Amino-terminal arginylation targets endoplasmic reticulum chaperone BiP for autophagy through p62 binding

Hyunjoo Cha-Molstad1,2, Ki Sa Sung2,3,12, Joonsung Hwang1,12, Kyoung A. Kim1,12, Ji Eun Yu1,4, Young Dong Yoo2, Jun Min Jang5,13, Dong Hoon Han6, Michael Molstad2, Jung Gi Kim1, Yoon Jee Lee2, Adriana Zakrzewska3, Su-Hyeon Kim1, Sung Tae Kim2,3, Sun Yong Kim1, Hee Gu Lee6, Nak Kyun Soung1, Jongs Seog Ahn9, Aaron Ciechanover2,10, Bo Yeon Kim1,14 and Yong Tae Kwon2,11,14

We show that ATE1-encoded Arg-transfer RNA transferase (R-transferase) of the N-end rule pathway mediates N-terminal arginylation of multiple endoplasmic reticulum (ER)-residing chaperones, leading to their cytosolic relocation and turnover. N-terminal arginylation of BiP (also known as GRP78), protein disulfide isomerase and calreticulin is co-induced with autophagy during innate immune responses to cytosolic foreign DNA or proteasomal inhibition, associated with increased ubiquitylation. Arginylated BiP (R-BiP) is induced by and associated with cytosolic misfolded proteins destined for p62 (also known as sequestosome 1, SQSTM1) bodies. R-BiP binds the autophagic adaptor p62 through the interaction of its N-terminal arginine with the p62 ZZ domain. This allosterically induces self-oligomerization and aggregation of p62 and increases p62 interaction with LC3, leading to p62 targeting to autophagosomes and selective lysosomal co-degradation of R-BiP and p62 together with associated cargoes. In this autophagic mechanism, Nt-arginine functions as a delivery determinant, a degron and an activating ligand. Bioinformatics analysis predicts that many ER residents use arginylation to regulate non-ER processes.

Substrate selectivity in proteolysis is governed by the interaction between degradation signals (degrons) and recognition components (recognins)1,2. A well-characterized class of degrons in the ubiquitin (Ub)-proteasome system (UPS) is defined by the amino-end rule pathway, in which a destabilizing N-terminal residue serves as a determinant of a class of degrons, called N-degrons1,2,3,4. N-degrons are recognized and bound by N-recognins, such as those containing UBR boxes5,6, leading to proteolysis by the proteasome7,8,9 (Fig. 1a). A functional N-degron is composed of a destabilizing N-terminal residue as a binding ligand, an internal lysine residue as a site of polyubiquitylation, and an appropriate secondary/tertiary structure10,11. In mammals, destabilizing N-terminal residues include arginine, lysine, histidine (type 1; positively charged), phenylalanine, tyrosine, tryptophan, leucine and isoleucine (type 2; bulky hydrophobic)12,15,16. These degradation determinants can be generated through endoproteolytic cleavages followed by post-translational modifications (PTMs; refs 7–9), such as N-terminal arginylation by arginyltransferase 1 (ATE1)-encoded Arg-transfer RNA transferases (R-transferases; EC 2.3.2) that transfer L-arginine from Arg-tRNA8,9 to protein N-termini17,18 (Fig. 1a). In eukaryotes, N-terminal (Nt)-aspartic acid and Nt-glutamic acid are the acceptors of N-terminal arginylation17,18. Nt-asparagine, Nt-glutamine and Nt-cysteine can also be arginylated following deamidation (of Nt-asparagine and Nt-glutamine19,20) or oxidation (of Nt-cysteine18,19; Fig. 1a). ATE1-knockout mouse embryos die with cardiovascular defects18,21,22. Recent studies have shown that Nt-methionine23 and N-terminally acetylated residues24 can also function as N-degrons.

Whereas PTM-generated degrons have been well characterized in the UPS (refs 1–6,12,25,26), little is known about the role of such degrons in proteolysis through the autophagy–lysosome

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system (hereafter called autophagy). Misfolded proteins that escape UPS-dependent quality control, including monomeric and oligomeric aggregates, may impair proteasomal and other cellular functions, and thus are redirected to macroautophagy, wherein cellular cargos are segregated by autophagosomes for lysosomal degradation. In macroautophagy, the autophagic adaptor p62 (also known as sequestosome 1, SQSTM1) selectively delivers Ub-conjugated cargoes to autophagosomes through self-aggregation and the interaction

**Figure 1** Bioinformatic analysis of the ER N-end rule pathway, and the generation of antibodies to the arginylated form of BiP. (a) A summary of this study that describes a dual role for the Nt-arginine residue as a degron for both the UPS and autophagy. The Nt-arginine residue generated through the N-end rule pathway is known to act as a degron for selective proteolysis by the UPS. In the Ub-dependent N-end rule pathway, specific recognition components, called N-recognins, recognize and bind the primary degron Nt-arginine and mediate ubiquitylation for targeting to the proteasome. The results of this study show that N-terminal arginylation of ER-residing chaperones generates a cis-acting delivery determinant and degron for lysosomal degradation along with associated cargoes. In this autophagic proteolysis, Nt-arginine of BiP and other ER proteins is recognized by a recognin, p62. The binding of Nt-arginine to p62 activates p62, leading to the delivery of p62, R-BiP, and its cargoes to autophagosomes. In definition, Nt-arginine also acts as a trans-acting degron for its cargoes. (b) A sequence alignment of the N-terminal regions of human BiP and its sequelogues. The red box indicates the N-terminal residues (P1’ sites) of mature BiP proteins from various species. Shown above are secondary structures (red solid line, α-helices; blue arrows, β-strands). (c) Generation of anti-R-BiP DH1 antibody. Shown is a peptide binding/competition assay. An 11-mer R-BiP peptide (R-BiP-peptide) corresponding to the N-terminal region of R-BiP was immobilized on a 96-well plate, followed by incubation with serially diluted anti-R-BiP antibody and, subsequently, anti-goat secondary antibody conjugated with horseradish peroxidase (HRP). The amounts of R-BiP antibody that bound to immobilized R-BiP peptide were determined on the basis of the optical density value at 450 nm of the secondary antibody. As a control, BiP-peptide, a 10-mer peptide corresponding to the N-terminal region of unarginylated BiP, was used. (d) A dot blotting analysis of R-BiP antibody using the peptides corresponding to the N-terminal region of unarginylated (left) or arginylated (right) BiP.
with the integral autophagosome component LC3 (also known as MAP1LC3A, microtubule-associated protein 1 light chain 3 alpha) (refs 32,33). One outstanding question in macroautophagy is how cargoes are selectively recognized by p62 and delivered to autophagosomes34,35.

**Proteomic analysis of the N-end rule pathway for endoplasmic reticulum (ER)-residing proteins**

Approximately one-third of the human proteome is destined for the Golgi secretory pathway36,37. The signal peptides of ER clients and residents are co-translationally and co-translocationally cleaved off by the signal peptide peptidase, exposing new N-terminal residues on mature peptides38. Translocated polypeptides undergo folding and other PTMs through the assistance of folding factors such as the ER chaperone BiP (also known as GRP78 and HSPA5, heat shock 70 kDa protein 5), GRP94, calreticulin (CRT), protein disulphide isomerase (PDI) and ERdJ5 (ref. 39). Whereas these folding factors are known to be metabolically stable and function in the lumen, lacking proteolytic machinery, they are also present in various non-ER compartments40,41.

We hypothesized that these non-ER species acquire PTMs to support their activities, locations and turnover. To characterize the N-end rule pathway for ER-residing proteins, we analysed N-terminal residues of 498 ER-targeted proteins listed in the Human ER Aperçu (Hera) database42. Approximately 27% acquired destabilizing N-terminal residues (Supplementary Tables 1–4). Here, we focused on two arginylation substrates (aspartic acid and glutamic acid) and three N-terminal residues (asparagine, glutamine and cysteine), whose PTMs confer arginylation through deamidation/arginylation (asparagine and glutamine) or oxidation/arginylation (cysteine; Fig. 1a). This arginylation-permissive group represented 9% (43 out of 498), amongst which 25 bore aspartic acid or glutamic acid (Supplementary Table 1). Of note, 11 retained arginylation permissiveness throughout evolution, including major Ca\(^{2+}\)-binding molecular chaperones (BiP, GRP94 and CRT) and oxidoreductases (PDI and ERdJ5; Fig. 1b and Supplementary Fig. 1 and Supplementary Table 3). All three proteins (BiP, PDI and CRT) that we further characterized are shown to be N-terminally arginylated, indicating that many ER proteins may use similar N-terminal PTMs to participate in non-ER processes.

**BiP is N-terminally arginylated by ATE1, and arginylated BiP (R-BiP) functions in the cytosol**

The arginylation-permissive PI’ residue, Nt-Glu 19, of human BiP is evolutionarily conserved across various species (Fig. 1b). In agreement with this evolutionary pattern, an earlier mass spectrometric analysis of proteins separated on 2D SDS–polyacrylamide gel electrophoresis (SDS–PAGE) identified arginylated forms of BiP and PDI (ref. 43). We raised R-BiP DH1 antibody that exclusively recognizes R-BiP using the peptide REEDKKEDVGC. The specificity was confirmed using immunoblotting, dot blotting and peptide binding/competition assays (Fig. 1c,d and Supplementary Fig. 2). R-BiP was not readily detected in various cell lines. By overexpressing recombinant BiP, we confirmed that BiP was arginylated at Nt-Glu 19 and that its arginylation was abolished by the Glu 19-to-Val mutation (Fig. 2a). To further characterize BiP arginylation, we employed the Ub fusion technique2, wherein Ub–Glu 19-BiP was co-translationally cleaved by ubiquitylating enzymes into Ub and Glu 19-BiP (Fig. 2b). The arginylated mutant, Arg–Glu 19-BiP, produced a stronger R-BiP signal when compared with Glu 19-BiP (Fig. 2c, lanes 1 versus 2) in a manner independent of ATE1 function (Fig. 2d, lanes 1 versus 2). Overexpressing ATE1\(^{1A7A}\), an isoform containing alternative axons 1A and 7A, promoted N-terminal arginylation of recombinant Glu 19-BiP in HEK293 cells (Fig. 2c, lanes 5 versus 2) and ATE1 \(^{1C}\) mouse embryonic fibroblasts (MEFs; Fig. 2d, lanes 5 versus 2) as well as endogenous BiP in HeLa cells (Fig. 2e, lanes 5 versus 2). A moderate activity was also detected with ATE1\(^{1BTR}\) (Fig. 2d, lanes 4 versus 2; Fig. 2e, lanes 4 versus 2). ATE1 knockdown inhibited N-terminal arginylation of endogenous BiP (Fig. 2f, lanes 4 versus 3). The ER stressor thapsigargin, an inhibitor of sarco/endoplasmic reticulum Ca\(^{2+}\) ATPase, did not readily induce BiP arginylation when compared with overexpressed ATE1\(^{1A7A}\) (Fig. 2g), suggesting that R-BiP functions outside the ER. Indeed, R-BiP was retrieved from the cytosol (Fig. 2h) and excluded from the ER as determined by KDEL immunostaining (Supplementary Fig. 3). Consistent with an apparent lumen-to-cytosol flux of BiP, BiP was short lived under translational inhibition (Fig. 2i).

**N-terminal arginylation of BiP is induced by cytosolic foreign DNA**

We found that N-terminal arginylation of BiP was induced by transient transfection of various double-stranded DNAs (dsDNAs), including plasmid DNA and PCR-amplified DNA fragments (Fig. 2j,k). RNA interference assays showed that DNA-induced R-BiP was generated by ATE1 (Fig. 2l, lanes 4 versus 2 and 8 versus 6) and accumulated in the cytosol (Fig. 2l, lane 6). Extracellular DNA did not show such activity (Fig. 2k). Comparative assays (Fig. 2k,m and Supplementary Fig. 4) with single-stranded DNA (ssDNA) and dsRNA suggested that the sensing of the double-helical structure of DNA was essential for the induction of R-BiP. Poly(dA:dT) dsDNA, an immunostimulatory reagent that mimics microbial dsDNA, strongly induced BiP arginylation (Fig. 2m–o) and nuclear localization of NF-\(\kappa\)B (Supplementary Fig. 5). Thus, BiP arginylation may be triggered when cells sense invading pathogenic dsDNA and activate innate immune responses.

**Multiple Ca\(^{2+}\)-binding ER chaperones undergo both constitutive and DNA-induced arginylation at their N-termini**

Bioinformatic analysis showed that many ER-targeted human proteins, including CRT, PDI, GRP94 and ERdJ5, co-translationally and co-translocationally acquire the potential to undergo N-terminal PTMs (Supplementary Tables 1–4). Their arginylation permissiveness is evolutionarily conserved across various species. Consistently, earlier studies showed that a portion of human CRT was present in the cytosol in an arginylated form36,39. To characterize N-terminal arginylation of these proteins, we raised antibodies to their arginylated forms, amongst which anti-R-PDI DH1 and anti-R-CRT antibodies to RDAPHEEHSV and REPAYVFSEQ, respectively, showed sufficient specificity and avidity (Supplementary Fig. 2). In vivo arginylation assays showed that ATE1\(^{1A7A}\) mediates N-terminal arginylation of PDI and CRT at Nt-Asp 18 and Nt-Glu 18, respectively, which are exposed on signal peptide cleavage (Fig. 2p). Unlike R-BiP, detectible amounts of R-PDI and R-CRT were constitutively generated in various cell lines (Fig. 2p), indicating their differential roles in the homeostasis of unstressed cells. Despite apparent differences among R-BiP, R-PDI
and R-CRT, their N-terminal arginylation was commonly triggered by cytosolic dsDNA (Fig. 2k,n,m,q) and proteasomal inhibition (see below), indicating a shared role in innate immune responses to invading microbes. These results suggest that the N-end rule pathway has a broad role in the turnover and functions of ER-residing proteins.

R-BiP is targeted to autophagosomes by way of p62 bodies

Immunoblotting analysis showed that DNA-induced arginylation of ER proteins correlated with the synthesis and activation of LC3 (Fig. 2k,o). Immunostaining showed that DNA-induced R-BiP formed cytosolic puncta with diameters of 0.1–1 µm that co-localized...
Figure 3 R-BiP is targeted to the autophagosome by way of p62 bodies. (a) Co-localization of cytoplasmic R-BiP puncta with p62 puncta in poly(dA:dT)-treated HeLa cells. (b) Co-localization of R-BiP puncta with LC3 puncta in HeLa cells stably expressing RFP–GFP–LC3 as determined by immunostaining of R-BiP in comparison with RFP signal (red), which represents LC3-positive autophagic vacuoles. (c) Three-colour co-localization analysis between R-BiP (blue), p62 (red) and LC3 (green) in poly(dA:dT)-treated HeLa cells. (d) HeLa cells expressing RFP–GFP–LC3 were subjected to three-colour co-localization analysis between R-BiP (blue), acid-resistant RFP (red) and acid-sensitive GFP (green). Most R-BiP puncta show a strong co-localization with LC3 puncta, which are positive for both RFP and GFP, indicating the delivery of R-BiP to autophagosomes. (e) Immunohistochemistry of total BiP and LC3 on sections of mouse embryonic hearts at embryonic day 13.5, which reveals BiP puncta that co-localize with LC3 puncta. (f) RNA interference assay of ATE1, BiP and p62 in poly(dA:dT)-treated HeLa cells, followed by co-localization analysis between R-BiP and p62. Note that knockdown of any of ATE1, BiP or p62 disrupts the targeting of both R-BiP and p62 to autophagic vacuoles. (g) RNA interference assay of ATE1 and BiP in poly(dA:dT)-treated HeLa cells expressing RFP–GFP–LC3, followed by co-localization analysis between R-BiP and LC3. (h) RNA interference assay of LC3 in poly(dA:dT)-treated HeLa cells, followed by co-localization analysis between R-BiP, p62 and LC3. LC3 knockdown does not affect significantly the delivery of R-BiP to p62 puncta. Scale bars, 10 µm.
Figure 4 The N-arginine residue of R-BiP is a delivery determinant to the autophagosome. (a) Schematic diagram showing that the Ub fusion protein Ub–X-BiP–GFP is co-translationally cleaved into Ub and X-BiP–GFP by Ub hydrolases. Also shown is how Ub–X-BiP<sup>19-124</sup>–GFP (Ub–X-BiP<sup>A</sup>–GFP) is co-translationally cleaved into Ub and X-BiP–GFP. (b) Co-localization analysis between X-BiP–GFP (X = Arg (an arginylated form of E-BiP, R–E-BiP) or Val (a glutamic acid-to-valine mutant, V-BiP–GFP)) and p62 in HeLa cells. X-BiP–GFP is produced in vivo from a precursor protein, Ub–X-BiP–GFP, which is elaborated in a. Scale bar, 10 µm. (c) An analogous co-localization assay with X-BiP–RFP and LC3. Scale bar, 10 µm. (d) Punctum co-localization analysis of X-BiP<sup>19-124</sup>–GFP (X-BiP<sup>A</sup>–GFP; X = Arg, Glu or Val) and p62 in +/+ and p62<sup>−/−</sup> MEFs. Note that the ability of X-BiP<sup>A</sup>–GFP to form cytosolic puncta not only follows the N-end rule but also requires p62. Scale bar, 10 µm. (e) Punctum co-localization analysis of X-BiP<sup>A</sup>–GFP and LC3 in +/+ and p62<sup>−/−</sup> MEFs, which shows that the co-localization of R-BiP puncta with LC3 depends on both the N-end rule and p62. Scale bar, 10 µm. (f) Quantification of d and e, indicating that the ability of R-BiP to form cytosolic puncta depends on p62. The graph shows the percentage of BiP<sup>A</sup>–GFP-positive cells that form BiP<sup>A</sup>–GFP puncta. Mean ± s.d. of n = 3 independent experiments, in which 200 cells were analysed per experimental point. Statistical significance was calculated using a one-way ANOVA test (NS, not significant, *P > 0.05; ***P < 0.0001).

with puncta containing p62 (Fig. 3a) as well as LC3 (Fig. 3b). Co-localization of R-BiP puncta with p62 and LC3 puncta was confirmed in three-colour co-staining analysis (Fig. 3c) as well as in HeLa cells stably expressing RFP–GFP–LC3 (where RFP is red fluorescent protein and GFP green fluorescent protein) (Fig. 3d and Supplementary Fig. 4). Within R-BiP<sup>+</sup>p62<sup>+</sup> and R-BiP<sup>+</sup>LC3<sup>+</sup> puncta, R-BiP puncta were smaller than and morphologically different from p62 and LC3 puncta, indicating that R-BiP is first targeted to p62 bodies and subsequently delivered to LC3-positive autophagosomes.

Autophagic delivery of BiP was also observed on paraffin sections of mouse embryonic hearts (Fig. 3e). RNA interference assays showed that both ATE1 and BiP were required for optimal formation of p62 bodies (Fig. 3f) and LC3-positive autophagosomes (Fig. 3g), indicating the role of R-BiP in the induction of p62-mediated autophagy in response to poly(dA:dT). Reciprocally, p62 knockdown perturbed R-BiP delivery to autophagic vacuoles (Fig. 3f). By contrast, LC3 knockdown did not significantly affect the co-localization of R-BiP with p62 puncta (Fig. 3h). These results suggest that R-BiP...
is targeted to autophagosomes by way of p62 bodies and that N-terminal arginylation and R-BiP play a role in p62 delivery to autophagosomes.

**Nt-arginine of R-BiP functions as a delivery determinant during R-BiP targeting to p62 and autophagosomes**

Little is known about the mechanism by which cargoes are selectively delivered to autophagy. Co-localization analyses showed that R-BiP–GFP, generated from Ub–R–BiP–GFP (Fig. 4a), formed cytosolic puncta that co-localize with p62 bodies (Fig. 4b) and LC3-positive autophagosomes (Fig. 4c). Glu 19-to-Val mutation abolished BiP co-localization with autophagic components. To determine whether Nt-arginine is an autophagic delivery determinant, we removed the ATPase and substrate binding domains from X-BiP–GFP, leaving the first 106-residue fragment, Ub–X-BiP<sub>19–124</sub>–GFP (X = Glu, Arg or Val; Fig. 4a). R-BiP<sub>19–124</sub>–GFP (R-BiP<sub>Δ</sub>–GFP) and E-BiP<sub>19–124</sub>–GFP (E-BiP<sub>Δ</sub>–GFP) were readily targeted to p62 and LC3 puncta (Fig. 4d–f). Autophagic targeting of R-BiP<sub>Δ</sub>–GFP and E-BiP<sub>Δ</sub>–GFP was abolished in p62<sup>−/−</sup> MEFs (Fig. 4d–f), indicating that R-BiP delivery to autophagosomes requires p62. Moreover, Glu 19-to-Val mutation abolished BiP co-localization with p62 and LC3 puncta (Fig. 4d–f). Thus, R-BiP Nt-arginine is a delivery determinant in p62-mediated macroautophagy.

**R-BiP binds p62**

To determine whether R-BiP Nt-arginine binds p62, we carried out X-peptide pulldown assays<sup>14</sup> using synthetic X-BiP peptides (X = Arg–Glu (permanently arginylated), Glu (native) or Val (control); Fig. 5a). R-BiP peptide, but not E-BiP or V-BiP peptide, pulled down endogenous p62 from HEK293 cell extracts (Fig. 5b). To further demonstrate that Nt-arginine is a binding ligand to p62, we used 11-mer model N-end rule peptides, X-nsP4 (X = Arg, Phe or Val), corresponding to the N-terminal region of the Sindbis virus polymerase nsP4 (ref. 9), p62 preferentially bound R-nsP4 when compared with F-nsP4 and V-nsP4 (Fig. 5c). Thus, R-BiP binds p62 through the N-end rule interaction of its Nt-arginine.

The **ZZ motif of p62 is the ligand-binding domain for Nt-arginine**

To identify the domain of p62 that binds BiP Nt-arginine, we constructed carboxy-terminally (D1–D4) or N-terminally (D5–D7) deleted p62 mutants (Fig. 5d). Both R-BiP (Fig. 5e) and R-nsP4 (Fig. 5f) peptides pulled down D2, D3, D4 and D5 but not D1, D6 or D7, suggesting that Nt-arginine binds a region spanning the ZZ domain, a poorly characterized zinc finger motif<sup>14</sup>. Consistently, a p62 mutant lacking the ZZ domain (residues 128–163) no longer bound R-nsP4 peptide (Fig. 5g,h). Glutathione S-transferase (GST) pulldown assays (Fig. 5i–k) showed that a 93-residue ZZ-only fragment (residues 83–175), p62<sub>ZZ</sub>–GST (Fig. 5i), was sufficient to bind R-BiP (Fig. 5k, lane 1). The interaction between p62<sub>ZZ</sub>–GST and BiP was abolished by Glu 19-to-Val mutation (Fig. 5k, lanes 2 versus 1). Similarly, the alanine mutation of the conserved Asp 129 residue within the ZZ domain abolished this binding activity of p62<sub>ZZ</sub> (Fig. 5k, lanes 3 versus 1). Peptide pulldown assays independently demonstrated that R-nsP4 peptide also binds p62<sub>ZZ</sub>–GST but not its D129A mutant (Fig. 5j). Immunostaining analysis of p62<sup>−/−</sup> MEF cells showed that a 93-residue ZZ-only fragment fused with RFP, p62<sub>ZZ</sub>–RFP (residues 83–175), formed puncta co-localizing with R-BiP<sub>Δ</sub>–GFP but not V-BiP<sub>Δ</sub>–GFP (Fig. 5l). Thus, the p62 ZZ motif is the ligand-binding domain for Nt-arginine.

**Nt-arginine induces p62 aggregation**

Little is known about the mechanism by which p62 aggregation is regulated during autophagic induction. To determine the biological consequences of Nt-arginine binding to p62, we added the dipeptide Arg–Ala (type 1) in comparison with other dipeptides bearing N-terminal histidine, lysine (type 1), phenylalanine, tryptophan, tyrosine (type 2) or alanine (stabilizing) to HEK293 cell extracts expressing full-length p62, and monitored the conversion of p62 into oligomers and aggregates using non-reducing SDS–PAGE. When compared with other N-end rule dipeptides, we found that Arg–Ala strongly and selectively induced the oligomerization and aggregation of p62 (Fig. 6a–c). Ala–Arg, a control dipeptide bearing arginine at the second position, did not show such activity. These results suggest that Nt-arginine binding to p62 induces self-oligomerization and aggregation of p62 in vitro, consistent with our data that ATE1 is required for optimal p62 punctum formation in response to poly(dA:dT) (Fig. 3f).

**Nt-arginine increases p62 interaction with LC3**

The mechanism by which the interaction of cargo-associated p62 with LC3 on autophagic membranes is regulated remains unclear<sup>14</sup>. To determine whether Nt-arginine binding to p62 increases p62 interaction with LC3, we carried out GST-pulldown-coupled enzyme-linked immunosorbent assay (ELISA), wherein HEK293 cell extracts ectopically expressing p62 were incubated with LC3–GST immobilized on the surface of glutathione-coated wells in the presence of the aforementioned dipeptides (Fig. 6d). When compared with other dipeptides, only Arg–Ala markedly increased p62 interaction with LC3 (Fig. 6d). Our in vitro (Figs 5 and 6) and in vivo (Figs 3 and 4) assays collectively suggest that Nt-arginine binding to p62 induces an allosteric conformational change, exposing the PB1 domain, which mediates self-oligomerization, and the LIR domain, which mediates the interaction with LC3 on autophagic membranes.

**Nt-arginine of R-BiP is an autophagic degron**

Little is known about PTM-generated degrons that mediate selective proteolysis by autophagy. To determine whether R-BiP is a selective substrate of macroautophagy, we monitored the decay of X-BiP<sub>Δ</sub>–GST generated from Ub–X-BiP<sub>Δ</sub>–GST (Fig. 6e–g). R-BiP<sub>Δ</sub>–GST exhibited a shorter half-life than V-BiP<sub>Δ</sub>–GST as determined by the levels of GST (Fig. 6f, lanes 2 versus 3). As quantified in Fig. 6e, the degradation of R-BiP<sub>Δ</sub>–GST was inhibited by p62 knockout (Fig. 6f, lanes 5 versus 2) or the pharmaceutical blockage of autophagy (lanes 8 versus 2). Autophagic degradation of R-BiP<sub>Δ</sub>–GST was further demonstrated by its accumulation in ATG5<sup>−/−</sup> MEFs (Fig. 6g, lanes 5 versus 2). Finally, cycloheximide degradation assays confirmed that R-BiP–Myc/His was short lived in MEFs and metabolically stabilized by ATG5 knockout alone and, synergistically, when combined with proteasomal inhibition (Fig. 6h,i). These results suggest that R-BiP is selectively degraded by autophagy through the activity of Nt-arginine as a cis-acting autophagic degron.

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**Figure 5** The Nt-arginine residue of R-BiP binds to the ZZ domain of p62. (a) The sequences of X-BiP peptides used to pull down p62. (b) The Nt-arginine residue of R-BiP binds to p62. X-BiP peptides crosslinked to beads were used to pull down p62 from HEK293 cell extracts transiently expressing X-BiP–Myc/His. (c) Similar to b except that X-nsP4 peptides (X = Arg, Phe and Val) were used. (d) A diagram showing C-terminally (D1–D4) and N-terminally (D5–D7) deleted p62 mutant proteins. (e) R-BiP peptide pulldown assay with serially deleted p62 mutants. (f) R-nsP4 peptide pulldown assay with serially deleted p62 mutants. IB, immunoblot. (g) A diagram showing wild-type p62 and a ZZ-deletion mutant (p62ΔZZ). (h) R-nsP4 peptide pulldown assay with wild-type p62 and p62ΔZZ. (i) A diagram showing a ZZ-only fragment (p62zzΔwnt), residues 83–175) and its mutant (p62zzΔwnt), in which the conserved Asp 129 was mutated to alanine. (j) R-nsP4 peptide pulldown assay using p62zzΔwnt and p62zzΔwntΔ129. (k) A GST pulldown assay combined with an X-BiP pulldown assay. A p62-GST fusion, p62zz-GST or p62zzΔ129-GST, immobilized on glutathione beads, was incubated with HEK293 extracts expressing Ub–X-BiP–Myc/His (X = Arg or Val), followed by centrifugation and immunoblotting analysis of X-BiP–Myc/His that bound to p62 ZZ fragments. Note that R-BiP–Myc/His binds to the wild-type ZZ-only fragment but not to the D129A-ZZ fragment. No binding was detected with V-BiP–Myc/His. (l) Co-localization assay of X-BiP with Ub–X-BiP–GFP (X = Arg or Val) and p62ΔZZ–RFP co-expressed in p62ΔZZ MEFs, X-BiP–GFP was co-translationally generated from Ub–X-BiP–GFP using the Ub fusion technique. Note that R-BiP–GFP, but not V-BiP–GFP, forms cytosolic puncta co-localizing with p62ΔZZ–RFP. Unprocessed original scans of blots are shown in Supplementary Fig. 8 (p. 4).

**N-terminal arginylation of ER proteins is signalled by and associated with cytosolic misfolded proteins destined for autophagy**

We tested whether N-terminal arginylation of ER proteins is induced by Ub conjugates destined for autophagy. Immunoblotting analysis showed that poly(dA:dT) co-induced ubiquitylation and R-BiP (Fig. 7a). Immunostaining analysis showed that poly(dA:dT)-induced Ub conjugates formed cytosolic puncta that co-localized with R-BiP as well as p62 (Fig. 7b). Consistently, a screening with various stressors identified prolonged proteasomal inhibition as a stress type that strongly induces R-BiP (Fig. 7c). The induction of R-BiP, R-CRT and R-PDI under proteasomal inhibition became synergistic when co-treated with thapsigargin (Fig. 7d,e), suggesting that ER stress accelerates the supply of the precursor, luminal BiP, for N-terminal...
arginylation. In addition to proteasomal inhibition and ER stress, geldanamycin, an HSP90 inhibitor that facilitates the formation of misfolded proteins, induced R-Bip (Fig. 7f). These results suggest that N-terminal arginylation of ER proteins is signalled by cytosolic misfolded proteins, which are tagged with Ub but redirected to autophagy.

Given the intrinsic ability of BiP to bind and deliver terminally misfolded proteins to ER-associated protein degradation36, we determined whether R-Bip is associated with yellow fluorescent protein (YFP)–CL1 (ref. 45), a model cytosolic substrate of spontaneous misfolding (Fig. 7g–i). In MEFs treated with MG132 and bafilomycin A1, YFP–CL1 formed cytosolic puncta that co-localized with R-Bip as well as p62 (Fig. 7h). A similar co-localization of YFP–CL1 and R-Bip with p62 and LC3 was observed in MEFs treated with poly(AD)T (Fig. 7h) or expressing Ub–R-Bip (Fig. 7i). Finally, the molecular interaction between R-Bip and YFP–CL1 was confirmed.

**Figure 6** The Nt-arginine residue induces oligomerization and aggregation of p62 in vitro. (a) In vitro oligomerization/aggregation assays of HEK293 cell lysates expressing p62–Myc/His. The dipeptide Arg–Ala (type 1) at a final concentration of 20 μM was added to the extracts (1 μg) in comparison with other dipeptides. (b,c) Similar to a, Arg–Ala was compared with Ala–Arg in time-dependent (b) and dose-dependent (c) manners. (d) The p62–LC3 interaction assay shows that the Nt-arginine of Arg–Ala promotes the interaction of p62 with LC3–GST. LC3–GST (3 μg) was immobilized in glutathione-coated wells and incubated with HEK293 cell extracts (20 μg) expressing p62. Following incubation with dipeptides for 2 h, bound p62 was detected using anti-p62 antibody. Quantification is of p62. Following incubation with dipeptides for 2 h, bound p62 was detected using anti-p62 antibody. Quantification is of p62 interaction assay shows that the Nt-arginine of Arg–Ala promotes the interac-

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Figure 7 R-BiP is induced by and associated with cytosolic misfolded proteins, and ATE1-deficient cells are hypersensitive to misregulation of protein quality control. (a) Immunoblotting analysis of R-BiP and Ub conjugates in HeLa cells treated with 0.4 μg/ml poly(dA:dT) for 16 h. (b) Co-localization assay with antibodies specific to Ub conjugates (FK2 antibody) p62 and R-BiP in HeLa cells treated with 1 μg/ml poly(dA:dT) for 21 h. Scale bar, 10 μm. (c) HeLa cells were treated with various stresses for 24 h as described in Methods and subjected to immunoblotting analysis of R-BiP and Ub conjugates using FK1 antibody. (d) HeLa cells were treated with 10 μM MG132 in combination with 200 nM thapsigargin or 200 nM bafilomycin A1 for 16 h. The cells were treated with 10 μg/ml cycloheximide, followed by immunoblotting assay. (e) Arginylation of ER proteins is synergistically induced by proteasomal inhibition and ER stress. HeLa cells were treated with 10 μM MG132 and 100 nM thapsigargin for 18 h. (f) Treatment with geldanamycin, an inhibitor of HSP90, for 17 h results in co-induction of autophagy with arginylation of ER-residing chaperones. (g) Measurement of the interaction between R-BiP and YFP–CL1, a model substrate that undergoes spontaneous misfolding. See Methods for experimental details. (h) Co-localization assay of YFP–CL1 with R-BiP and p62. MEFs ectopically expressing YFP–CL1 were treated with 1 μg/ml poly(dA:dT) alone for 18 h or 10 μM MG132 and 200 nM bafilomycin A1 for 6 h, followed by immunostaining of endogenous R-BiP and p62 in comparison with YFP–CL1 fluorescence. Scale bar, 5 μm. (i) Ub–R-BiP–RFP was co-expressed with YFP–CL1 or GFP in HeLa cells, followed by fluorescence analysis. (j) Punctum formation assay of p62 in +/+ and ATE1−/− MEFs treated with 10 μM MG132 and/or 0.2 μM bafilomycin A1 for 6 h. (k) Control and ATE1-knockdown cells were treated with 10 μM MG132 and/or 0.2 μM bafilomycin A1 for 18 h, followed by immunoblotting analysis. (l) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay of control and ATE1-knockdown cells treated with various stresses, including 10 μM MG132. Mean ± s.d. of n=3 independent experiments in which 10,000 cells in a 24-well plate were analysed per experimental point. Statistical significance was calculated using a two-way ANOVA test (*P < 0.05; ** P < 0.01). Unprocessed original scans of blots are shown in Supplementary Fig. 8 (pp. 5, 6).
**Figure 8** A model illustrating the role of the N-end rule pathway in N-terminal arginylation of ER-residing proteins and the ligand-mediated regulation of autophagy in stressed cells. In this model, ER residents and clients acquire co-translationally and co-translocationally the degrons of the N-end rule pathway, which normally remain separated from cytosolic N-end rule machinery. N-terminal arginylation of ER-residing proteins, especially BiP, is induced by various stress signals such as cytosolic misfolded proteins that are initially tagged with Ub but cannot be readily processed by the proteasome (Step 1). One physiological stress signal that induces N-terminal arginylation of ER proteins is the presence of cytosolic foreign dsDNA, which triggers innate immune responses. N-terminal arginylation is thought to occur when the N-terminal residue of the substrate is exposed to the cytosolic surface of the ER membrane (Step 2), which may facilitate the cytosolic relocation of arginylated ER proteins. We do not exclude the possibility that N-terminal arginylation occurs after the substrates complete their cytosolic relocation. Arginylated ER proteins relocated to non-ER compartments seem to have both shared and distinct functions and metabolic fates. At least a subpopulation of cytosolic R-BiP seems to be associated with its cargoes such as cytosolic misfolded proteins (Step 3). R-BiP, alone or loaded with its cargo, binds to the ZZ domain of p62 through the N-end rule interaction of its Nt-arginine (Step 4). This induces an allosteric conformational change of p62, exposing PB1 and LIR domains. The PB1 domain promotes self-oligomerization and aggregation of p62 (Step 5), together with cargoes such as cytosolic misfolded proteins (Step 6). The LIR domain mediates the interaction of p62 with LC3 on the autophagic membranes (Step 7). In this model, the Nt-arginine residue acts as an autophagic delivery determinant to autophagosomes for BiP and its cargoes, an activating ligand to p62, and an autophagic degron for BiP and the cargoes of R-BiP and p62.

**Arginylation-deficient cells are hypersensitive to the misregulation of protein quality control**

To determine the physiological importance of Nt-arginine in stress responses to misregulation of protein quality control, we treated +/+ and ATE1−/− MEFs with MG132 for 6 h and monitored the formation of p62 puncta. Consistent with the finding that Nt-arginine is an autophagic delivery determinant (Fig. 4) as well as an activation ligand to p62 (Figs 5 and 6), immunostaining analysis showed that ATE1−/− MEFs were impaired in the formation of p62 puncta under proteasomal inhibition (Fig. 7j), suggesting that Nt-arginine plays an important role in p62-dependent autophagic delivery of cytosolic misfolded proteins. In addition, ATE1 knockdown rendered MEFs hypersensitive to prolonged proteasomal inhibition (Fig. 7l), resulting in apoptotic death through the cleavage of caspase 3 and PARP (Fig. 7k). These results indicate that N-terminal arginylation by ATE1 is essential for coping with cellular stresses caused by excessive misfolded proteins.

**DISCUSSION**

Although many ER-residing proteins have been found in various cellular compartments, the mechanisms underlying their functions remain poorly understood. Here, we show that a large number of ER-targeted proteins acquire the potential to undergo N-terminal arginylation and other N-end rule PTMs on signal peptide cleavage. These include major Ca2+-binding folding factors whose arginylation permissiveness is conserved throughout evolution. By raising arginylation-specific antibodies, we show that BiP, PDI, and CRT are the physiological substrates of ATE1-encoded R-transferase, suggesting that the N-end rule pathway may have a broad role in the regulation of ER proteins. One fundamental question not addressed here is whether these arginylation substrates are released into the cytosol through a translocon-like retrotranslocation channel and/or as endosome-like vesicles.

In part because of their low abundance in unstressed cells, it has been challenging to characterize the metabolic fates of ER-derived proteins in non-ER compartments. We now demonstrate that cytosolic R-BiP is selectively delivered to autophagic vacuoles by way of p62 bodies (Figs 3 and 4). The autophagic targeting requires p62 (Figs 3f and 4d,e) and involves the binding of R-BiP Nt-arginine to p62 (Fig. 5a–c). The autophagic/signalling adaptor p62 is known to exert its functions through multiple domains such as PB1 (for self-oligomerization), ZZ, TB (TRAF6 interacting), LIR (LC3 interacting) and UBA (Ub interacting). Although these domains have been extensively studied in signalling and autophagic processes, the functions of ZZ, a C2-H2 zinc finger domain, have remained murky. Our X-peptide and GST-binding assays (Fig. 5d–k) and co-localization assays (Fig. 5l) now show that ZZ is the ligand-binding domain for Nt-arginine, making p62 an N-recognin in...
autophagy. Moreover, our aggregation and binding analyses (Fig. 6) suggest that Nt-arginine binding to p62 induces self-oligomerization and aggregation and increases p62 interaction with LC3. These biochemical consequences are consistent with our data that R-BiP is delivered to autophagosomes and degraded by lysosomal hydrolases (Fig. 6e–i). Thus, Nt-arginine, a well characterized degron in the UPS (refs 1–4, 12), is an autophagic degron as well. One intriguing observation with R-BiP turnover was that, whereas a short N-terminal fragment of R-BiP was readily degraded by autophagy (Fig. 6e–g), endogenous R-BiP exhibited longer half-life (Fig. 2i). The apparent difference could be because full-length R-BiP has multiple domains that interact with other molecules, resulting in cytotoxic retention before targeting for lysosomal degradation. In summary, we propose a model (Fig. 8) that the binding of R-BiP Nt-arginine to the ZZ domain of p62 ‘activates’ p62 through an allosteric conformational change, which exposes the PB1 and LIR domains. In this autophagic regulatory mechanism, Nt-arginine is a delivery determinant to autophagic vacuoles, an activating ligand to p62 and a trans-acting autophagic degron to BiP and other arginylated ER proteins. Given the emerging role of Nt-arginine in autophagic degradation, another important question concerns the identity of cargos of arginylated ER proteins. We demonstrate that one signal that co-induces N-terminal arginylation of ER proteins together with ubiquitylation and autophagy is cytotoxic misfolded proteins destined for p62-dependent macroautophagy, which are generated during cellular stress responses such as innate immune responses to cytosolic foreign dsDNA (Fig. 7a–f). Consistently, we find that R-BiP associates with cytotoxic misfolded proteins in vitro and in autophagic vacuoles (Fig. 7g–i), indicating the role for R-BiP in autophagy-mediated homeostasis of proteins and non-proteinaceous cargos.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
Bioinformatic analyses of N-end rule degrons on ER proteins were carried out by M.M.; antibodies to arginylated ER proteins were generated by D.H.H.; immunoblotting analyses of arginylated ER proteins were carried out by K.A.K., V.Y., H.C.-M., K.S.S., J.H., J.G.K. and J.E.Y. immunostaining of arginylated ER proteins was carried out by H.C.-M., J.E.Y., Y.L. and N.K.S.; DNA-induced innate immune responses were characterized by K.S.S., H.C.-M., A.Z., S.H.K., and S.T.K.; the domain of p62 that binds to Nt-arginine was determined by J.M.J. and H.C.-M.; the relationship of arginylated ER proteins with misfolded proteins and proteasomal inhibition was investigated by H.C.-M. and S.Y.K.; X-peptide pulldown assay with R-BiP was carried out by H.C.-M. and J.E.Y. and p62 aggregation assay was carried out by H.C.-M. and J.E.Y. H.C.-M., S.Y.K., J.H. and K.A.K. contributed equally to this work. H.G.L., J.S.A. and B.Y.K. provided guidance, specialized reagents and expertise. Y.T.K., H.C.-M., B.Y.K. and A.C. supervised personnel and/or wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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METHODS

Plasmids and other reagents. The plasmids encoding ATE1 R-transferase isoforms are described (12, 24). To construct a plasmid encoding full-length BiP–Flag–KDEL, in which the C-terminal KDEL ER retention signal was replaced with Flag–KDEL, the BiP–Flag–KDEL vector was amplified from pENTR/hBiP–Flag–KDEL (Invitrogen) using PCR and inserted into the pCDNA3.1–Mycl/His vector at the EcoRV–Xhol site. The plasmids encoding Ub–X-BiP–GFP and Ub–X-BiP^{EEEDKKEDVGC}–GFP were constructed into pEGFP-N3 at the BamHI–HindIII site using PCR amplification. To construct complementary DNA encoding full-length BiP–Mycl/His, the BiP fragment was amplified from pENTR/hBiP–Flag (Invitrogen) using PCR and inserted into the pCDNA3.1–Mycl/His vector at the EcoRV–Xhol site. The plasmids encoding Ub–X-BiP–Mycl/His were constructed into pCDNA3.1–Mycl/His at the BamHI/Xhol site using PCR amplification. To construct a plasmid encoding full-length human p62 tagged with Myc and His, a 1,320-base pair cDNA fragment was PCR-amplified from the hMU012675 clone (21C Frontier Human Gene Bank), digested with restriction enzymes EcoRI and Xhol and subcloned into pCDNA3.1–Mycl–His. To construct a series of deletion mutants (D1–D7, CD1–CD9 and ND1–ND6) of human p62, we amplified appropriate DNA fragments from the hMU012675 clone and subcloned them into pCDNA3.1–Myc–His through a similar strategy. These constructed plasmids were confirmed by sequencing.

The following plasmids are commercially available: pEGFP-N3 (Addgene, catalogue no 6800-1, Addgene, catalogue no 6768, pkR5–HA–Ub–WT), ub–mt–HA (Addgene, 17603; pkR5–HA–Ub–KO), YFP–CL1 (Addgene, 11950), IgG–kinase fusion (Addgene, 24068, pkR5–HA) or pkR5–HA (Promega). The NIH–mGFp plasmid was a gift from N. Hoshikawa (Kyoto University, Japan). Other reagents used in this study were purchased as follows: thapsigargin, Sigma (T9033); digoxigenin, Sigma (D411); bafilomycin A1, Sigma (B7937); geldanamycin, Sigma (G3381); MG132 Calbiochem (474970); polyclonal anti-apolipoprotein B (Abcam, ab290); mouse monoclonal anti-p62 (Santa Cruz, sc-28359). The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11029, 1:200), Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A21249, 1:200), Alexa Fluor 633 goat anti-rabbit IgG (Invitrogen, A2104, 1:200), anti-rabbit IgG–HRP (Cell Signaling, 7074, 1:10,000), and anti-mouse IgG–HRP (Cell Signaling, 7076, 1:10,000).

Antibodies. The antibodies used here are as follows: rabbit monoclonal anti-BiP (Cell Signaling, catalogue no 3177, 1:1,000), rabbit polyclonal anti-GFP (Abcam, ab290, 1:3,000), mouse monoclonal anti-CL3 (Santa Cruz, sc-271625, 1:2,000), rabbit polyclonal anti-GAPDH (Santa Cruz, sc-25778, 1:2,000), mouse monoclonal anti-Flag (Sigma, F1804, 1:5,000), mouse monoclonal anti-A1E1 (Santa Cruz, sc-271220, 1:500), rabbit monoclonal anti-IRF-3 (Cell Signaling, 11904P, 1:500), rabbit monoclonal anti-phospho-IRF-3 (Ser96) (Cell Signaling, 4947S, 1:500), rabbit polyclonal anti-NFkB p65 (Santa Cruz, SC-7178, 1:50), mouse monoclonal anti-AK (Abcam, ab21223, 1:200), mouse monoclonal anti-calreticulin/CRT (BD Biosciences, 612136, 1:5,000), mouse monoclonal anti-polypeptidyltransferase (FK2 clone (Millipore, 04-263), rabbit polyclonal anti-PDI (Cell Signaling, 2446S, 1:1,000) and mouse monoclonal anti-p62 (Santa Cruz, SC-28359). The following secondary antibodies were used: Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, A11034, 1:200), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11029, 1:200), Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A21249, 1:200), Alexa Fluor 633 goat anti-rabbit IgG (Invitrogen, A21071, 1:200), Alexa Fluor 633 goat anti-mouse IgG (Invitrogen, A21052, 1:200), anti-rabbit IgG–HRP (Cell Signaling, 7074, 1:10,000), and anti-mouse IgG–HRP (Cell Signaling, 7076, 1:10,000).

To identify chemical stressors that induce R-BiP, HeLa cells were treated with the following reagents for 24 h: 500 μM H2O2; 2 μg/ml−1 A21387, calcium ionophore; 10 μM carbonyl cyanide m-chlorophenyl hydrazone, a chemical inhibitor of oxidative phosphorylation; 100 nM thapsigargin; 5 μM MG132; 5 μM bortezomib (PS-341/Velcade), a proteasome inhibitor, and 0.1 μM bafilomycin A1, an autophagic inhibitor. To identify chemical stressors that induce R-BiP, HeLa cells were treated with the following reagents for 24 h: 500 μM H2O2; 2 μg/ml−1 A21387, calcium ionophore; 10 μM carbonyl cyanide m-chlorophenyl hydrazone, a chemical inhibitor of oxidative phosphorylation; 100 nM thapsigargin; 5 μM MG132; 5 μM bortezomib (PS-341/Velcade), a proteasome inhibitor, and 0.1 μM bafilomycin A1, an autophagic inhibitor.

To determine the interaction of R-BiP with YFP–CL1, a model substrate of spontaneous misfolding, HeLa cells exogenously expressing Ub–R-BiP–Myc–His were lysed in a native purification buffer (50 mM NaH2PO4, pH 8.0, 250 mM NaCl and 10 mM imidazole with a cocktail of protease and phosphatase inhibitors (Roche)) through two cycles of freezing in liquid nitrogen and thawing in a 42 °C water bath. Genomic DNA was sheared by passing the extracts through a 24-gauge needle four times. The resulting lysates were centrifuged at 13,000rpm for 15 min to pellet the cellular debris. A total of 1 mg of proteins was incubated with 50 μl of 50% Ni–NTA beads for 4 h at 4 °C with gentle agitation, and then the beads were washed with wash buffer (50 mM NaH2PO4, pH 8.0, 250 mM NaCl and 20 mM imidazole (Roche)) for 10 min at 4 °C with gentle agitation; washing was repeated three times. The beads were then incubated with HeLa cell extracts (500 μg protein) containing either exogenously expressed GFP in YFP–CL1 in binding buffer (20 mM Tris–HCL at pH 7.6, 125 mM NaCl, 15 mM imidazole and 1% Nonidet P-40 with a cocktail of protease and phosphatase inhibitors (Roche)) for 3 h with gentle agitation. The beads were washed with the binding buffer for 5 min at 4 °C with gentle agitation; washing was repeated three times. The proteins bound to His–NTA agarose beads were dissociated in 2× SDS sample buffer, heated at 100 °C for 5 min and separated on SDS–PAGE.

Protein degradation assays. For pulse chase analysis, cells at 80% confluence were treated with 100 nM thapsigargin for 6 h to induce the transcription of BiP and pulse labelled with [35S]-methionine/cysteine (35S-Express protein labelling mix;
Perkin-Elmer) for 12 min, followed by a chase in the presence of cycloheximide, preparation of extracts, immunoprecipitation, NuPAGE 10%-Bis-TRIS SDS–PAGE (Invitrogen), autoradiography and quantification of 35S (Molecular Imager FX system, Bio-Rad). For cycloheximide-chase degradation assay, cells at 80% confluence were treated with 10µg ml–1 cycloheximide. At indicated times, cells were lysed on ice for 30 min in RIPA buffer containing a protease inhibitor cocktail, followed by centrifugation for 20 min at 15,000g. After centrifugation, 10 µg of total protein was subjected to immunoblotting.

**RNA interference analysis.** Cells in a six-well plate (1 × 10^6/well) were transfected with 100 pmol of siRNA using the RNAiMax reagent (Invitrogen). Pre-designed Silencer Select siRNAs (Invitrogen) were used to knock down ATE1, BIP and p62 using the following sequences: ATE1 siRNA no 204 (HS117-204-sense, 5′-ACC CACCAUCUUGUUCUCCAAA-3′; HS117-204-antisense, 5′-UUUGGG GAAACAAAGAUGGGGU-3′), GRP78/Bip siRNA (sense, 5′-AGUGUGGA AGAUUCUGATT-3′; antisense, 5′-AUCAAGAUUCUCAAACUTT-3′) and SQSTM1/p62 siRNA (HS113117-sense, 5′-AUAGUUCUGUGUCUGCAGGAGC CG-3′; HS113117-antisense, 5′-CAGGGUCUCUGACGACAAAGAUAU-3′).

**Immunocytochemistry.** Immunocytochemistry of an arginylated form of ER-residing proteins in cultured cells was carried out using a standard procedure49. Briefly, the cells were washed with PBS twice, fixed in 4% paraformaldehyde at room temperature for 10 min, treated with ice-cold methanol for 2.5 min and incubated in blocking solution (5% BSA in PBS), followed by incubation with primary antibody and subsequently Alexa Fluor-labelled goat anti-rabbit/mouse IgG (Invitrogen). BIP was stained using goat anti-rabbit IgG conjugated with an Alexa Fluor 488 (Fig. 3a), an Alexa Fluor 555 (Fig. 7b) or an Alexa Fluor 633 (Figs 3b–d, f–h and 7b). Confocal microscopy images were taken using a 510 Meta laser scanning confocal microscope (Zeiss) and analysed with the Zeiss LSM Image Browser (Version 4.2.0.121). To determine the intracellular localization of recombinant proteins, cells grown on coverslips were transfected with poly(dA:dT) (X-IFSTIEGRTYK–biotin) bearing N-terminal Arg–Glu (permanently arginylated), Glu (native) or Val (glutamic acid to valine mutant) residues were crosslinked through C-terminal biotin to streptavidin agarose resin (Thermo Scientific). Alternatively, we used a set of 12-mer BIP-derived peptides (X-EEKKDENVG–biotin) bearing N-terminal Arg–Glu (permanently arginylated), Glu (native) or Val (glutamic acid to valine mutant) residues were crosslinked through C-terminal biotin to streptavidin agarose resin (Thermo) with a ratio of 0.5 mg peptide per 1 ml settled resin. Following dilution in 5× PBS and incubation with cell extracts overnight at 4°C, the protein-bound beads were collected by centrifugation at 4,600g for 3 min and washed by PBS three times. Protein extracts were prepared using HEK293 cells transiently expressing p62 mutant proteins. 24 h after transfection, cells were collected by centrifugation and lysed in a hypotonic buffer (10 mM HCl, 1.5 mM MgCl_2 and 10 mM HEPES at pH 7.9), followed by incubation on ice for 30 min. Cell lysates were obtained through five rounds of freezing–thawing cycles or using Dounce homogenization, followed by centrifugation at 15,900g at 4°C for 10 min. For the X-peptide pulldown assay, 150–200µg total proteins in soluble extracts (30 µl) were diluted in 300 µl binding buffer (0.05% Tween 20, 10% glycerol, 0.2 M KC1 and 20 mM HEPES at pH 7.9) and mixed with 50 µl (in packed volume) of X-peptide beads. The mixtures were gently rotated at 4°C for 2 h. The beads were collected by centrifugation at 4,600g for 30 s, washed five times each with 1 ml of binding buffer at 4°C for 20 min, resuspended in 20 µl SDS sample buffer and heated at 100°C for 5 min, followed by SDS–PAGE and immunoblotting.

**In vitro p62 oligomerization assays.** HEK293 cells were transiently transfected with a plasmid encoding p62–Myc/His using Lipofectamine 2000 following the manufacturer’s instructions. Approximately 24 h after transfection, cells were lysed with cell lysis buffer (50 mM HEPES at pH 7.4, 0.15 M KC1, 0.1% Nonidet P-40, 10% glycerol, and a mixture of protease inhibitors and a phosphatase inhibitor). Following a cycle of freezing and thawing, the cell suspension was incubated on ice for 1 h and centrifuged at 15,900g for 20 min at 4°C. Protein concentration was determined using the Bradford assay. For p62 oligomerization assays, 1 µg of protein was incubated in the presence or absence of dipeptides and in the presence of 100 µM Bestatin at room temperature for 2 h. Samples were mixed with a non-reducing loading buffer containing 4% lithium dodecyl sulphate, heated at 95°C for 10 min and resolved on a 3% stacking and 12% separating SDS–PAGE. Immunoblotting analysis using a mixture of anti-p62 and anti-Myc antibodies was employed to monitor the conversion of p62 monomers into oligomers and aggregates.

**Measurement of p62–LC3 interaction on the basis of ELISA.** To determine the specificity of the ELISA, p62−/− MEFs were transfected with a plasmid encoding full-length p62 or a truncated p62 fragment (p62-D3) using Lipofectamine 2000. 24 h after transfection, cells were harvested and lysed in a lysis buffer (20 mM HEPES at pH 7.6, 0.15 M KC1, 0.1% Nonidet P-40, 10% glycerol and a protease inhibitor cocktail) followed by centrifugation at 15,900g for 20 min at 4°C. Total cell extract (20 µg protein) was incubated with GST-tagged LC3 recombinant protein (Enzo Lifesciences, BML-UW1155) that was immobilized on the glutathione-coated plates (Pierce, 15140) in the absence or presence of various concentrations of type-1 and type-2 dipeptides for 1.5 h at room temperature. Bound p62 was detected by incubation with anti-p62 antibody for 1 h at room temperature followed by incubation with HRP-conjugated secondary antibody for 45 min at room temperature. After washing three times with PBS, 3,3′,5,5′-tetramethylbenzidine (TMB) (TMB) substrate (Pierce, 34021) was added to each well, and colour was developed in the dark at room temperature for 10 min. TMB stop solution, 2 N H_2SO_4, was added to stop the colour reaction. Absorbance was measured on a plate reader at 450 nm.

**Statistical analysis.** For all experiments shown, n is indicated in the figure legends. Each point value represents the mean ± s.d. from independent experiments unless specified otherwise and depending on the nature of experimental settings. P-values were determined using two-way ANOVA (for Fig. 7f) or one-way ANOVA (for Figs 4f and 6d,e) tests. For the count of R-Bip cytosolic puncta in comparison with those positive for LC3 and p62, at least five different confocal microscopy images were randomly selected, and positive puncta were examined on the images with identical brightness and contrast conditions. The total number of cells on images was determined using 4,6-diamidino-2-phenylindole (DAPI) (Sigma, D9542) counterstaining. Quantification as a percentage was carried out by counting at least 200 cells per group as indicated in the figure legends. No exclusion criteria were used; data were not excluded. No randomization or blinding of samples was used here. For the representative images (Figs 1c,d, 2a,c–q, 3a–h, 4b–e, 5b,c,e,f,i,j–l, 6a–c,f–h and 7a–k), the results were reproduced in at least three independent experiments.
**Supplementary Figure 1** Sequence alignments of the N-terminal regions of ER-residing chaperone molecules (CRT, PDI, GRP94, and ERdJ5) that acquire evolutionarily conserved Nt-destabilizing residues after the cleavage of their signal peptides. Red boxes indicate P1' residues after the cleavage by the signal peptide peptidase.
Supplementary Figure 2 Peptide binding/competition assays using antibodies specific to the arginylation forms of BiP (a), CRT (b) and PDI (c). (a) An 11-mer R-BiP peptide, which corresponds to the N-terminal region of the arginylated form of BiP, was immobilized on a 96-well plate. A 10-mer peptide (E-BiP peptide) corresponding to an unarginylated form of BiP was used as an N-end rule control. The immobilized peptide was incubated with serially diluted anti-R-BiP antibody, and the amounts of R-BiP antibody bound to immobilized R-BiP peptide were determined using anti-goat secondary antibody conjugated with horseradish peroxide. (b) Similar to (a) except that the binding of R-CRP and E-CRP peptides to anti-R-CRT antibody was determined. (b) Similar to (a) except that the binding of R-PDI and E-PDI peptides to anti-R-PDI antibody was determined.
Supplementary Figure 3  R-BiP is present in the cytosol and shows a mutually exclusive localization with the KDEL immunostaining signal which represents the ER. HEK293 cells (2.5 X 10^5/well) were incubated in the absence or presence of 200 nM thapsigargin for 6 hrs, followed by immunostaining of R-BiP in comparison with antibody to the KDEL sequence, the latter representing the ER. Scale bar, 5 μm. (Right) Enlarged views corresponding to the areas indicated by rectangles. Scale bar, 2 μm.
**Supplementary Figure 4** Puncta formation and colocalization analysis of R-BiP with LC3 and p62 in HeLa cells treated with poly(dA:dT) dsDNA or 5’-PPP dsRNA. (a) HeLa cells stably expressing RFP-GFP-LC3 were treated with poly(dA:dT) dsDNA or 5’-PPP dsRNA, followed by immunostaining analysis to determine the formation and colocalization of R-BiP with LC3 puncta as visualized by RFP fluorescence. (b) HeLa cells were treated with poly(dA:dT) dsDNA or 5’-PPP dsRNA, followed by immunostaining analysis to determine the formation and colocalization of R-BiP with p62 puncta.
Supplementary Figure 5 Immunostaining of NF-kB p50 in HeLa cells (3 X 10⁶/well) treated with 0.5 μg/well poly(dA:dT) dsDNA for 16 hours. Upon activation, NF-kB p50 is dissociated from IkB and enters the nucleus to induce the transcription of its target genes, including interferons. Scale bar, 5 μm.
Supplementary Figure 6 Puncta formation and colocalization analysis of p62 in comparison with LC3 which is produced from RFP-GFP-LC3. HeLa cells expressing RFP-GFP-LC3 were treated with poly(dA:dT) dsDNA, followed by immunofluorescence analysis of acid-resistant RFP-LC3 and acid-sensitive GFP-LC3. Note that most LC3 puncta are positive for both RFP and GFP, indicating that they represent autophagosomes.
Supplementary Figure 7 R-BiP is induced by prolonged proteasomal inhibition and colocalizes with ubiquitin conjugates in autophagic vacuoles.

(a) R-BiP is induced by prolonged proteasomal inhibition. HeLa cells were treated with a proteasomal inhibitor or other stressors, followed by immunoblotting analysis of R-BiP and ubiquitin conjugates (as visualized using FK1 antibody). A23187, calcium ionophore; CCCP, carbonyl cyanide m-chlorophenylhydrazone (Protonophore (H+ ionophore) and uncoupler of oxidative phosphorylation in mitochondria). (b) R-BiP colocalizes with ubiquitin conjugates in autophagic vacuoles. Poly(dA:dT)-treated HeLa cells were subjected to immunostaining analysis of R-BiP and p62 with ubiquitin conjugates as visualized by FK2 antibody. This assay reveals that ubiquitin-positive puncta are invariably positive for both R-BiP and p62. Scale bar, 10 µm. (c) ATE1-knockdown inhibits Nt-arginylation of R-BiP as well as autophagic induction in HeLa cells treated with both 10 µM MG132 and 200 nM thapsigargin. (d) BiP-knockdown inhibits Nt-arginylation of R-BiP as well as autophagic induction in HeLa cells treated with both 10 µM MG132 and 200 nM thapsigargin. (e) R-BiP induced by proteasomal inhibition and ER stress is mainly retrieved from the cytosolic fraction.
Supplementary Figure 8 The unprocessed original scans.
Supplementary Figure 8 continued
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Supplementary Figure 8 continued
**Supplementary Table Legends**

**Supplementary Table 1** Sequences of the N-terminal regions of ER-residing proteins that carry arginylation-permissive destabilizing N-terminal residues, Asp and Glu. Shown in red are the signal peptides that are removed by the signal peptide peptidase complex upon translocation into the ER lumen. The resulting Asp and Glu residues exposed on the N-termini of mature proteins are indicated in blue.

**Supplementary Table 2** Sequences of the N-terminal regions of ER-residing proteins that carry tertiary destabilizing N-terminal residues (Asn, Gln, and Cys). Shown in red are the signal peptides that are removed by the signal peptide peptidase complex upon translocation into the ER lumen. The resulting Asn, Gln, and Cys residues exposed on the N-termini of mature proteins are indicated in blue.

**Supplementary Table 3** Sequences of the N-terminal regions of ER-residing proteins whose N-terminal Asp and Glu residues are evolutionarily conserved. Shown in red are the signal peptides that are removed by the signal peptide peptidase complex upon translocation into the ER lumen. The resulting Asp and Glu residues exposed on the N-termini of mature proteins are indicated in blue.

**Supplementary Table 4** Sequences of the N-terminal regions of human ER-residing proteins whose arginylation-permissive residues, Asp and Glu, are not evolutionarily conserved. Shown in red are the signal peptides that are removed by the signal peptide peptidase complex upon translocation into the ER lumen. The resulting Asp and Glu residues exposed on the N-termini of mature proteins are indicated in blue.