. p62 binds to both type-1 and type-2 N-end rule peptides. Type-1 N-degrons have positive charged R-group (Arg, His and Lys). Type-2 N-degrons have an aromatic side chain and hydrophobic R-group (Tyr, Phe, Trp, Leu and Ile). Endogenous UBR2, p62 and overexpressed p62 deletion mutant (D3) bind type-1 and type-2 N-end rule peptides. UBR2 shows almost equivalent binding affinity to each type-1 and type-2 peptide, but p62 binds strongly to R-11, moderately to K-11 and weakly to H-11. Endogenous p62 also binds to three out of four type-2 N-end rule peptides, F-11, W-11 and Y-11 but don't bind to L-11.

3.3 The ZZ type zinc finger domain is responsible for the binding to N-end rule N-termini.

Our results show that p62 bind to N-end rule peptides similar to UBR box E3 ligases. UBR box E3 ligase have two domains, the UBR box and the N-domain. The N-domain which found in UBR1 and UBR2 recognize type-2 N-end rule peptides. The UBR box is a canonical substrate recognition zinc finger domain with 70 residues present in the N-end rule E3 family termed UBR1 through UBR7 and is well conserved throughout evolution. This domain is responsible for the recognition of type-1 N-end rule peptides. p62 also is a multi domain protein which contains PB1, LIR, UBA and ZZ domain. To determine whether the ZZ domain of p62 resembles a UBR box, the primary sequence of mammalian p62-ZZ domain was aligned with the UBR domain of UBR E3 ligases from different organism using a ClustalW program. When compared, C2H2 zinc finger fold of the UBR box is well conserved in the p62 ZZ domain. (Fig 6A).

To identify and confirm the region of p62 that recognizes destabilizing N-end rule N-termini, we performed an X-peptide pull down assay by using p62 domain deletion mutants. Deletion mutants were constructed to delete C-terminal and N-terminal domains (PB1, ZZ, TB, LIR, UBA) serially, and inserted into pcDNA3.1(myc-his tag). 8 deletion mutant plasmids were transiently transfected into HEK293 cells. The total protein lysates were mixed with R-11(Type-1) or W-11(Type-2) peptides and analyzed by immunoblotting (His-tag rabbit antibody MBL, myc-tag rabbit polyclonal antibody abcam). The expression of all constructs

except D8 in HEK293 cells were confirmed and consistent with our previous results. Full length p62 showed strong binding affinity for R-11 and W-11 peptides. Of the eight p62 deletion mutants, only 4 deletion mutants (D2, D3, D4, D5) bound to R-11(Type-1) and W-11(Type-2) peptides (Fig 6B). These 4 deletion mutants generally exhibited stronger binding affinity to R-11(Type-1) than W-11(Type-2) and only the deletion mutants(D2, D3, D4, D5) that contained the zinc finger domain(yellow box) bound X-peptides. It means that the ZZ domain containing the zinc finger structure is important to p62 in its role as an N-recognin of the N- end rule pathway.

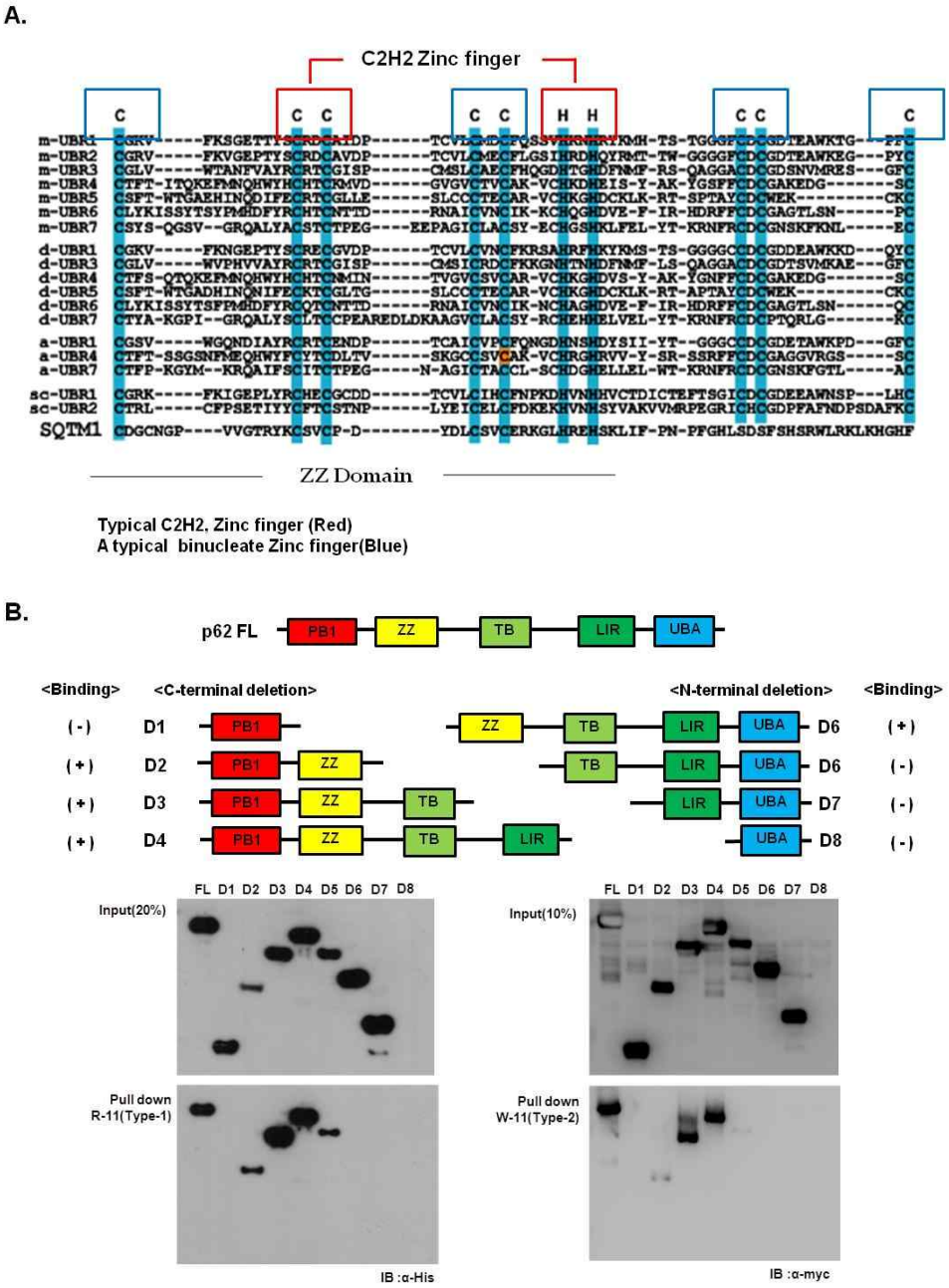


Figure 6. The X-peptide pull down assay for p62 domain deletion mutants. A, The UBR box is well conserved in the p62 zinc finger domain (ZZ domain). The

red box indicates C2H2 zinc finger amino acids and the blue box indicates internal conserved Cys residues. **B,** The X-peptide pull down assay using p62 deletion mutants. The p62 mutant constructs were transiently expressed in HEK293 cells and total cell lysates were used in the assay. D1 thorough D4 represent serial C-terminal domain deletions. And D5 through D8 are serial N-terminal deletion mutants. R-11 and F-11 peptide beads were used in the pull down assay. And myc-his tag antibody was used in immunoblot detection.

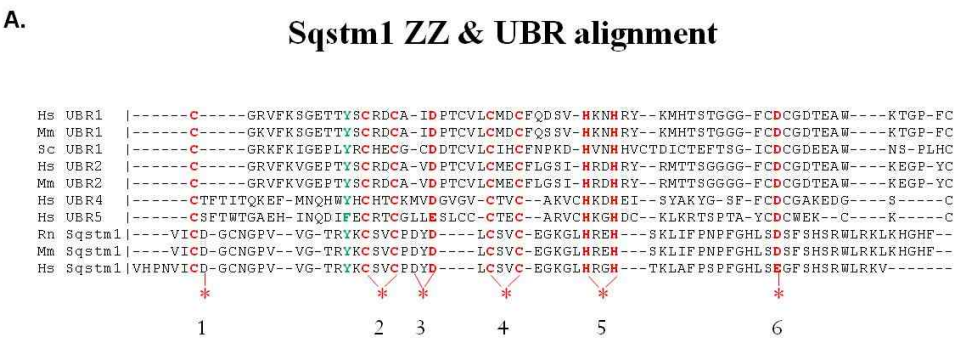
3.4 The amino acids components of the C2H2 Zinc finger structure are important for binding of Type-1 and Type-2 N-end rule substrates.

The defined p62-ZZ domain spans from a.a 129 to a.a 163, and contains the typical ZZ coordinating residues (C142, C145, H160, H163) flanking a couple of cysteine residues (C151, 154), which are evolutionally conserved and identifical to the UBR box. The p62 ZZ domain also contains most of the residues that are present in the atypical Zinc finger domain of the UBR box, but they are arranged differently. The UBR domain contains well conserved three aspartic acids (D118, D150 and D153) which are essential for binding of the N-terminal destabilizing residue of a substrate. The p62 ZZ domain also contains three aspartic acids, including D129, D147 and D147.

To determine the functional importance of the identical and conserved ZZ-residues that were identified in the alignment, we constructed site-directed mutagenesis of the p62 ZZ domain and performed an X-peptide pull down assay. pcDNA 3.1 vectors containing p62 point mutants (D129A, C142 and C145A, D147 and D149A, C151 and C154A, H160 and H163A, E177A) (Fig 7A), were transiently transfected into HEK293 and incubated for 24 hrs. After we checked the total protein lysates, we performed an X-peptide pull down assay using R-11(Type-1) and W-11(Type-2) peptide beads and analysed the results by SDS-PAGE and western blotting. We saw that the point mutants (D129A, C142-145A, D147-149A, C151-154A, H160A) lost the binding affinity for R-11(Type-1) N-end rule peptides (Fig 7B). Interestingly, alanine mutation of E177, which is conserved in

all sequences but outside of ZZ domain, showed no effect on p62 binding to type 1 (R-11) and type 2 (W-11) N-end rule N-terminal residues. These point mutants also exhibited the same binding affinity for W-11(Type-2) but weaker than R-11(Type-1). This suggests that the C2H2 residues in the p62 Zinc finger domain play a key role in the binding of N-end rule substrates and the Zinc finger domain of p62 is related to the binding of type-1 and type-2 N-end rule substrates. CDGC (a,a 128 to 131) in the p62 ZZ domain is reminiscent of CDCG (a,a 149 to 153) in the UBR box. Interestingly, the a,a D150 has been shown to be a very critical a,a for the substrate recognition of UBR1. We think that is why the mutation at the D129 kills p62 binding affinity while E177A did not abolish binding.

A.



Label
1: ZZ-D129A
2: ZZ-C142A_C145A
3: ZZ-D147A_D149A
4: ZZ-C151A_C154A
5: ZZ-H160A_H163A
6: ZZ-E177A

B.

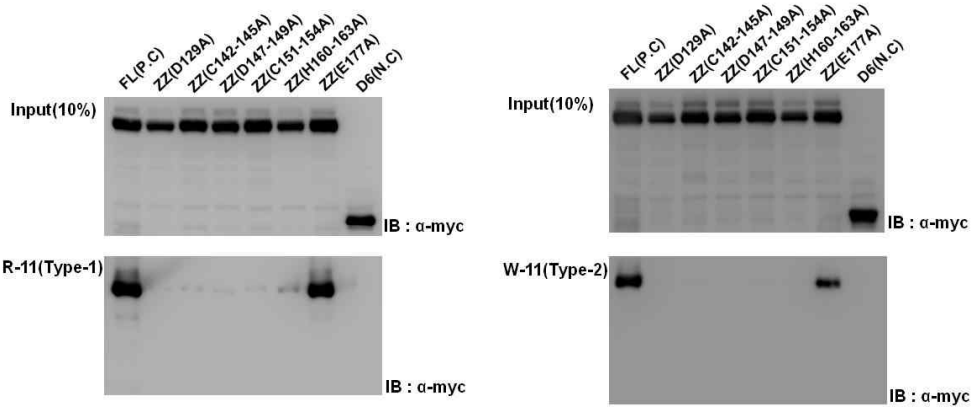


Figure 7. X-peptide pull down assay for p62 point mutants. A, The typical C2H2 zinc finger fold of the UBR box is well consserved in the p62 ZZ domain while the atypical binucleate zinc finger fold of UBR box is partially shared by p62-ZZ. We conducted site-directed mutagenesis to generate 6 mutants of human p62, in which internal Cys, Asp a,a were changed to Alanine. **B,** The p62 mutant

were transiently expressed in HEK293 cells transiently and total cell lysates were used in the assay. D6 deletion mutant was used as a Negative control.

3.5 The Zinc finger domain of p62 is important of binding to Type-1 and Type-2 N end rule N-termini.

To identify the specific region of the ZZ domain needed for the binding of type-1 and type-2 N-end rule termini, we further deleted the C-terminal and N-terminal region of the D3 p62 mutant. The effect of these deletions on the binding to N-end rule N-termini was examined employing X-peptide pull down assay. C-terminal deletion mutants were based on the p62 deletion mutant(D3) which has similar binding affinity to N-end rule peptides as full length p62. pcDNA 3.1 vectors, which contains p62 serial ZZ deletion mutants, (CD1~CD9 are C-terminal deletion mutants and ND1~ND6 are N-terminal deletion mutants), were transiently transfected into HEK293 and incubated for 24 hrs. After incubation and washing, peptide beads were analyzed by SDS-PAGE and western blotting. Of the overexpressed ZZ C-terminal deletion mutants, CD_1~6 exhibited binding affinity for R-11 and W-11 but CD_7~9 didn't (Fig 8A). The difference between CD6 and CD7 falls into the boundary of C2H2 zinc finger of ZZ domain and showed a similar pattern between type-1 and type-2 binding. Of the overexpressed ZZ N-terminal deletion mutants, ND_1~3 exhibited binding affinity for R-11 and W-11 but ND_4~6 didin't (Fig 8B). This data suggests that the core C2H2 zinc finger of p62 has the ability to bind to both type-1 and type-2 N-end rule substrates which is in contrast to the current understanding of the UBR box and N-domain.

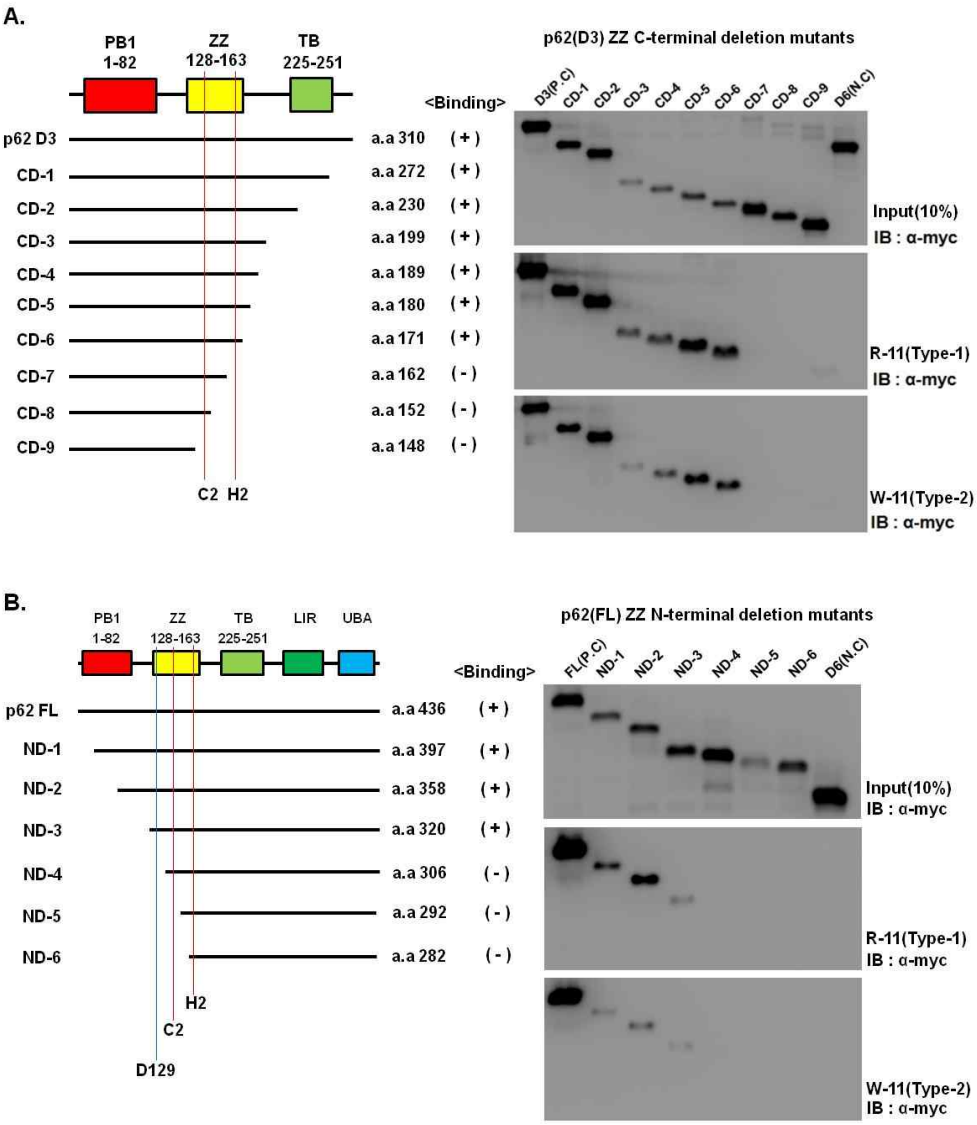
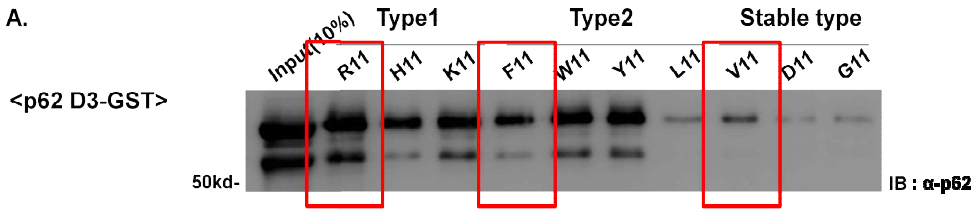


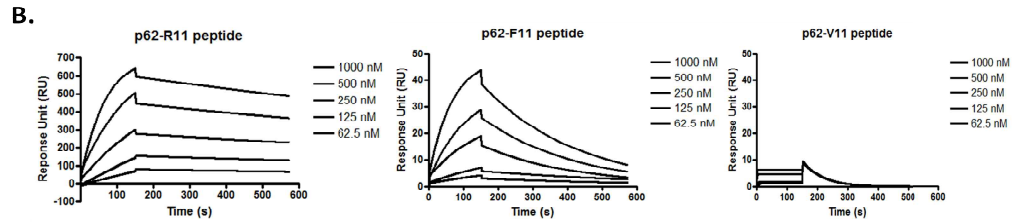
Figure 8. X-peptide pull down assay of p62 serial C-terminal, N-terminal deletion mutants. A, The X-peptide pull down assay using p62 C-terminal deletion mutants. The p62 mutant constructs were expressed in HEK293 cells and total cell lysates were used in the assay. The identification number for p62

fragments are shown on the left. R-11 and W-11 peptide beads were used in pull down assay and myc tag antibody was used in immunoblot detection. **B,** The X-peptide pull down assay using p62 N-terminal deletion mutants.

3.6 Surface Plasmon resonance (Biacore) analysis of the interaction between p62-D3-GST (E.coli expressed) and X-peptide (X=Arg, Phe or Val).

Next we determined the binding affinity and kinetics between the p62-GST (E.coli expressed) and various N-terminal amino acids using surface Plasmon resonance biosensors (Biacore). X-peptide pull-down assays demonstrated that p62 bound strongly to R-11 peptides and weakly to F-11 peptides but did not bind to V-11 peptides. To measure the binding constant of p62 to the destabilizing and stabilizing N-termini, first we performed X-peptide pull down assay using expressed p62-D3-GST construct in total cell lysate. As we expected, E.coli expressed p62-D3-GST bound to all types of N-end rule peptides similar to endogenous p62 (Figure 9A). Next, we purified p62-D3-GST to a purity of 50%. Biotinylated X-peptides were immobilized on the streptavidin-coated sensor chip, and p62-D3-GST was injected over the immobilized peptides. Consistent with our results obtained from X-peptide pull-down assay, p62-D3-GST showed strong binding to R-11 peptides, weak binding to F-11 peptide but no binding to V-11 peptide. The KD values of R-11 and F-11 peptides are 30nM and 300mM, respectably (Fig 9B). These results again demonstrated the interaction between p62 and destabilizing N-end rule N-termini.





Sample	ka (1/s)	kd (1/MS)	KD (M)	R_{max} (RU)
R-11 peptide	1.646E+4	4.953E-4	3.008E-8	657.7
V-11 peptide	1.457E+5	0.01574	1.080E-7	13.15
F-11 peptide	1.180E+4	0.003539	3.000E-7	54.66

Figure 9. Surface Plasmon Resonance (Biacore) analysis of the interaction between p62-D3-GST and X-peptides. **A,** X-peptide pull down assay of total lysates of expressing p62-D3-GST. **B,** Biacore sensogram illustrating the binding of p62-D3-GST to R-11 and F-11 peptide but not to V-11.

3.7 GRP78 ER chaperone protein is a new N-end rule substrate.

The fact that our findings support the hypothesis that N-end rule ligand binding to the p62-ZZ domain induces a conformational change which facilitates p62 aggregation, leading to enhanced autophagosome formation, thereby resulting in autophagy stimulation, prompted us to search for a biological N-end rule ligand of p62. First, known and putative N-end rule substrates were reviewed. In mammals, known N-end rule substrates include RAD21, GNG2, BRCA1, RGS4, RGS5, and RGS16 and putative N-end rule substrates include Bip and PDI, ER chaperones.

First we made R-11, E-11 and V-11 GRP78 peptide beads which are based on the first 10 mer sequence of the GRP78 chaperone protein and performed a pull down assay. Compared to the X-nsP4 peptides, p62 showed less binding affinity to R-GRP78 peptide (Fig 10). Next we performed a pull down assay using p62 deletion mutants. The deletion mutants showed similar binding pattern to R-GRP78 peptides as compared to R-nsP4 peptides. These results suggest that GRP78 ER chaperone protein may be a new substrate of the N-end rule pathway in autophagy.

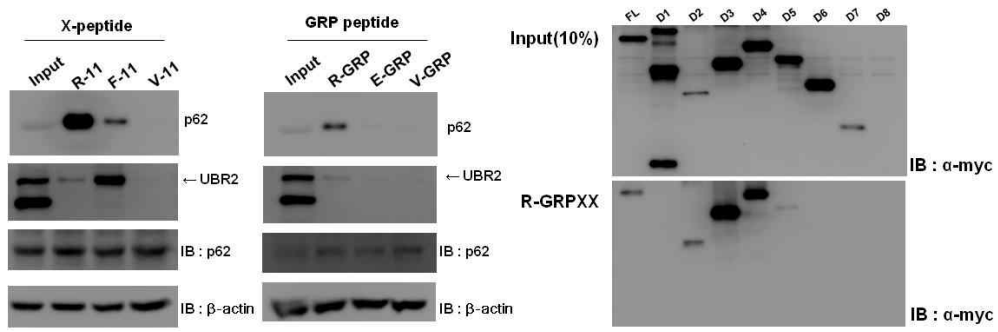


Figure 10. GRP78 peptide pull down assay. GRP78 peptides were constructed based on the first 10 amino acids of the GRP78 ER chaperone protein and the n-terminal addition of the amino acid was R, E or V. IB:p62 is a loading control which are 10% of the total pull down binding volume. The results of pull down assay for the p62 deletion mutants shows similar pattern compared to X-nsP4 pull down assay. We used myc -tag antibody for immunoblot detection.

4. Discussion

Although the physiological functions and domains of the UBR family E3 ligases and p62 have been extensively study, the function of the ZZ domain of p62 has remained elusive. Our study started with proteomic screen for N-degron binding proteins using X-peptide pull down assays. We identified p62/SQSTM1, which is known to mediate autophagic degradation of aberrant proteins as a new recognition component of the N-end rule pathway. We first saw that p62 strongly binds R-11 peptides, weakly binds F-11 peptides and didn't have any binding affinity for V-11 peptides (Fig. 4C). UBR box E3 ligases have two domains, the UBR box and the N-domain. The N-domain which is present in UBR1 and UBR2 recognizes type-2 N-end rule peptides. The UBR box is a canonical substrate recognition zinc finger domain of 70 residues present in the N-end rule E3 family termed UBR1 through UBR7 and is well conserved throughout evolution [16]. The ZZ domain of p62 structurally and functionally resembles the UBR box of UBR E3 ligases. But, unlike the UBR boxes of E3 ligases which can bind only type 1 N-degrons, the ZZ domain of p62 can bind both type1 and type 2 N-degrons (Fig 5). p62 does not contain any obvious E3 ligase domains such as the RING and HECT domains which are present in UBR E3 ligases. To identify and confirm the region of p62 that recognizes destabilizing N-end rule N-termini, we performed an X-peptide pull down assay using p62 domain deletion and point mutants. Like the UBR box, the typical and atypical zinc finger motifs of the ZZ

domain function as important structural elements, and its three aspartic acids with negative charges are also essential for binding to N-end rule N-termini (Fig 7). This means that the ZZ domain containing the zinc finger structure is important to p62 in its role as an N-recognin of the N- end rule pathway and the C2H2 residues of the p62 zinc finger domain play a key role in the binding of type 1 and type 2 N-end rule of substrates to the ZZ domain of p62. According to our Biacore assay, Arg N-termini display high binding affinity for the p62 ZZ domain with the KD of 30 nM, which is about 10,000 times greater than its affinity to UBR box domains (Fig. 9). This data hints that to be an effective autophagy inducer, a ligand should have high affinity for the ZZ domain. The selectivity of p62 for Arg-N-degron interaction is strengthened by the identification of Arg-Bip as a biological N-end rule ligand of p62 in innate immune system. Solving the three dimensional structure of these proteins will help us to understand the substrate specificity of the ZZ domains of p62 .

In mammals, known N-end rule substrates include RAD21, GNG2, BRCA1, RGS4, RGS5, and RGS16 and putative N-end rule substrates include Bip and PDI, ER chaperones [13]. GRP78 chaperone protein as an Arg N-degron substrate of the N-end rule pathway recognized by this new N-recognin (Fig 10). These results suggest that GRP78 ER chaperone protein may be a new substrate of the N-end rule pathway in autophagy. In our ongoing study, we present another function of N-terminal Arg as a positive regulator of autophagy via its ability to induce p62 aggregation leading to autophagy stimulation (unpublished data). Next time we

anticipate more findings about p62 functions as an autophagic N-recognin of the Arg/N-end rule pathway, providing a new potential therapeutic target for diseases associated with innate immunity.

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요약(국문초록)

아미노 말단 법칙 경로는 포유동물에서 불안정한 아미노 말단 잔기 {유형-1 (아르기닌, 라이신, 히스티딘) 그리고 유형-2 (페닐알라닌, 트립토판, 타이로신, 루신, 아이소루신)}가 분해신호 (N-degron) 역할을 하는 단백질 분해 시스템이다. 이 경로에 알려진 기능들은 유비퀴틴-프로테아좀 시스템을 통한 수명이 짧은 단백질의 분해 조절을 포함한다. 지금까지 알려진 아미노 말단-인식 구성요소(N-recognins)는 분해신호 (N-degron)를 인식하는데 필요로 하는 진화적으로 보존된 72 잔기의 UBR Box 를 포함하는 UBR1, UBR2, UBR4 그리고 UBR5 가 있다. 우리는 이 경로의 인식 구성요소 (N-recognins)를 정제하기 위해 아미노 말단 불안정 잔기를 가진 합성 펩타이드를 사용하였다. N-degrons 에 붙는 단백질들에 대한 우리의 프로테오믹 스크리닝에서 비정상적인 단백질의 자가포식 분해를 중재하는 것으로 알려진 p62/SQSTM1 을 아미노 말단 법칙 경로의 새로운 인식 구성요소로 규명하였다. 이 연구에서, 우리는 p62 의 결합 특이성과 다른 생화학적 특성들을 아미노 말단 법칙 경로의 잠재적인 아미노 말단-인식 구성요소 (N-recognins)로 특정 지었다. 합성 아미노 말단 법칙 펩타이드를 사용한 pull-down 분석은 그 기능이 불분명한 그것의 ZZ 도메인이 아미노 말단 법칙의 아미노 말단 결합에

필요하다는 것을 보여준다. p62 는 아미노 말단의 아르기닌과 다른 유형-1 펩타이드(라이신과 히스티딘) 뿐만 아니라 루신과 아이소루신에는 아니지만 유형-2 펩타이드 (페닐알라닌, 트립토판 그리고 타이로신)에도 결합한다. 부위 특이적 돌연변이는 특정 잔기가 아미노 말단 법칙 펩타이드와의 결합에 중요하고 p62 와 알려진 인식 구성요소 (N-recognins)에 보존되어 있다는 것을 보여준다. 또한 표면 플라즈몬 공명 (Biacore assay)는 p62 와 불안정한 아미노 말단 법칙의 아미노 말단과의 상호작용을 다시 한번 증명해 준다. 우리의 결과는 p62 단백질이 아미노 말단 법칙 경로의 새로운 인식 구성 요소가 될 수 있다는 것을 제안하고 ZZ 도메인과의 강한 친화력을 가져야 하는 효과적인 자가포식 유도인자임을 암시한다.

주요어 : 아미노-말단 법칙 경로, UBR, 자가포식, p62 (sequestosome-1)

학 번 : 2011-23066