1 **Materials and Methods**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse mAb ANTI-FLAG® M2 antibody	Sigma-Aldrich	Cat#F1804-1MG
Goat Anti-Mouse IgG (H+L), HRP Conjugate	TransGen Biotech	Cat#HS201-01
Goat Anti-Rabbit IgG (H+L), HRP Conjugate	TransGen Biotech	Cat#HS101-01
Mouse mAb Anti-alpha-tubulin	DSHB	Cat#12G10
Mouse mAb *ProteinFind*® Anti-β-Tubulin	TransGen Biotech	Cat#HC101
Rabbit pAb SAPK/JNK Antibody	Cell Signaling Technology	Cat#9252
Rabbit mAb Phospho-SAPK/JNK (Thr183/Tyr185) (81E11)	Cell Signaling Technology	Cat#4668
Bacterial and virus strains		
DH5α	Sangon Biotech	Cat#B528413
OverExpress™ *E. coli* C43(DE3)	Weidi Biotechnology	Cat#EC1040
Biological samples		
Coding sequence of Raw	Synthesized (Vigene Bioscience)	GeneBank NM_164836
Chemicals, peptides, and recombinant proteins		
DMEM (Dulbecco's Modified Eagle Medium)	Gibco	Cat#12800-017
insect culture medium (UK1000)	Union Biotech	Cat#UN24G18F
Union-293	Union Biotech	Cat#UP1000
Trypsin-EDTA	Gibco	Cat#25200072
Penicillin/Streptomycin solution	Gibco	Cat#15140122
Fetalbovine semm	PAN	Cat#P30-3302
Polyethylenimine (PEI)	Polysciences	Cat#24765-1
Lipofectamine 2000	Gibco	Cat#12800-017
X-tremeGENE HP DNA	Roche	Cat#06366236001
NAD	Sigma-Aldrich	Cat#N8410
NMN	Sigma-Aldrich	Cat#N3501
cADPR	Lee and Aarhus(1)	N/A
ADPR	Lee et al. (16)	N/A
NGD	Sigma-Aldrich	Cat#N5131
NHD	Sigma-Aldrich	Cat#N6506
8-Br-cADPR	Bruzzone et al.(2)	N/A
8-Br-ADPR	Bruzzone et al.(2)	N/A
CZ-48	Kwong et al.(3)	N/A
Perchloric acid	Sigma-Aldrich	Cat#PX0396D
Tri-n-octylamine	TCI AMERICA	Cat#T0502
Chloroform	Sigma-Aldrich	Cat#288306
Alcohol dehydrogenase (ADH)	Sigma-Aldrich	Cat#A600194
Resazurin	Sigma-Aldrich	Cat#A600485
Diaphorase	Sigma-Aldrich	Cat#D5540
FMN	Sigma-Aldrich	Cat#F6750
Nicotinamide	Sigma-Aldrich	Cat#N0636
ADP-ribosyl cyclase	Lee and Aarhus(1)	N/A
Protease inhibitor cocktail	Roche	Cat#11697498001
Digitonin	Sigma-Aldrich	Cat#D141
Triton X-100	Sigma-Aldrich	Cat#T8787
Tris	Sangon Biotech	Cat#A600194

NaCl	Sangon Biotech	Cat#A610476
EDTA	Sigma-Aldrich	Cat#E9884
Amylose Resin	NEB	Cat#E8021S
Strep-Tactin XT	IBA	Cat#2-5010-010
Imidazole	Sangon Biotech	Cat#A600277
D-Biotin	Sangon Biotech	Cat#A600078
TRI reageent^{TM} solution	Thermo	Cat#00670193
PerfectStart Green qPCR SuperMix	TransGen Biotech	Cat#AQ602-02-V2
Critical commercial assays		
First-Strand cDNA Synthesis SuperMix for qPCR	TransGen Biotech	Cat#AT341-02
T7 High Efficiency Transcription Kit	TransGen Biotech	Cat#JT101-01
ClonExpress Ultra One Step Cloning Kit	Vazyme	Cat#115-01
Deposited data		
RNA-seq, Raw data	This paper	SRA: PRJNA1230788
Experimental models: Cell lines		
D. melanogaster: Cell line S2: S2-DRSC	BioVector NTCC	Cat#CVCL_Z992
HEK-293T	ATCC	Cat#CRL-3216
HEK-293F	ATCC	Cat#AC340697
Experimental models: Organisms/strains		
Drosophila, *Sd*-Gal4;	BDSC	Cat#8609
Drosophila, elav-Gal4	BDSC	Cat#8765
Drosophila, UAS-*mCherry*-RNAi	BDSC	Cat#35785
Drosophila, UAS-*raw*-RNAi	VDRC	Cat#KK101255
Drosophila, UAS-*ask1*-RNAi-#1	BDSC	Cat#35331
Drosophila, UAS-*ask1*-RNAi-#2	VDRC	Cat#GD34891
Drosophila, UAS-*raw RA*	Collins et al.(4)	N/A
Drosophila, UAS-*raw RB*	Collins et al.(4)	N/A
Drosophila, raw^{dcp-1}	Collins et al.(4)	N/A
Drosophila, 5×UAS-*dSarm^{eGFP}*	Collins et al.(4)	N/A
Oligonucleotides		
RT-qPCR primers (See Supplementary Table S3)	This paper	N/A
raw RNAi primers (See Supplementary Table S3)	This paper	N/A
raw mutation construction primers (See Supplementary Table S3)	This paper	N/A
Recombinant DNA		
pAc5.1 V5-His B	Invitrogen	Cat#V411020
Plasmid: pAc5.1 V5-Flag-dt-Raw	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawE396A	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawE422A	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawA532D	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawR949C	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawY953N	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawTIR1	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-RawTIR2	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-Raw△N	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-Raw△TIR1	This paper	N/A
Plasmid: pAc5.1 V5-Flag-dt-Raw△TIR2	This paper	N/A
Plasmid: pAc5.1 V5-dSarm-His	This paper	N/A
Plasmid: pLenti-puro	Addgene	Cat#39481
Plasmid: pLenti-Flag-dt-dSarm	This paper	N/A
Plasmid: pLenti-Flag-dt-dSarm^{TIR}	This paper	N/A

Plasmid: pLenti-Flag-dt-Raw	This paper	N/A
Plasmid: pLenti-Flag-dt-Sarm1	This paper	N/A
Plasmid: pLenti-Flag-dt-Olrn-1	This paper	N/A
Plasmid: pRHIS_MBP-Raw	This paper	N/A
Plasmid: pET28a-Raw	This paper	N/A
Plasmid: pET28a-Raw△N	This paper	N/A
Software and algorithms		
GraphPad Prism version 9.5	GraphPad Software	Version 9.5
Image J	Image J Software	https://imagej.net/ij/
FastQC	FastQC website(5)	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
Cutadapt	Martin(6)	https://github.com/marcelm/cutadapt/
HISAT2	Kim et al.(7)	https://daehwankimlab.github.io/hisat2/download/
DESeq2	Love et al.(8)	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
R(v4.4.0)	R Development Core Team	https://www.r-project.org/
GSEA	Mesirov et al.(9)	https://www.gsea-msigdb.org/gsea/downloads.jsp
PSI-BLAST	National Institutes of Health	Version 2.13.0+
BLASTP	National Institutes of Health	Version 2.13.0+
BLASTCLUST	National Institutes of Health	https://ftp.ncbi.nih.gov/blast/documents/blastclust.html
KALIGN	Lassmann(10)	Version 3.3.2
MUSCLE	Edgar(11)	Version v3.8.1551
PROMALS3D	Pei et al. (12)	http://prodata.swmed.edu/promals3d/promals3d.php
JPRED	Cole et al. (13)	https://www.compbio.dundee.ac.uk/jpred/
CHROMA	Goodstadt et al. (14)	https://www.llew.org.uk/chroma/
Adobe Illustrator	Adobe	Adobe 2024
Microsoft Word	Microsoft	Version 2501
AlphaFold 2	DeepMind(15)	https://colab.research.google.com/github/sokrypton/ColabFold/blob/main/AlphaFold2.ipynb#scrollTo=kOblAo-xetgx
AlphaFold3	DeepMind(16)	https://alphafoldserver.com/
PAE Viewer	Elfmann C, Stülke J.(17)	https://pae-viewer.uni-goettingen.de/
PyMOL	Schrödinger	Version 3.1
CLANS	Bioinformatics Toolkit	https://toolkit.tuebingen.mpg.de/tools/clans

General culture conditions of cells and flies

HEK-293F cells were obtained from ATCC and cultured in serum-free SMM-293TII medium (Sino Biological) at 37°C in a humidified incubator with 5% CO_2,

shaking at 150 rpm. HEK-293 and HEK-293T cells were obtained from ATCC and cultured in a static incubator at 37°C with 5% CO_2 in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

S2 cells were obtained from BioVector NTCC and maintained in insect culture medium (UK1000, Union Biotech) supplemented with 0.5% penicillin-streptomycin (Gibco) solution at 25-28°C.

The fly strains, *elav*-Gal4 and UAS-*ask1*-RNAi#1 were obtained from BDSC, UAS-*raw*-RNAi and UAS-*ask1*-RNAi#2 were obtained from VDRC, and *Sd*-Gal4(18), UAS-Raw RA, UAS-Raw RB, $raw^{dcp\text{-}1}$, 5×UAS-$dSarm^{eGFP}$(4), UAS-*mCherry*-RNAi were gifted by Prof. Zizhang Zhou (Shandong Agricultural University), Prof. Wenzhe Li (Tongji University), and Prof. Catherine Collines (Case Western Reserve University). Flies were maintained at 22-25°C under a 12-hour light/dark cycle with 60-70% humidity. They were reared in ventilated vials or bottles containing standard cornmeal-yeast-sugar-agar medium supplemented with methylparaben and were transferred to fresh food every 1-2 weeks to prevent overcrowding. For experiments investigating developmental events, the maintenance temperature was increased to 29°C to accelerate growth.

Protein sequence and structural analysis.

To make a comprehensive collection of *Raw* homologous sequences, we started with *Drosophila melanogaster Raw* protein (NP_723416.1) to search against NCBI nr protein database using the iterative sequence profile search program, PSI-BLAST(19). A cut-off *e*-value of 0.0001 was used as a significant threshold. Similarity-based clustering was conducted by BLASTCLUST, a BLAST score-based single-linkage clustering method (https://ftp.ncbi.nih.gov/blast/documents/blastclust.html). Multiple sequence alignments (MSA) were constructed using the KALIGN(10), MUSCLE(11), and PROMALS3D(12) programs, followed by careful manual adjustments based on the profile-profile alignment and the secondary structure information generated by the JPRED program(13). The conservation pattern of the MSA was calculated using a

consensus method based on different categories of amino acid physicochemical properties developed by Taylor in 1986(20). The consensus was determined by examining each column of the MSA to determine if a threshold fraction (80%) of the amino acids belong to a defined category. Then, the MSA was colored using the CHROMA program(14) based on the calculated consensus sequence and further modified using Adobe Illustrator or Microsoft Word.

AlphaFold2(15) and AlphaFold3(16) programs were utilized to predict the 3D structure of the Raw proteins and the binding between Raw and cADPR. The model accuracy was accessed with PAE (Predicted Aligned Error) plots(17). Further structure analysis and visualization were carried out using PyMOL program.

Sequence network clustering analysis.

We collected representative sequences from various TIR families via PSI-BLAST program(19) with default parameter, including Raw-TIR family, animal TLR-TIR family, animal SEF-TIR family, SARM1-TIR family, plant TIR family, and several TIR sequences from bacteria, and combined them to run the CLANS program(21). CLANS program performs all-against-all BLASTP comparisons with scoring matrix BLOSUM62 and an *e*-value cutoff of 0.0001 and generates a two-dimensional graph in which nodes represent sequences while edges represent detected pairwise similarities between sequences. The layout of the graph is rearranged by the Fruchterman and Reingold force-directed graph drawing algorithm(22). Additionally, the clustering graph was colored and labeled using Adobe Illustrator.

Plasmid construction

To construct pAc-dSarm-His, the *dsarm* coding sequence (CDS) was amplified by PCR from fly cDNA. For the construction of pAc5.1 V5-Flag-dt-Raw, Raw TIR1, Raw TIR2, and Raw△N, the inserts were amplified from synthetic *raw* CDS. All fragments were seamlessly cloned into the pAc5.1 V5-His B vector (Invitrogen) using the ClonExpress Ultra One Step Cloning Kit (Vazyme, Nanjing). Point mutations, including pAc5.1 V5-Flag-dt-RawE396A, E422A, A532D, R949C, and Y953N, as well as truncations pAc5.1 V5-Flag-dt-Raw△TIR1 and Raw△TIR2, were generated by

PCR amplification of the pAc-Raw construct, with primers designed to introduce the corresponding mutations or truncations. The primer sequences are listed in **Supplementary Table S3**. Subcloning into the pLenti-puro (#39481, Addgene) and pET28 (Novagen) vectors was performed using the same seamless cloning method.

dsRNA synthesis and interference assay

To synthesize dsRNA for the RNA interference assay, DNA fragments were amplified by PCR using *raw* DNA containing plasmid as the template and specific primers containing the T7 promoter sequence (TAATACGACTCACTATAGGG) at their 5' ends. Three pairs of primers were designed to target different regions of *raw* mRNA, while an additional pair of primers targeting EGFP was used as a control. *In vitro* RNA synthesis was performed according to the manufacturer's instructions (Transgen). The sequences of the primer pairs are provided in **Supplementary Table S3**.

The RNA interference assay for S2 cells in this study was conducted through dsRNA incubation. S2 cells were cultured in 6-well plates until reaching approximately 90% confluence. After replenishing the medium, dsRNAs were directly added to the culture medium at a final concentration of 20 μg per well. The cells were harvested for cell cycle analysis and qRT-PCR after 2–4 days of incubation.

Transient transfection

Transient transfection of HEK-293T cells was performed using PEI, following the manufacturer's instructions. Transfection of S2 cells was carried out using X-tremeGENE HP DNA transfection reagent (Roche) according to the manufacturer's protocol. Briefly, prior to transfection, cells were cultured in 6-well plates until reaching 80–90% confluence. For each well, 2 μg of plasmid DNA was used, with a reagent-to-DNA ratio of 3:1. For co-transfection experiments, 1 μg of each plasmid was used, and the total DNA amount in single-transfection wells was adjusted to 2 μg per well by including 1 μg of control plasmid (pAc). Unless otherwise specified, cells were incubated for 48 hours post-transfection before being harvested for Western blotting or nucleotide extraction.

Measurement of cellular NAD or cADPR levels

Cells were pelleted by centrifugation and lysed in 0.6 M perchloric acid. After centrifugation, the supernatant was used to measure cADPR or NAD levels using a previously described cycling assay(23). The pellets were re-dissolved in 1 M NaOH and protein concentrations were determined using the Bradford assay (Quick Start™ Bradford Kit, BIO-RAD). The results were expressed as picomoles of cADPR or NAD per milligram of total proteins.

Preparation of Raw-containing cell lysate

The recombinant Raw protein, along with its truncations or mutations, was expressed in S2 cells and extracted using 0.1% Triton X-100 in PBS supplemented with a protease inhibitor cocktail (Roche). The cell lysates were subjected to ultrafiltration (10 kDa cutoff; Amicon Ultra-15) to achieve a 1,000-fold dilution of small molecules, thereby minimizing the influence of endogenous NAD. Lysates from S2 cells transfected with an empty vector, prepared under the same conditions, were used as negative controls. To normalize protein levels, Raw expression levels were quantified by Western blot. Lysates containing different forms of Raw were balanced with S2 lysates to ensure that each sample contained the same proportion of Raw protein. Equal amounts of total protein were subsequently used in the enzymatic assays.

Purification of recombinant proteins

The prokaryotic expression vector pRHIS, encoding Raw fused with an N-terminal Maltose-Binding Protein (MBP), was transformed into *E. coli* C43(DE3). Transformed bacteria were cultured at 16°C in a shaking incubator until OD_{600} reached 0.6, followed by an additional 12-hour incubation. Cells were harvested by centrifugation, and the MBP-Raw protein was purified using Amylose Resin (NEB) according to the manufacturer's instructions. The purified recombinant protein was used in the enzymatic assays.

The gene encoding recombinant dSarm, Raw, Raw-Y953N and hSARM1, tagged at the N-terminus with a tandem Strep-tag II and Flag-tag for purification, was

cloned into the pLenti-CMV-puro-Dest vector (Addgene) as previously described(24). HEK-293F cells were transduced with lentivirus encoding FLAG-2×Strep-tagged dSarm/hSarm1/Raw/Raw-Y953N and selected with 1 μg/mL puromycin. Strep-dSarm and Raw/Raw-Y953N was released with 0.1% Triton X-100, and strep-hSARM1 was released with 100 μM digitonin(24), followed by immunoprecipitation with Strep-Tactin XT (IBA, #2-5010-010). The resin was washed four times with Buffer W (20 mM Hepes, pH 7.4; 150 mM NaCl; 1 mM EDTA) and eluted with 50 mM D-biotin in Buffer W. Recombinant human SARM1 was prepared. Protein concentration was further validated through Western blot.

Western blots

Proteins were extracted from S2 and HEK-293T cells using ice-cold lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1 mM EDTA; 0.5% Triton X-100) with a protease inhibitor cocktail (Roche). After centrifugation, the supernatants or purified proteins were mixed with SDS loading buffer, boiled at 100°C for 10 minutes, and subjected to SDS-PAGE and Western blot analysis with the indicated antibodies. Signals were developed using ECL (Abvansta) and detected/quantified with a Chemidoc MP system and ImageLab software (BIO-RAD).

Proteins from wing discs were extracted by dissecting third-instar female larvae in cold PBS. Five pairs of discs were lysed in 200 μL ice-cold PBS using ultrasonic crushing (25 kHz, 300 W; XM-650T; Xiaomeichaosheng) with a pulse cycle of 3 seconds on and 5 seconds off for 1 minute. The samples were mixed with SDS loading buffer, boiled at 100°C for 10 minutes, and subjected to Western blot analysis using anti-JNK (CST: #9252,1:2000) and anti-phospho-JNK (CST: #4668,1:2000) antibodies (Cell Signaling Technology).

Michaelis-Menten kinetics

Recombinant WT and Y953N mutant Flag-Strep-Raw (0.13 nM) were incubated with varying concentrations of cADPR in PBS buffer at room temperature. Reactions were stopped after 3 minutes by adding an equal volume of 0.6 M HCl, followed by neutralization with an equal volume of NaOH. Residual cADPR was quantified using

the cycling assay. The substrate concentrations and initial reaction velocities were plotted to generate a Michaelis-Menten curve, and the best-fit K_m and k_{cat} (best fit) values were derived from nonlinear regression using the Michaelis-Menten model and were further validated by the Lineweaver-Burk plot.

RNA Extraction and qRT-PCR

Total RNAs were isolated from 3 × 10^6 S2 cells seeded in a 6-well plate or 15 pairs of wing discs dissected from third-instar male larvae using TRIzol reagent (Thermo: #00670193), followed by chloroform extraction and centrifugation to separate the aqueous phase. RNA was precipitated with sodium acetate and isopropanol, incubated at -20°C for 1 hour, and pelleted by centrifugation. The RNA pellet was washed twice with 70% ethanol, air-dried, and resuspended in RNase-free water. RNA concentration and purity were assessed using NanoDrop.

For qRT-PCR, mRNA was reverse transcribed using the First-Strand cDNA Synthesis SuperMix for qPCR kit. The resulting cDNA was diluted and used as a template for qRT-PCR with PerfectStart Green qPCR SuperMix, performed on the QuantStudio 7 Pro system according to the manufacturer's protocol. Primers are listed in **Supplementary Table S3**.

RNAseq and data analysis

Total RNAs were extracted from S2 cells to construct a cDNA library and perform RNA-seq. Quality control was applied by FastQC (Version 0.12.0)(5). Adapter trimming was done by cutadapt(6). Sequence alignment to genome was done by HISAT2(7). Feature counting was done by featureCounts(25). Gene expressions were quantified using counts per million (CPM) and the trimmed mean of M values (TMM) methods.

Principal Component Analysis (PCA) was performed using the expression data of all genes. Differentially expressed genes (DEGs) were identified using DESeq (Version 1.46.0)(8). A heatmap was generated based on the top DEGs, normalized using TMM. Both the PCA and heatmap visualizations were created by Chiplot (https://www.chiplot.online/). A significance threshold was set at the Q-value, an

adjust *P*-value accounting for the False Discovery Rate (FDR), of 0.05 and a $\log_2$(fold change) of 1 for DEGs.

Gene enrichment analyses were conducted for significant DEGs using the Gene Ontology (GO)(26, 27) and Kyoto Encyclopedia of Genes and Genomes (KEGG)(28) database, implemented via Metascape(29). Furthermore, Gene Set Enrichment Analysis (GSEA)(9) was performed to identify enriched pathways, utilizing gene sets from the GO and KEGG databases. For statistical hypothesis testing, the *Q*-value, the adjusted *P*-value accounting for FDR, was utilized.

Analysis of *Drosophila* wing morphology

Experimental lines were generated by crossing *Sd*-Gal4 virgin females with males of the following genotypes: wild type (WT, control), UAS-*raw*-RNAi; UAS-*mCherry*-RNAi (Raw knockdown), and two independent double knockdown lines (UAS-*raw*-RNAi; UAS-*ask1*-RNAi-#1 and UAS-*raw*-RNAi; UAS-*ask1*-RNAi-#2). All crosses were maintained at 29 °C to ensure optimal Gal4 activity.

After eclosion, male wings were dissected in PBS under a Nikon SMZ745T stereomicroscope and mounted dorsal side up on glass slides using 25% glycerol/25% isopropanol. Wing images were acquired with a Nikon SMZ18 stereomicroscope, and wing areas were measured manually using ImageJ. Data visualization was performed as bar graphs or scatter plots using GraphPad Prism v9.5.

Analysis of *Drosophila* development

Virgin *Sd*-Gal4 females were crossed with males of the following genotypes: wild type (WT, control), 5×UAS-$dSarm^{eGFP}$; UAS-*mCherry*-RNAi (dSarm overexpression), and two independent double-manipulation lines (5×UAS-$dSarm^{eGFP}$; UAS-*ask1*-RNAi-#1 and #2, dSarm overexpression with *ask1* knockdown). All progenies were cultured at 29 °C. Parental flies were transferred to fresh food vials every 24 hours to maintain synchronized progeny development.

Developmental stages were scored on day 9 after egg laying (AEL), and both the number of pupae and total animals per vial were recorded. Pupariation rate was calculated as the percentage of pupae relative to total animals. Data are shown as

bar graphs or scatter plots generated using GraphPad Prism v9.5.

Molecular phylogenetic analysis

Phylogenetic relationships were reconstructed using the Maximum Likelihood (ML) method with the JTT model in the MEGA7 program(30). The tree with the highest log likelihood is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories). The rate variation model allowed for some sites to be evolutionarily invariable. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The tree diagram was generated using FigTree v1.4 program (http://tree.bio.ed.ac.uk/software/figtree/) and further modification was performed by Adobe Illustrator. Species abbreviations used in the Fig. 7 are as follows: Dmel: *Drosophila melanogaster*; Agam: *Anopheles gambiae*; Cqui: *Culex quinquefasciatus*; Tcas: *Tribolium castaneum*; Pcau: *Priapulus caudatus*; Sech: *Saccostrea echinata*; Mcal: *Mytilus californianus*; Spur: *Strongylocentrotus purpuratus*; Apla: *Acanthaster planci*; Skow: *Saccoglossus kowalevskii*; Bbel: *Branchiostoma belcheri*; Bmal: *Brugia malayi*; Cele: *Caenorhabditis elegans*; Pinf: *Phytophthora infestans*; Lgig: *Lagenidium giganteum*; Ctet: *Cymbomonas tetramitiformis*; Pbac: *Pseudomonadota bacterium*; Bbac: *Bacteroidales bacterium*; CSba: '*Candidatus* Shapirobacteria bacterium'; Cbac: *Chloroflexota bacterium*; Abac: *Anaerolineae bacterium*.

Data analysis for biological experiments

Statistical analyses were conducted utilizing GraphPad Prism 9.5. Data values were derived from a minimum of three independent experiments, each including three technical replicates per condition. For comparisons among multiple groups, one-way (Figures 2I, 3B-C, 4E-H, 5A-C, 5G, 6B, 6D, and 7C) or two-way ANOVA (Figures 2H, and 4C-D) was used as appropriate, followed by prespecified post hoc multiple-comparisons tests (Bonferroni). For two-group comparisons, Student's *t*

tests were applied (Figures 2C, 3D-E, 3H, and 6F). All statistical tests were two-tailed, with a *P*-value threshold of less than 0.05 deemed significant. The significance levels are denoted as follows: ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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