

Reporting Summary

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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                                                                                                                                                                                                                                                                                      |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The exact sample size ( <i>n</i> ) for each experimental group/condition, given as a discrete number and unit of measurement                                                                                                                               |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly                                                                                                                                    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> The statistical test(s) used AND whether they are one- or two-sided<br><i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i>                                                               |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> A description of all covariates tested                                                                                                                                                                                                                                |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons                                                                                                                                        |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> 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) |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. <i>F</i> , <i>t</i> , <i>r</i> ) with confidence intervals, effect sizes, degrees of freedom and <i>P</i> value noted<br><i>Give P values as exact values whenever suitable.</i>                     |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings                                                                                                                                                                      |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes                                                                                                                                                |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Estimates of effect sizes (e.g. Cohen's <i>d</i> , Pearson's <i>r</i> ), 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 | We collected ribosome profiling and RNA-Seq data using Illumina Casava 1.8 software. Fastp version 0.21.0 facilitated the 3' adapter trimming. A custom script was employed for library sorting, which was based on the barcode sequence, and for unique molecular index trimming (available at <a href="https://github.com/ingolia-lab/RiboSeq">https://github.com/ingolia-lab/RiboSeq</a> ). Alignments of rRNA and other non-coding RNA were carried out using STAR version 2.7.0a. We also utilized STAR version 2.7.0a for aligning reads to the corresponding genome sequence (human, hg38; mouse, mm10; C. elegans, WBcel235). UMI suppression in Thor-Ribo-Seq was performed using UMIttools (version 1.1.2). For the quantification, we used another custom script (available at <a href="https://github.com/ingolia-lab/RiboSeq">https://github.com/ingolia-lab/RiboSeq</a> ). Gene annotation was done with the GENCODE Human Release 32 reference or Mouse Release 23 reference, obtained through the UCSC Genome Browser ( <a href="https://genome.ucsc.edu/index.html">https://genome.ucsc.edu/index.html</a> ). For C. elegans, the reference was obtained through the WormBase ( <a href="https://wormbase.org/#012-34-5">https://wormbase.org/#012-34-5</a> ). |
| Data analysis   | We analyzed all RNA sequence data using DESeq2 version 1.32.0 and R software version 4.1.1, both operated within the RStudio interface version 2021.09.0+351. For the data analysis for Ribo-Seq, RNA-Seq, and MitolP-Thor-Ribo-Seq data, we used deposited codes in Zenodo ( <a href="https://zenodo.org/records/5675739">https://zenodo.org/records/5675739</a> and <a href="https://zenodo.org/records/11348425">https://zenodo.org/records/11348425</a> ), which used a reported custom script ( <a href="https://github.com/ingolia-lab/RiboSeq">https://github.com/ingolia-lab/RiboSeq</a> ).                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |

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 standard Ribo-Seq, RNA-Seq, and MitolIP-Thor-Ribo-Seq data (GSE222998) obtained in this study were deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database. This study also used the reported data for the retapamulin-assisted mitoribosome run-off assay (GSE237154). The details of the experiments on the ISSa and on the ground (OSD-425, OSD-936, OSD-955, and OSD-956) were also found in the NASA Open Science Data Repository (OSDR). Source data are provided with this paper. The metabolomic data obtained in this study were deposited in the Metabolomics Workbench (PR003099).

## 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

*Use the terms sex (biological attribute) and gender (shaped by social and cultural circumstances) carefully in order to avoid confusing both terms. Indicate if findings apply to only one sex or gender; describe whether sex and gender were considered in study design; whether sex and/or gender was determined based on self-reporting or assigned and methods used. Provide in the source data disaggregated sex and gender data, where this information has been collected, and if consent has been obtained for sharing of individual-level data; provide overall numbers in this Reporting Summary. Please state if this information has not been collected. Report sex- and gender-based analyses where performed, justify reasons for lack of sex- and gender-based analysis.*

### Reporting on race, ethnicity, or other socially relevant groupings

*Please specify the socially constructed or socially relevant categorization variable(s) used in your manuscript and explain why they were used. Please note that such variables should not be used as proxies for other socially constructed/relevant variables (for example, race or ethnicity should not be used as a proxy for socioeconomic status). Provide clear definitions of the relevant terms used, how they were provided (by the participants/respondents, the researchers, or third parties), and the method(s) used to classify people into the different categories (e.g. self-report, census or administrative data, social media data, etc.) Please provide details about how you controlled for confounding variables in your analyses.*

### Population characteristics

*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*

### Recruitment

*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*

### Ethics oversight

*Identify the organization(s) that approved the study protocol.*

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](https://www.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

Basically, for the standard Ribo-seq, RNA-Seq, and MitolIP-Thor-Ribo-seq analyses, we analyzed two or three independent biological replicates in line with the standard protocols in the field. This sample size was sufficient for the statistical analysis conducted by DESeq2 to estimate the dispersion of the data. Detailed information about the sample numbers for each experiment is provided in the manuscript. No statistical methods were employed to predetermine the sample size.

### Data exclusions

No data were excluded from the analysis.

### Replication

The reproducibility of our experiments was basically confirmed, with two replicates tested for deep sequencing experiments. Cell viability assay, cell adhesion assay, oxygen consumption rate assay, qPCR, and flow cytometry were conducted with triplicates. FUNCAT and WB were basically conducted with triplicates, and the reconstituted mammalian mitochondrial translation system was conducted with nine replicates.

|               |                                               |
|---------------|-----------------------------------------------|
| Randomization | Randomization was not required in this study. |
| Blinding      | Blinding was not required in this study.      |

## 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    |                                                                 | Methods                             |                                                    |
|-------------------------------------|-----------------------------------------------------------------|-------------------------------------|----------------------------------------------------|
| n/a                                 | Involved in the study                                           | n/a                                 | Involved in the study                              |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  | <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Eukaryotic cell lines       | <input type="checkbox"/>            | <input checked="" type="checkbox"/> Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology and archaeology          | <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging    |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |                                     |                                                    |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |                                     |                                                    |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Dual use research of concern           |                                     |                                                    |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Plants                                 |                                     |                                                    |

## Antibodies

|                 |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Antibodies used | <p>Western blotting: Anti-<math>\beta</math>-actin (MEDICAL &amp; BIOLOGICAL LABORATORIES [MBL], M177-3, 1:1000), anti-GAPDH (Cell Signaling Technology [CST], 2118, 1:1000), anti-FAK (CST, 3285, 1:1000; Thermo Fisher Scientific, AHO0502, 1:1000), anti-p-FAK397 (CST, 3283, 1:200), anti-TOMM20 (CST, 42406, 1:1000), anti-RAC1 (CST, 8631, 1:200), anti-GST (CST, 2625, 1:1000), anti-PAK1 (CST, 2602, 1:1000), anti-p-PAK1S144/p-PAK2S141 (CST, 2606, 1:200), anti-BAD (CST, 9292, 1:200), anti-lipoic acid (Abcam, ab58724, 1:1000), anti-DLAT (Abcam, ab172617, 1:1000), anti-MRPS22/mS22 (Thermo Fisher Scientific, PA5-52249, 1:1000), anti-Mal-K (PTM Bio, PTM-201, 1:1000), MCAT (Santa Cruz Biotechnology, sc-390858, 1:1000), OXSM (Thermo Fisher Scientific, PA5-32132, 1:1000), and anti-MECR (PROTEINTECH, 51027-2-AP, 1:1000) were used as primary antibodies. IRDye800CW anti-rabbit IgG (LI-COR Biosciences, 926-32211, 1:10000), IRDye680RD anti-rabbit IgG (LI-COR Biosciences, 925-68071, 1:10000), and IRDye680RD anti-mouse IgG (LI-COR Biosciences, 925-68070, 1:10000) were used as secondary antibodies.</p> <p>Flow cytometry: anti-TOMM20 antibody (Abcam, ab209606, 1:100) was used.</p> <p>MitoIP: an anti-TOMM22 antibody from the Mitochondria Isolation Kit, Human (Miltenyi Biotec) was used.</p> |
| Validation      | Antibody was validated by the manufacturer (CST, <a href="https://www.cellsignal.com/about-us/cst-antibody-validation-principles">https://www.cellsignal.com/about-us/cst-antibody-validation-principles</a> ).                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |

## Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

|                                                                   |                                                                                                                                                                                                                                                                                                                                                                                                  |
|-------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Cell line source(s)                                               | HEK293 cells (ATCC, CRL-1573, female), C2C12 cells (ATCC, CRL-1772, female), 3T3 cells (kind gifts from Shinichi Nakagawa's laboratory, male), naïve HAP1 cells (Horizon Discovery, C631, male), BAD KO HAP1 cells (Horizon Discovery, 29-bp deletion, HZGHCO05959c002, male), naïve MEF cells (ATCC, CRL-2991, gender unspecified), and OPA1 KO MEF cells (ATCC, CRL-2995, gender unspecified). |
| Authentication                                                    | Cells were authenticated via periodic morphology check using a microscope.                                                                                                                                                                                                                                                                                                                       |
| Mycoplasma contamination                                          | Cell lines were routinely tested for mycoplasma.                                                                                                                                                                                                                                                                                                                                                 |
| Commonly misidentified lines (See <a href="#">ICLAC</a> register) | No misidentified cell line was used in this study.                                                                                                                                                                                                                                                                                                                                               |

## Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

|                    |                                                                                            |
|--------------------|--------------------------------------------------------------------------------------------|
| Laboratory animals | Adult male (7 weeks old) C57BL/6J mice were purchased from Sankyo Lab Service Corporation. |
| Wild animals       | NA                                                                                         |
| Reporting on sex   | We used male mice for all experiments.                                                     |

Field-collected samples NA

Ethics oversight All animal experiments were authorized and approved by the Animal Care and Use Committee of The University of Tokyo.

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

## 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, mosaicism, off-target gene editing) were examined.

## Flow Cytometry

### Plots

Confirm that:

- ☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☐ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

**Sample preparation** For mitochondrial membrane potential assessment, cells were incubated with 200 nM MitoTracker Red CMXRos and 150 nM MitoTracker Green FM at 37°C for 25 min and then washed with PBS supplemented with 5% FBS. The cells were dissociated from the dish with 0.05% trypsin, neutralized, collected by centrifugation at 300 × g for 3 min, resuspended in PBS supplemented with 5% FBS, and then subjected to flow cytometry.

For mito-FUNCAT FACS, cells were cultured in methionine-free medium supplemented with 100 μM HPG and 100 mg/ml anisomycin for 3 h before cell harvesting. After washing with PBS, the cells were dissociated from the dish with 0.05% trypsin. The collected cells were washed, prepermeabilized with 0.0005% digitonin in mitochondrial protective buffer for 5 min at room temperature, fixed in 4% PFA for 15 min at 4°C, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 5 min at room temperature. Subsequently, nascent peptides within mitochondria were labeled with 1 μM azide-conjugated Cy3 with a Click-it Cell Reaction Buffer Kit. Mitochondria were immunostained with Alexa Fluor 647 Anti-TOMM20 antibody in Intercept Blocking Buffer for 1 h at 4°C. The cells were washed three times with PBS and then analyzed via flow cytometry.

**Instrument** FACSaria II Special Order system (BD)

**Software** FACSDiva ver. 6.1.3 (BD)

**Cell population abundance** Cell lines we used were all homogeneous and contained one population.

**Gating strategy** Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.