nature portfolio

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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	LC-MS/MS proteomic data were collected with an Ultimate 3000 RSLCnano attached to an Q Exactive mass spectrometer using the Xcalibur software (v4.4.16.14). Immunoblots were imaged using a Davinch Chemisystem (CAS-4000SM) with Davinch v0.0.4.5 software.
Data analysis	Raw data collected by LC-MS/MS were searched with Proteome Discoverer v2.4, MSFragger v4.0 and FragPipe v21.1. AlphaPeptDeep v1.1.5 for MS2 and RT model training and prediction. Statistics and visualization in Rstudio v2024.09.1 Build 394 and R v4.4.2 and Cytoscape v3.9.1. Following R packages were used to analyze the data: clusterProfiler (v4.12.6), dagLogo (v1.28.1), protti (v0.9.1), crmn (v0.0.21). DeepLoc v2.0 was used to annotate localizations of identified proteins.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The MS proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD058868 and

PXD058872. The data was also deposited in KPOP (Korea ProteOme rePository, https://kbds.re.kr/KPOP) with the accession ID KAP241007. Upon publication, the data will be made available to the general audience. The code for the ML-based filtering and data analyses is available at GitHub (https://github.com/syju1984/NtarginylationFiltering) and Zenodo (DOI: 10.5281/zenodo.17247948).

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	N/A
Reporting on race, ethnicity, or other socially relevant groupings	N/A
Population characteristics	N/A
Recruitment	N/A
Ethics oversight	N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	All proteomic experiments were performed with at least 2 biological replicates to demonstrate the reproducibility of the developed method.
Data exclusions	No data were excluded from the analyses.
Replication	Replicates were used as indicated in figure legends, method section, and text.
Randomization	Samples were allocated into experimental groups based on treatment conditions: DMSO (control), thapsigargin, and thapsigargin + MG132. Random allocation was not applicable, as this study used cultured HeLa cells subjected to defined chemical treatments under controlled conditions. All experiments were performed using identical cell passage numbers and culture conditions to minimize variability. No randomization is needed as this study involves user-designed experiments only.
Blinding	Blinding was not applied in this study because all experiments involved predefined chemical treatments of cultured HeLa cells (DMSO, thapsigargin, and thapsigargin + MG132) followed by mass spectrometry–based proteomic analysis. Sample processing and data acquisition were performed in an automated and unbiased manner, and data analysis was conducted using objective computational pipelines without investigator influence. Therefore, blinding was not relevant to the experimental design or outcomes.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

The antibodies used are as follow: rabbit polyclonal anti-HSPA5 (Cell Signaling Technology, #3183, lot 11, 1:5000), rabbit polyclonal anti-R-HSPA5 (Sigma-Aldrich, #ABS2103, lot 4117365, 1:5,000), rabbit polyclonal anti-CRT (courtesy of Dr. Yong Tae Kwon, 1:2000), rabbit polyclonal anti-R-CRT (courtesy of Dr. Yong Tae Kwon, 1:2,000), rabbit polyclonal anti-PDI (courtesy of Dr. Yong Tae Kwon, 1:1000), rabbit polyclonal anti-R-PDI (courtesy of Dr. Kwon Yong Tae, 1:2000), rabbit monoclonal anti-ATF4 (Cell Signaling Technology, 11851, lot 6, 1:2,000), rabbit polyclonal anti-caspase-3, cleaved form (Cell Signaling Technology, #9661, lot 47, 1:500), rabbit polyclonal anti-GAPDH (Abcam, #ab9485, lot 1064471-1, 1:10,000), mouse monoclonal anti-alpha-tubulin (Santa Cruz Biotechnology, #sc-5286, lot D2310, clone B-7, 1:1,000), rabbit monoclonal anti-cytochrome c (Cell Signaling Technology, #4280, lot 3, clone 136F3, 1:1,000), mouse monoclonal anti-ATE1 (Santa Cruz Biotechnology, #sc-271220, lot F2921, clone E-6, 1:2,000), mouse monoclonal anti-beta-actin (Sigma-Aldrich, #A1978, lot 0000227449, clone AC-74, 1:30,000), mouse monoclonal anti-Myc (Santa Cruz Technology, #sc-40, lot C2224, clone 9E10, 1:2000), horse anti-mouse IgG-HRP (Cell Signaling Technology, #7076, lot 38, 1:5000) and goat anti-rabbit IgG-HRP (Cell Signaling Technology, #17492, lot 33, 1:100,000).

Validation

The antibodies obtained from Cell Signaling Technology were validated by the manufacturer. The validation statements are available on the manufacturer's website (https://www.cellsignal.com/about-us/our-approach-process/antibody-validation-western-blotting).

For the antibodies provided by Dr. Yong Tae Kwon, they were validated in publication (Cha-Molstad, H. et al. Amino-terminal arginylation targets endoplasmic reticulum chaperone BiP for autophagy through p62 binding. Nat Cell Biol 17, 917-929 (2015).)

The antibodies used for R-catcher pulldown assay were validated in publication (Seo, T. et al. R-catcher, a potent molecular tool to unveil the arginylome. Cell Mol Life Sci 78, 3725-3741 (2021).)

Eukaryotic cell lines

Policy information about cell lines and Sex and Gender in Research

Cell line source(s)

HeLa cells were obtained from the ATCC (Cat# CCL-2). Wild-type (+/+) and ATE1-knockout (ATE1-/-) mouse embryonic fibroblasts (MEFs) were originally generated in Dr. Alexander Varshavsky's laboratory and later provided by Dr. Yong Tae Kwon for use in this study.

Authentication

The authenticity of HeLa cells was guaranteed by the supplier. The authenticity of MEFs was validated in previous publications (Kwon, Y. T. et al. An essential role of N-terminal arginylation in cardiovascular development. Science 297, 96-99 (2002), Seo, T. et al. R-catcher, a potent molecular tool to unveil the arginylome. Cell Mol Life Sci 78, 3725-3741 (2021).)

Mycoplasma contamination

Mycoplasma contamination was not assessed; however cells were handled under standard sterile conditions.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified cell lines were used.

Plants

Seed stocks

Report on the source of all seed stocks or other plant material used. If applicable, state the seed stock centre and catalogue number. If plant specimens were collected from the field, describe the collection location, date and sampling procedures.

Novel plant genotypes

Describe the methods by which all novel plant genotypes were produced. This includes those generated by transgenic approaches, gene editing, chemical/radiation-based mutagenesis and hybridization. For transgenic lines, describe the transformation method, the number of independent lines analyzed and the generation upon which experiments were performed. For gene-edited lines, describe the editor used, the endogenous sequence targeted for editing, the targeting guide RNA sequence (if applicable) and how the editor was applied.

Authentication

Describe any authentication procedures for each seed stock used or novel genotype generated. Describe any experiments used to assess the effect of a mutation and, where applicable, how potential secondary effects (e.g. second site T-DNA insertions, mosiacism, off-target gene editing) were examined.