Supporting information

Methods

Cells culture and Genes

HeLa cell lines and HCT116 cells were cultured in DMEM (Sigma-Aldrich, D6429) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 1% penicillin-streptomycin (Thermo Fisher Scientific). All cells were cultured at 37°C in a humidified atmosphere with 5% CO2.

Protein expression and purification

Recombinant proteins were expressed in BL21 (DE3) E. coli cells, induced with 200 μM IPTG at 16°C for 15 hours. The bacterial cell pellets were resuspended in binding buffer (50 mM Tris, 500 mM NaCl, and 5 mM imidazole, pH 7.9), and lysed using an ultrahigh pressure homogenizer (FB-110XNANO, Shanghai Litu Machinery Equipment Engineering Co., Ltd.). The lysate was then centrifuged at 17,000 rpm (35,000 g) for 30 minutes to remove the pellet fraction. All proteins were purified first using Ni Sepharose™ 6Fast Flow (GE Healthcare,17-5318-03). Each recombinant protein was further purified by size-exclusion chromatography (Superdex 75 or 200 26/60 column; GE Healthcare), equilibrated with a buffer containing 20 mM Tris, 100 mM NaCl, and 1 mM DTT, pH 7.5.

For the crystallization chimera, Trx-Tollip (1-27)-3xSG-Tom1 (215-318) was expressed in BL21 (DE3) E. coli cells, and the N-terminal Trx tag was cleaved using 3C protease. The cleaved tag was then removed via a HisTrap Excel column (GE Healthcare, 17371206).

Analytical gel filtration chromatography

Purified proteins were loaded onto a Superose 200 Increase 10/300 GL (GE Healthcare, 28990944) or Superdex 75 10/300 GL column (GE Healthcare, 29148721), which was equilibrated with freshly prepared column buffer containing 20 mM Tris, 100 mM NaCl, and 1 mM DTT, pH 7.5. Analytical gel filtration chromatography was performed using an AKTA FPLC system (GE Healthcare). The results were then analyzed using Origin 8.5 software.

Multi-angle light scattering

For the multi-angle light scattering measurement, purified Tollip (1-27)-3xSG-Tom1 (215-308) chimera was injected into an AKTA FPLC system (GE Healthcare) equipped with Superdex 75 10/300 GL column. The column buffer contained 20 mM Tris-HCl, 100 mM NaCl and 1 mM DTT at pH 7.5. The system was connected to a miniDawn static light scattering detector and an Optilab refractive index detector (both from Wyatt Technology). Data were recorded at 0.5-second intervals with a 0.5 mL/min flow rate. The results were analyzed using ASTRA 6 software and plotted with Origin 8.5 software.

Isothermal titration calorimetry (ITC) assay

ITC measurements were conducted using a Microcal PEAQ-ITC calorimeter (Malvern) at 25°C. All protein samples were prepared in the same buffer. Concentrated 10 μM proteins were loaded into the cell, and 100 μM proteins were loaded into the syringe. Titration was performed by injecting the proteins from the syringe into the cell at 2-minute intervals, ensuring that the titration peaks returned to baseline between injections. The resulting data were analyzed using the Malvern MicroCal PEAQ-ITC analysis program and fitted using the one-site binding model.

NMR spectroscopy

The ^{15}N-labelled protein samples for NMR studies were concentrated to ~0.1 mM for titration experiments in a buffer containing 50 mM NaH_2PO_4/Na_2HPO_4 (pH=6.5), 50 mM NaCl, and 1 mM DTT. NMR spectra were acquired at 25 °C on an Agilent 800 MHz spectrometer equipped with an actively z gradient shielded triple resonance cryogenic probe.

Protein crystallization and structure determination

The crystals of Tollip (1-27)-3xSG-Tom1 (215-308) chimera were obtained using the sitting-drop vapor-diffusion method at 16 °C. The purified Tollip (1-27)-3SG-Tom1 (215-308) chimera (20 mg/mL) was mixed with equal volumes of reservoir solution containing “0.1 M BIS-TRIS pH 6.5; 28% w/v Polyethylene glycol monomethyl ether 2,000”. A 1.37 Å resolution X-ray data set for the Tollip (1-27)-3xSG-Tom1 (215-308) chimera was collected at the beamline BL19U1 of the Shanghai Synchrotron Radiation Facility [38]. The diffraction data were processed upon autoPROC [39]. The phase problem of the Tollip/Tom1 complex was solved by molecular replacement method using the apo-form Tom1 GAT structure (PDB ID: 2N9D) as the search model with PHASER [40]. The initial structural models were rebuilt manually using Coot [41] and then refined through Phenix[42] . Further manual model building and adjustments were completed via Coot [41]. The qualities of the final models were validated by MolProbity[43] . The final refinement statistics of solved structures in this study were listed in Table Supplementary1. All the structural diagrams were prepared using the program PyMOL (http://www.pymol.org/).

Generation of knock-out and relative stable cell line

The TOLLIP gene was knocked out in HeLa cells using the CRISPR/Cas9 system upon a sgRNA targeting the exon 2 of TOLLIP gene, 5’-CATCACGCCCACACAGCAGC-3’. The TOM1 gene was knocked out in HeLa cells using the CRISPR/Cas9 system upon a sgRNA targeting the exon 3 of TOM1 gene, 5’-GCAGTAAAGAAGAGAATCGT-3’. The guide sequence was designed using Benchling and cloned into the LentiCRISPR v2 vector. HEK293T cells were co-transfected with the constructed LentiCRISPR v2 vector, pMD2.G and psPAX2 using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, 11668019). HeLa cells were incubated with virus-containing medium Supplemented with 8μg/mL polybrene (Sigma-Aldrich) and filtered through a 0.45 μm-pore syringe filter. Following one day

incubation, transfected HeLa cells were treated with 1.5 μg/mL puromycin (InvivoGen, ant-pr-1) to select for positive clones. Monoclonal expansion was performed in a 96-well plate by serial dilution. Expanded single colonies were screened by western blot using a specific Tollip antibody (Proteintech, 68170-1-Ig, 1:1000) or Tom1(Santacruz, sc-514430), and positive clones were further confirmed by DNA sequencing. For the generation of relatively stable cell lines, AcGFP1-tagged wild-type or L22R mutant Tollip was cloned into the pMSCV-Blasticidin vector and co-transfected into Platinum-E cells with VSV.G and gag/pol using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, 11668019). Notably, the sgRNA-targeting region of TOLLIP in the pMSCV-Blasticidin vector was synonymously mutated to avoid being targeted again by the Cas9 enzyme. TOLLIP-knockout cells were similarly incubated with virus-containing medium and 8 μg/mL polybrene, followed by filtration through a 0.45 μm-pore syringe filter. Stable polyclonal cell lines were obtained after selection with 2μg/mL blasticidin (InvivoGen, ant-bl-05) for approximately two weeks.

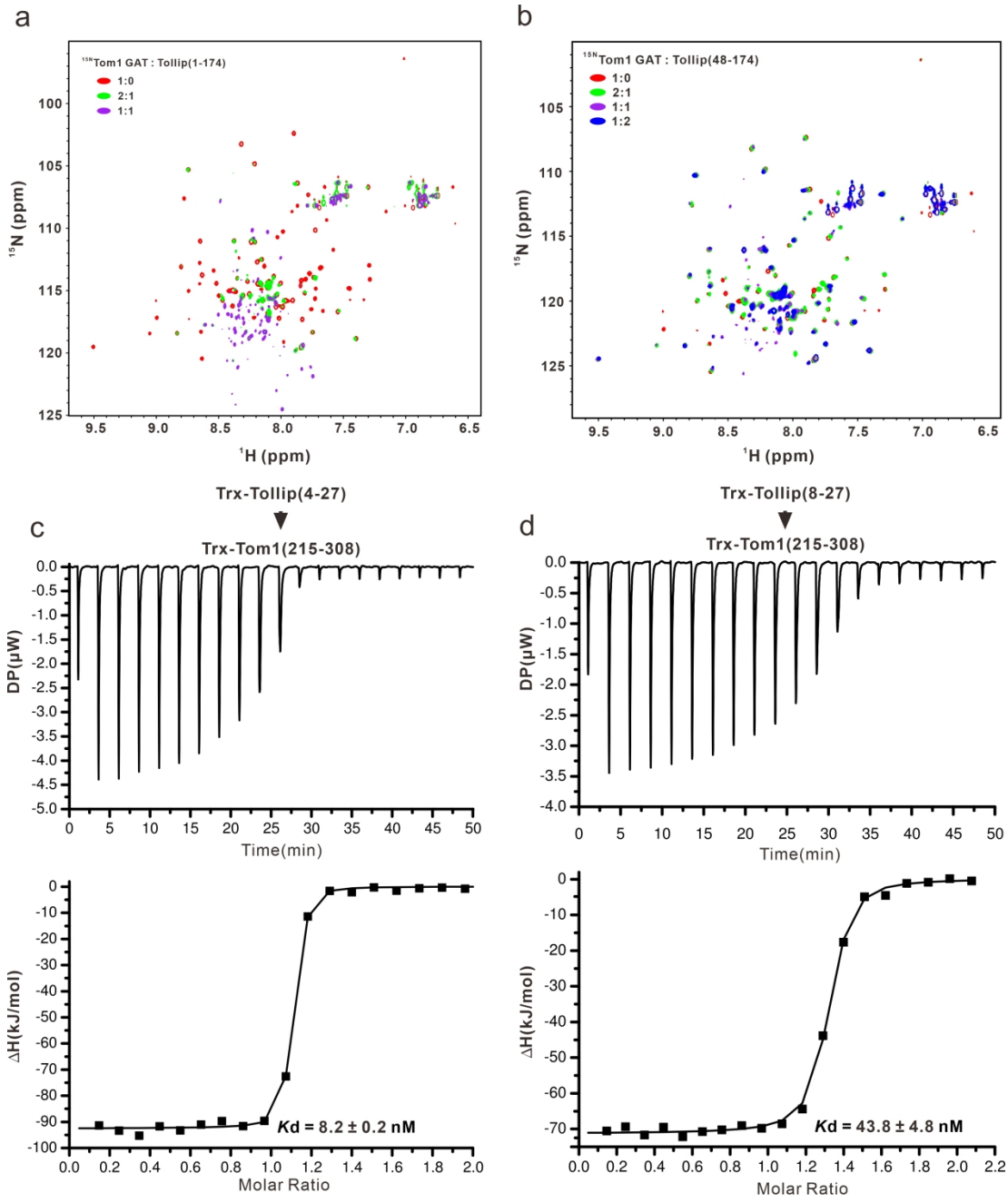
Mitochondria stress induction

Cells were separately seeded on 12-well plate. The following day, cells were changed to fresh medium or medium with different drugs and incubated for 2 hours. For HeLa cells, 20 μM Oligomycin A/5 μM Antimycin A, 20 μM Oligomycin A/5 μM Antimycin A plus 400 nM Bafilomycin A1 (Selleck, S1413), 10 μM CCCP (MCE, HY-100941), and 10 μM CCCP with 400 nM Bafilomycin A1. For western blotting experiment, cells were digested and re-suspended with the SDS-PAGE sample buffer and boiled for 10 minutes at 100 °C after stress treatment. The samples were detected by western blot using specific Tollip antibody (Proteintech, 68170-1-Ig, 1:1000), Tom20 antibody (Proteintech, catalog no. 81501-1-RR, 1:2000), β-actin antibody (Proteintech, catalog no. 66009-1-Ig, 1:5000) and HA antibody (MBL, catalog no. M180-3, 1:2000). The data are presented as mean±S.E.M. from 3 independent experiments. Statistical analyses were performed in GraphPad Prism 9 by two-way ANOVA followed by Bonferroni multiple comparison test and P value. For immunofluorescent experiment, cells were separately seeded on 12-well plate with Circular Cover Glasses (Fisherbrand, 12-541-005) at each well. And same drug action concentration as western blotting experiment.

Gene Ontology term and KEGG Pathway Enrichment analysis

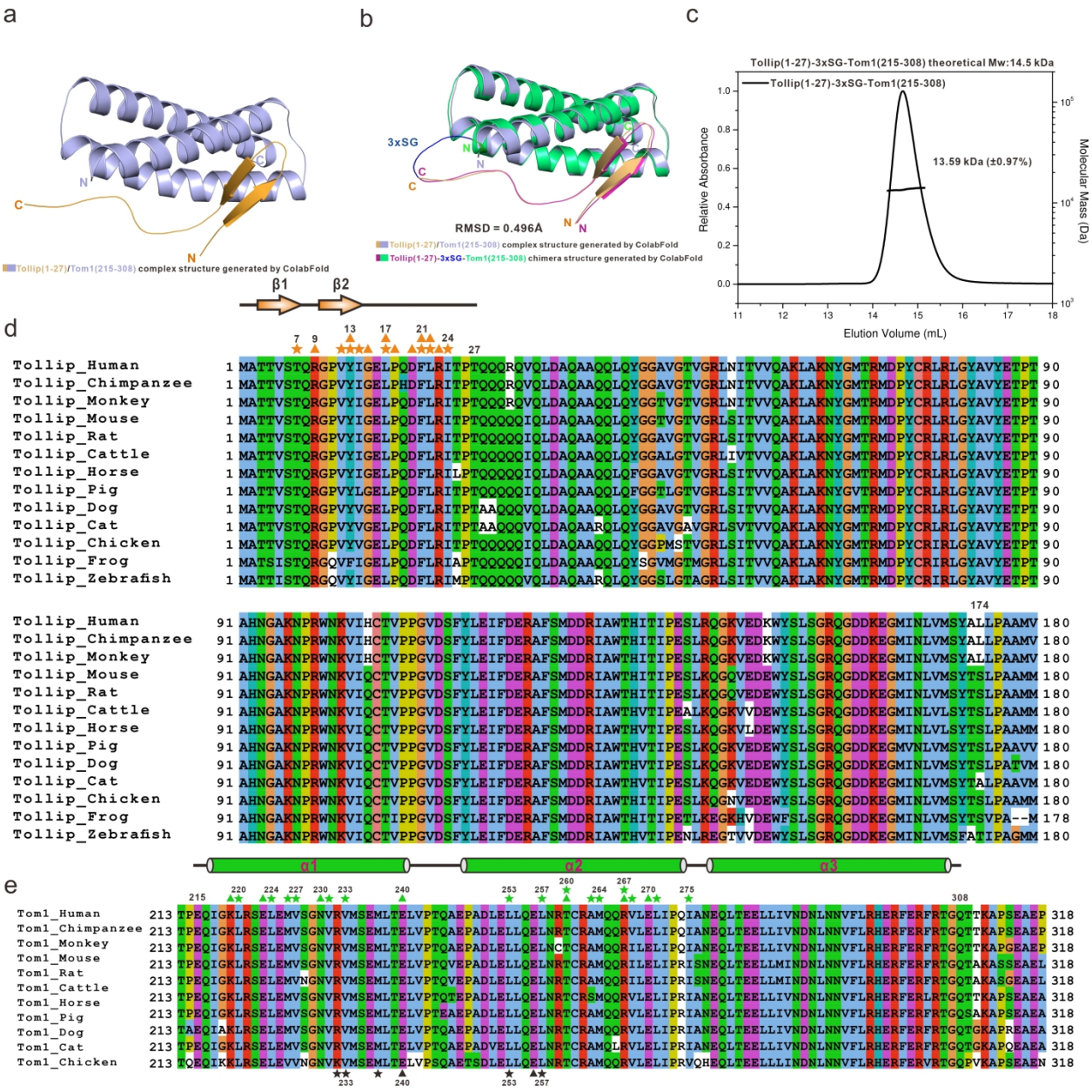
Heatmap was plotted by https://www.bioinformatics.com.cn (last accessed on 10 Dec 2024), an online platform for data analysis and visualization.

Figures



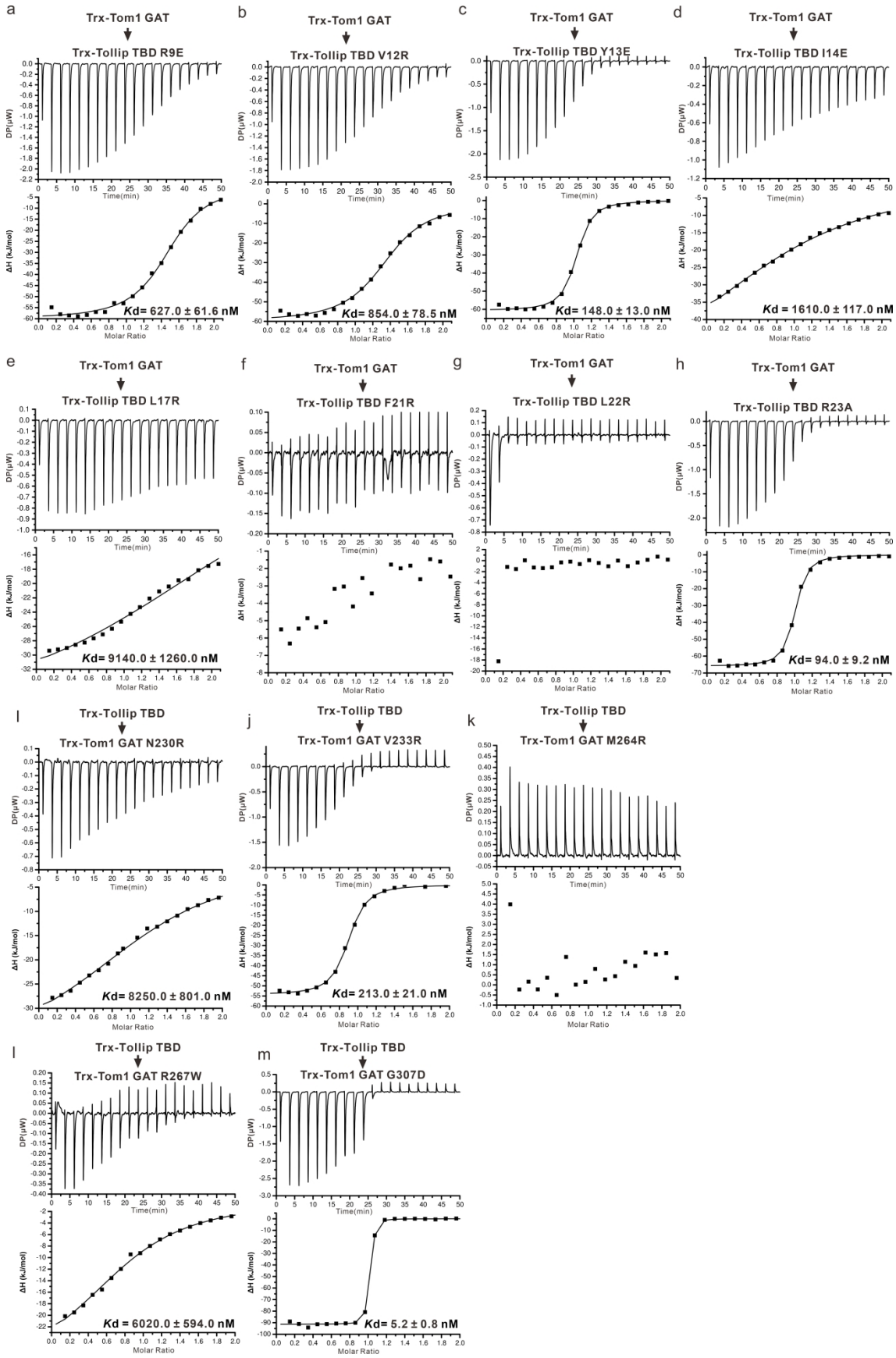
Supplementary Fig. 1| Nuclear Magnetic Resonance (NMR) spectroscopy and quantitative ITC-based mapping characterize the interaction between Tom1 and Tollip a Superposition plots of the 1H-15N HSQC spectra of Tom1 (215-308) titrated with the un-labeled TBD and C2 of Tollip proteins at different molar ratios. b Superposition plots of the 1H-15N HSQC spectra of Tom1 (215-308) titrated with the un-labeled C2 domain of Tollip proteins at different molar ratios. **c-d** ITC-based validations of the key Tom1 interface residues observed in the Tom1/GAT-Tollip/TBD complex structure. **c** Binding analysis of Tollip (4 - 27) with Tom1/GAT, **d** Binding analysis of Tollip (8 - 27) with Tom1/GAT. "DP" refers to the differential power recorded by the ITC while "ΔH" denotes the enthalpy change determined through ITC

measurements. The dissociation constants "*K*d" values are calculated from the data using a one-binding-site model, with the corresponding standard errors indicated.

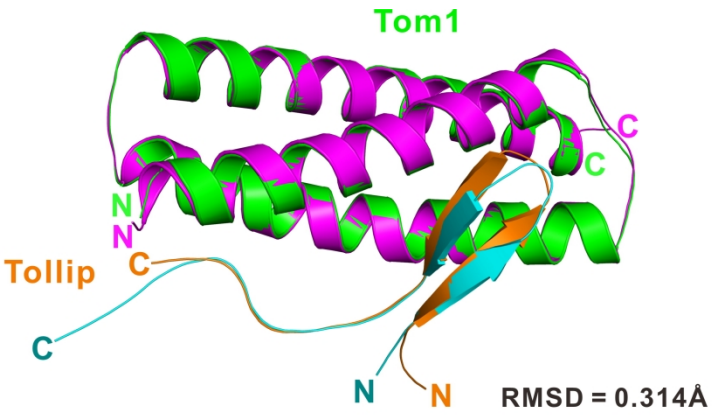


Supplementary Fig. 2| Structural and biochemical results of Tom1-Tollip complex. a apo-form Tollip and Tom1 protein predict complex. **b** Comparison of the structures of the apo-form Tollip/Tom1 complex and the chimera Tollip-3xSG-Tom1 protein. The root-mean-square deviation (RMSD) value between the two structures is 0.496. **c** Multi-angle light scattering (MALS) analysis of Tollip-3xSG-Tom1. The MALS analysis of purified chimera Tollip-3xSG-Tom1 protein shows the relative light scattering signal as a function of elution volume. The derived molecular mass of the Tollip-3xSG-Tom1 complex is indicated in black, with the molecular mass error (in brackets) obtained from data analysis software. **d-e** Structure-based sequence alignment analyses of the Tollip/TBD and Tom1/GAT across different species. d Conserved residues are highlighted in color using Jalview version 2.10.5 (http://www.jalview.org/). Conserved interface residues involved in interactions with Tollip are marked with orange stars (hydrophobic interactions) and triangles (polar interactions), respectively. **e** Conserved interface residues that participate in interactions with Tom1/GAT are marked with green stars (indicating hydrophobic interactions) and triangles (indicating polar interactions). Conserved interface residues that participate in

interactions with Ubiquitin are marked with black stars (indicating hydrophobic interactions) and triangles (indicating polar interactions).


a
Trx-Tom1 GAT
Trx-Tollip TBD R9E
Kd= 627.0 ± 61.6 nM
b
Trx-Tom1 GAT
Trx-Tollip TBD V12R
Kd= 854.0 ± 78.5 nM
c
Trx-Tom1 GAT
Trx-Tollip TBD Y13E
Kd= 148.0 ± 13.0 nM
d
Trx-Tom1 GAT
Trx-Tollip TBD I14E
Kd = 1610.0 ± 117.0 nM
e
Trx-Tom1 GAT
Trx-Tollip TBD L17R
Kd= 9140.0 ± 1260.0 nM
f
Trx-Tom1 GAT
Trx-Tollip TBD F21R
g
Trx-Tom1 GAT
Trx-Tollip TBD L22R
h
Trx-Tom1 GAT
Trx-Tollip TBD R23A
Kd= 94.0 ± 9.2 nM
i
Trx-Tollip TBD
Trx-Tom1 GAT N230R
Kd= 8250.0 ± 801.0 nM
j
Trx-Tollip TBD
Trx-Tom1 GAT V233R
Kd= 213.0 ± 21.0 nM
k
Trx-Tollip TBD
Trx-Tom1 GAT M264R
l
Trx-Tollip TBD
Trx-Tom1 GAT R267W
Kd = 6020.0 ± 594.0 nM
m
Trx-Tollip TBD
Trx-Tom1 GAT G307D
Kd= 5.2 ± 0.8 nM
DP(μW)
Time(min)
ΔH (kJ/mol)
Molar Ratio

Supplementary Fig. 3| ITC-based validations of key interface residues in the Tom1/GAT-Tollip/TBD complex structure. (a-h) ITC-based measurements of the binding affinities of Trx-Tom1 GAT with Trx-Tollip mutants. **a** Tollip R9E mutant, **b** Tollip V12R mutant, **c** Tollip L17R mutant, **d** Tollip Y13E mutant, **e** Tollip I14E mutant, **f** Tollip F21R mutant, **g** Tollip L22R mutant, **h** Tollip R23A mutant. i**-m** ITC-based measurements of the binding affinities of Trx-tagged Tollip TBD with Trx-tagged Tom1 mutants. **(l)** Tom1 N230R mutant **j** Tom1 V233Q mutant **k** Tom1 M264R mutant l Tom1 R267W mutant **m** The disease-associated Tom1 G307D mutant. "DP" refers to the differential power recorded by the ITC while "ΔH" denotes the enthalpy change determined through ITC measurements. The dissociation constants "*K*d" values are calculated from the data using a one-binding-site model, with the corresponding standard errors indicated.



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889 **Supplementary Fig. 4| Comparative structure of the Tom1-Tollip complex and Tom1 G307D-Tollip**
890 **complex.** Predict by AlphaFold3. The Tom1-Tollip complex (orange and green) and the predicted Tom1
891 G307D-Tollip complex (celadon and purple) structural differences are minimal.

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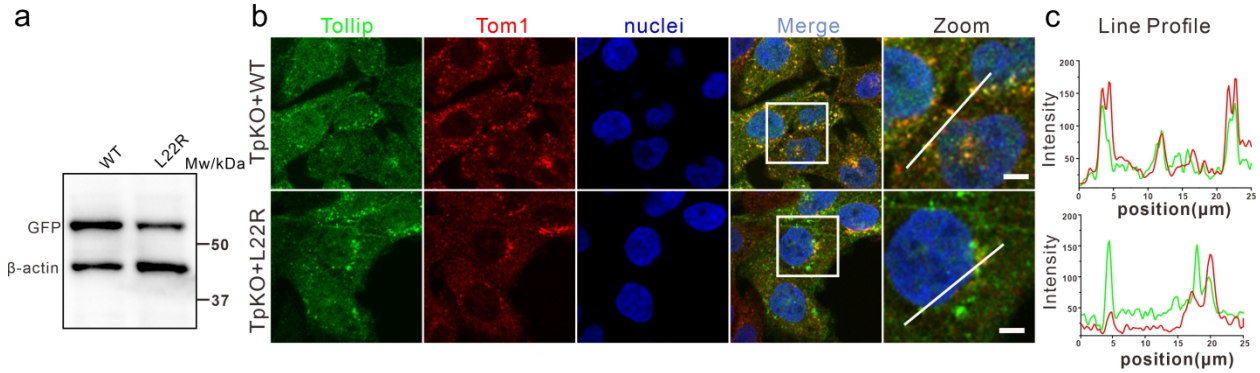

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894 **Supplementary Fig. 5| Co-localization of Tom1/Tollip in Tollip rescued cells. a** Immunoblotting
895 results of Tollip rescued cells. B Cell co-localization experiments and fluorescence intensity line
896 measurement in TpKO+WT or TpKO+L22R cells. **b** magnified view of the merged image is shown in the
897 zoomed-in panel. Red signals represent endogenous Tom1. Green signals represent Tollip. Scale bars,
898 5μm. **c** Fluorescence intensity profiles along lines in the zoomed region were measured to assess the
899 overlap between AcGFP-Tollip and endogenous Tom1.

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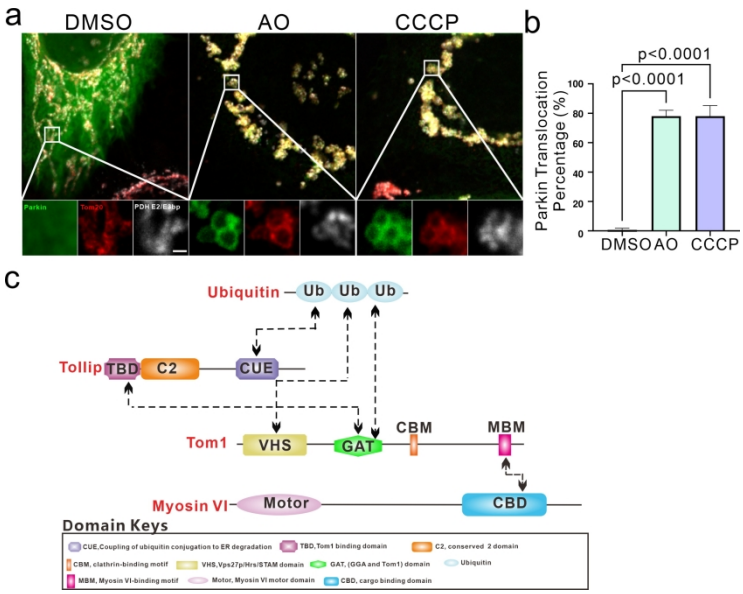
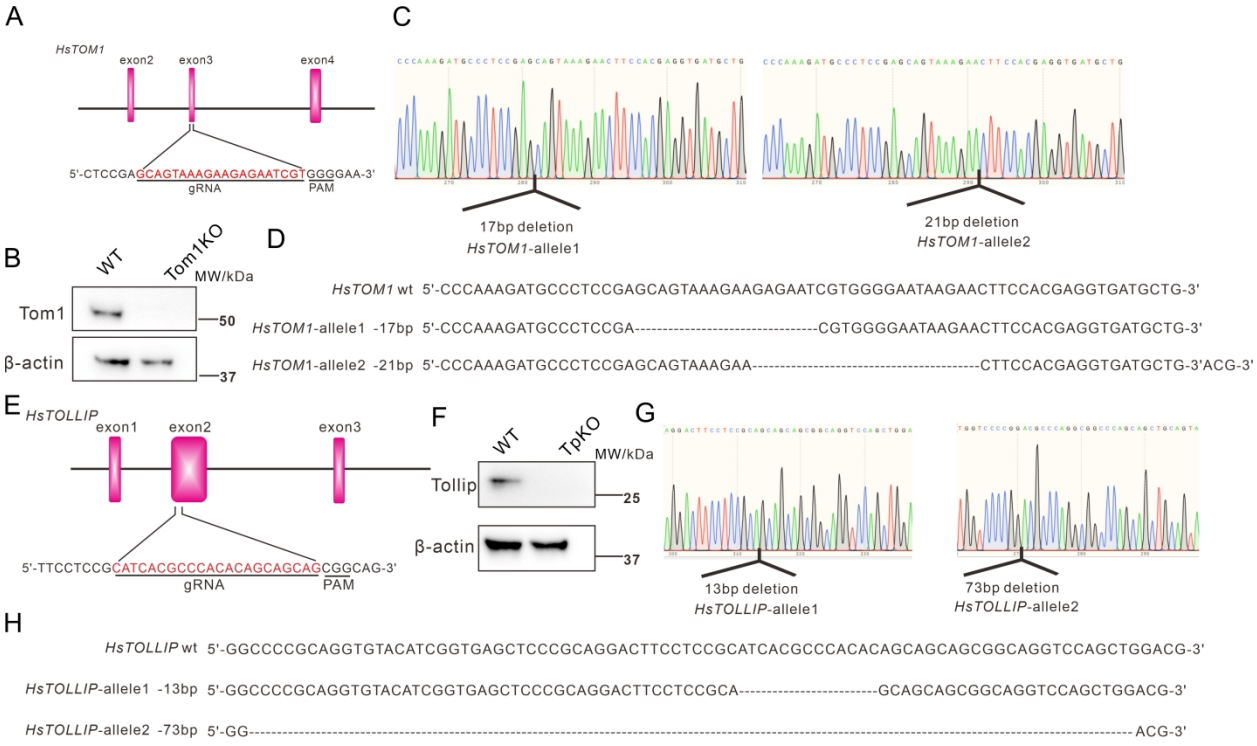
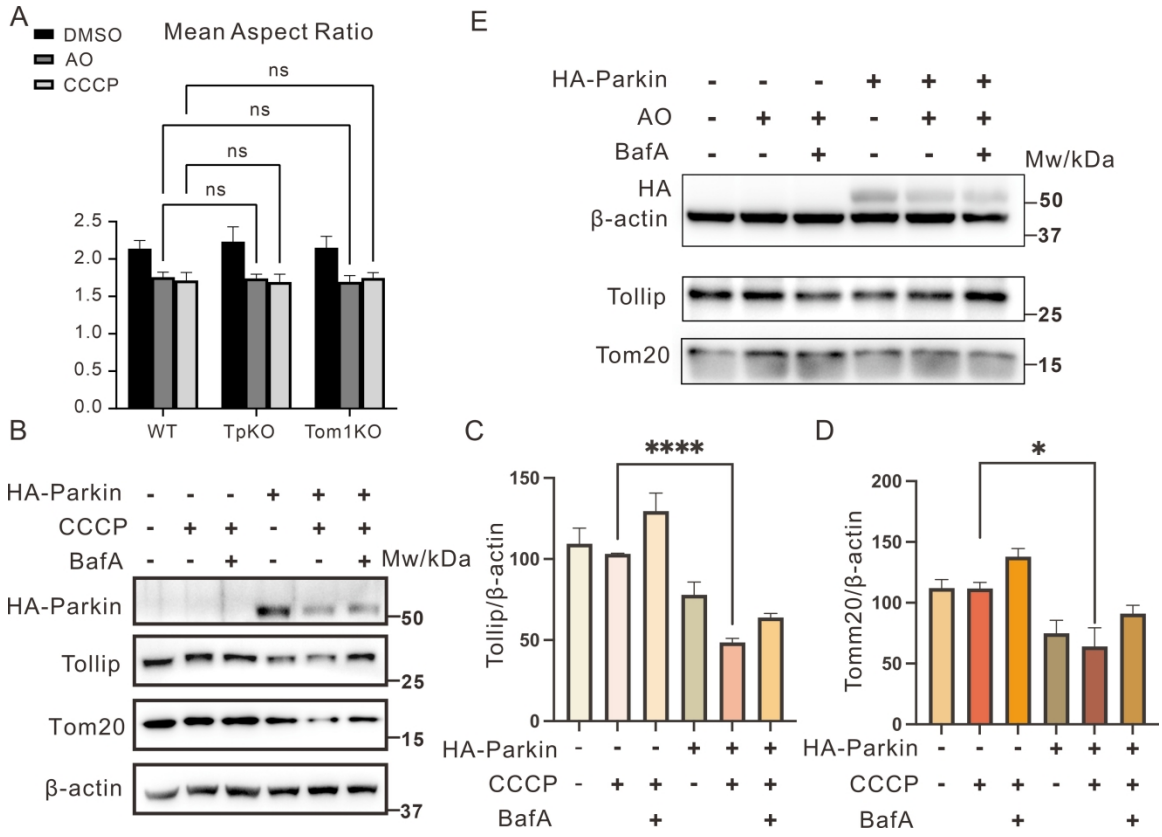


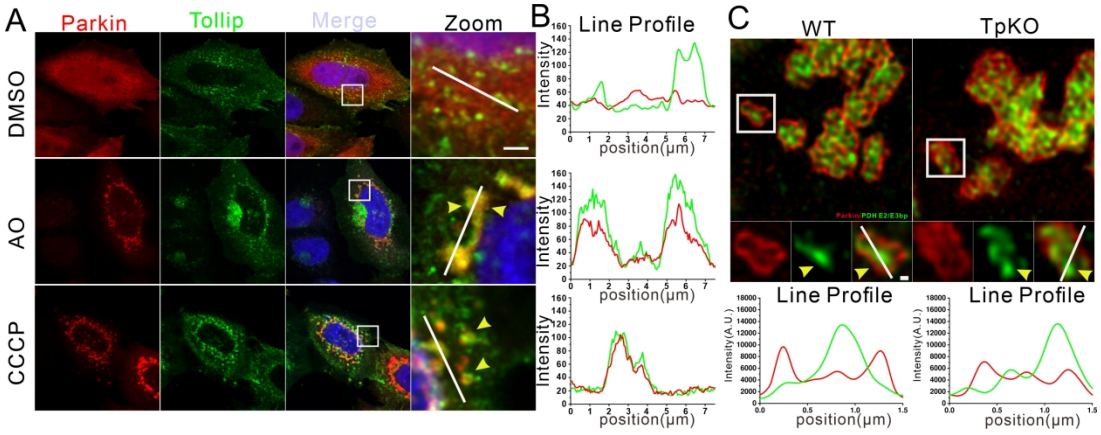
Supplementary Fig. 6| Parkin is recruited to dysfunctional mitochondria which contained both inner and outer membrane proteins. a Hela cells were transfected with HA-Parkin for 24hours, then treated with AO or CCCP for 2 hours. Scale bars:500nm. **b** Quantification of Parkin translocation Rate to damaged mitochondria after 2h of AO or CCCP treatment, as defined by complete colocalization of Parkin with the mitochondrial marker Tom20. (n=50). **c** Schematic diagram showing the interaction among Tom1, Tollip and MyosinVI.



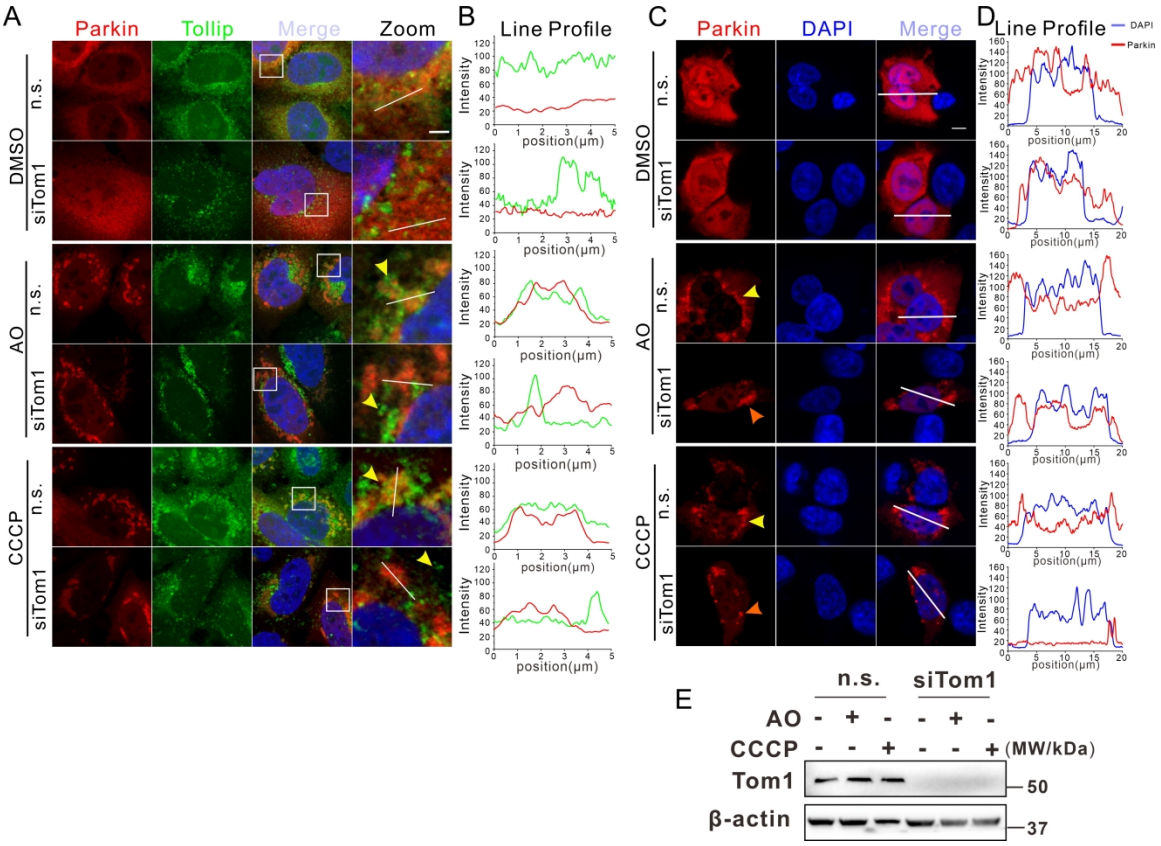
Supplementary Fig. 7| Construction of Tom1 and Tollip knockout KO HeLa cell line using the CRISPR/Cas9 system. a Schematic of Tom1 knockout strategy using the CRISPR/Cas9 system, with the gRNA targeted to exon 3 of the human TOM1 gene. B Immunoblot results of Tom1 protein in Tom1 KO cells. (**c-d**) DNA sequencing results showing the genomic sequences of TOM1 alleles in Tom1 knockout HeLa cells. **e** Schematic of Tollip knockout strategy using the CRISPR/Cas9 system, with the gRNA targeted to exon 2 of the human TOLLIP gene. **f** Immunoblot results of Tollip protein in Tollip knockout cells. (**g-h**) DNA sequencing results showing the genomic sequences of TOLLIP alleles in Tollip knockout HeLa cells.



Supplementary Fig. 8| Mitochondria Morphology and Immunoblot results in mitochondria stress conditions. a Wild type, TpKO and Tom1 KO HeLa was transfected in mCherry-Parkin, Parkin expression cells were selected and endogenous Tom20 was stained for mitochondria morphology measure. Image J Plugins Mitochondrial Analysis was used(n=30). **b** HeLa cells transfected in HA-Parkin in CCCP or not for 24 hours and treated in 10 μM CCCP or 10 μM CCCP plus 400 nM Bafilomycin A1, then cells were harvest for immunoblot. (**c-d**) The levels of Tollip and β-actin in panel A were also quantified in ImageJ and normalized to the HeLa cells in normal condition. And the levels of Tom20 and β-actin in panel A. Data are both presented as mean±s.e.m. from 3 independent experiments. Statistical analyses were both performed in GraphPad Prism 9 by two-way ANOVA. (n=3, **P<0.01, *P<0.0410). **e** HeLa cells were transfected in HA-Parkin or not for 24 hours and treated in AO or AO plus Bafilomycin A1, then cells were harvest for immunoblot.



Supplementary Fig. 9| Tollip was recruited to Parkin under mitochondrial stress conditions. a AcGFP-Tollip signals show co or around with Parkin in HeLa cells transfected with mCherry-Parkin, under 2 hours treatment of AO or CCCP. **b** Fluorescence intensity line measurement of AcGFP-Tollip and mCherry-Parkin under 2 hours treatment of AO or CCCP. **c** Immunofluorescence fluorescence intensity line measurement shown mitochondria inner proteins leakage in Tp-KO cells. Scale bar:200nm.



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940 **Supplementary Fig. 10| Tollip signals and Parkin translocation in mitochondria stress conditions.**
941 **a** TpKO+WT cells were transfected with Tom1 or scramble siRNAs for 12h, then follow transfecting with
942 mCherry-Parkin for 24hs, after that cells were treated AO or CCCP for 2hours and IF experiment was
943 done as Method described. **b** Fluorescence intensity Tollip and mCherry-Parkin was measured. **c**
944 HCT116 cells was transfected with Tom1 or scramble siRNAs for 12h, then follow transfecting with
945 mCherry-Parkin for 24 hours. After that cells were treated AO or CCCP for 2 hours and IF experiment was
946 done as Method described. mCherry signal was detected for Parkin. **d** Fluorescence intensity of Parkin
947 and DAPI was measured for normal translocation. e Immunoblot results of Tom1 in HCT116 cells from
948 Fig Supplementary7C.

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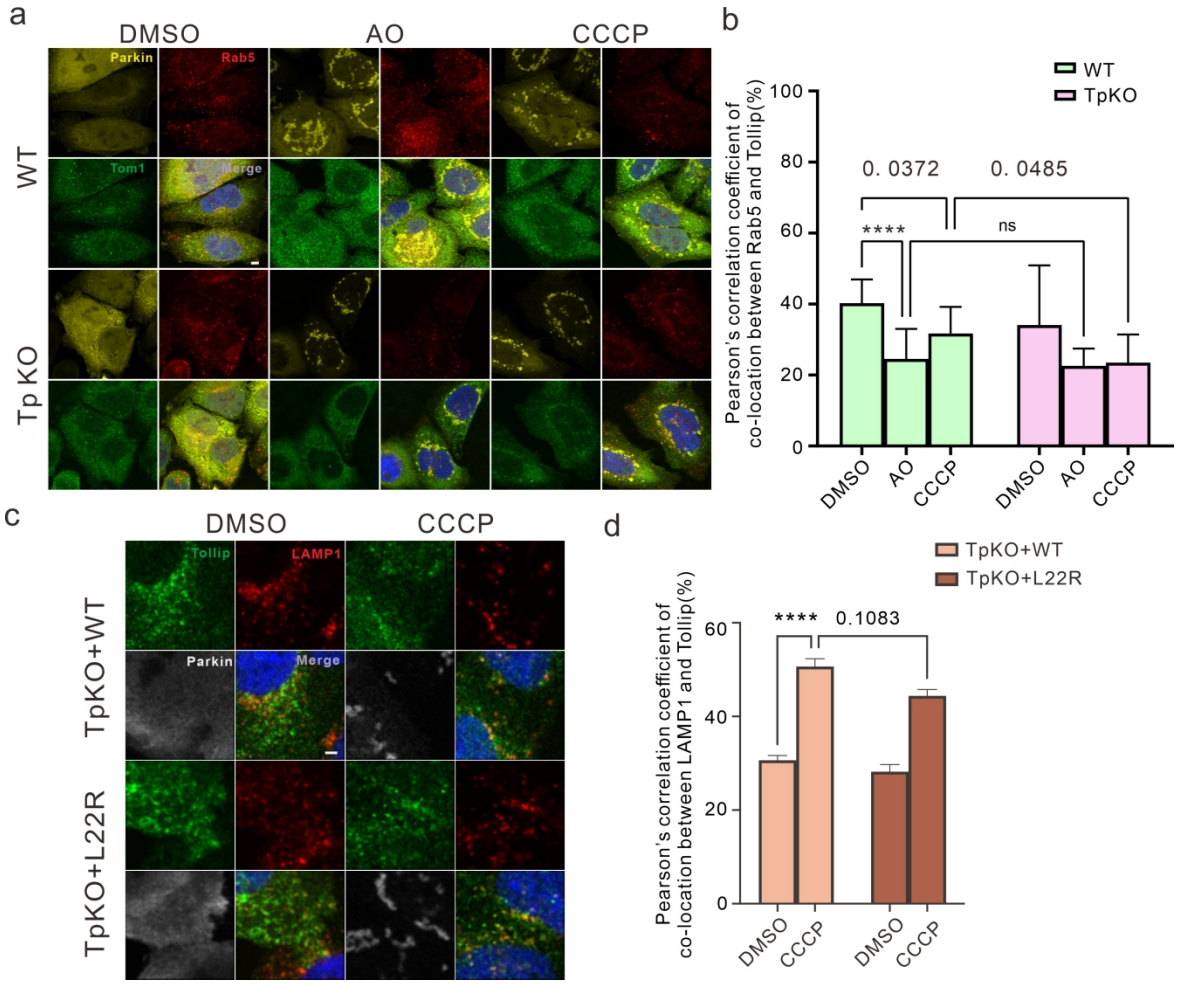
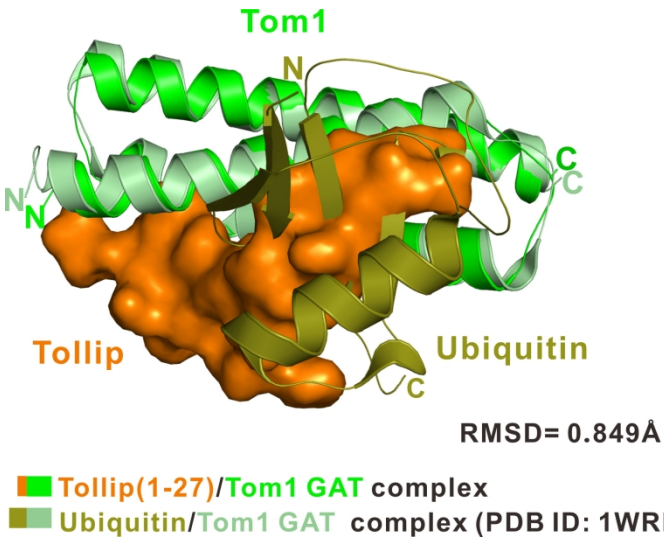
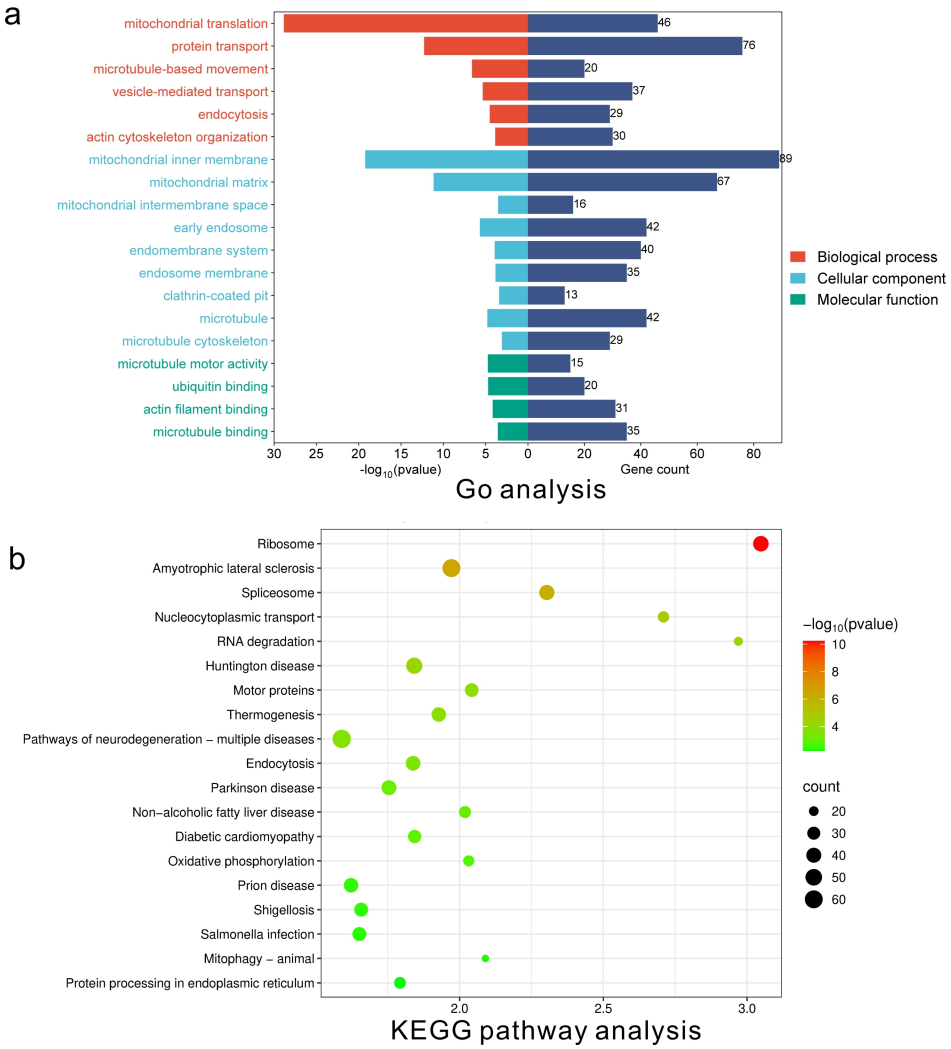


Supplementary Fig. 11| Tom1 and Rab5 colonization rate in wild or TpKO cells and Tom1, Tollip and Myosin VI form ternary complex. a mCherry-Parkin was transfected into TpKO cells. After 24 hours, the cells were treated with drugs as described in the methods. Rab5 and Tom1 was visualized by immunofluorescence staining of Rab5 and Tom1 antibody. Scale bars: 5μm. **b** Quantification of Pearson's correlation coefficient between Rab5 and Tom1. (n = 10 cells. ****p<0.0001, *p<0.05). **c** LAMP1 and Parkin was visualized by immunofluorescence staining of LAMP1 antibody and HA antibody. Scale bars: 2μm. **d** Quantification of Pearson's correlation coefficient between LAMP1 and Tollip. (n = 20 cells. ****p<0.0001). Statistical analyses were both performed in GraphPad Prism 9 using two-way ANOVA followed by Bonferroni multiple comparison test.



Supplementary Fig. 12| Comparative structure of the Tom1-Ub complex and Tom1-Tollip complex. The Tom1-Tollip complex (green) and the solved human Tom1-Ub complex (grass) are compared to highlight structural differences. In the Tom1-Tollip complex, Tom1 is colored green, while in the Tom1-Ub complex, Tom1 is depicted in grass (PDB: 1WRD). The interaction of Tom1 with ubiquitin (Ub) in the Tom1-Ub complex and with the Tollip protein in the Tom1-Tollip complex are contrasted to show distinct binding modes and structural adaptations.



Supplementary Fig. 13| GO function histogram analysis and dot plot of the KEGG pathway enrichment analysis of Tom1. Flag-Tom1 or Flag vector expressed in HEK293T cells for 24 hours, IP of Flag beads, then IP proteins was washed with Flag peptide and for LC-MS experiment. a Biological process, cellular component and molecular function analysis was done for de novo proteins. BP is marked by dark cyan; CC is marked by steel blue and MF is marked by grass green. B KEGG analysis shows Tom1 associated with mitochondrial dysfunction diseases. The horizontal axis represents the gene ratio, while the vertical axis represents the enriched pathway name. The color scale indicates different thresholds of the p-value, and the size of the dot indicates the number of genes corresponding to each pathway.

Supporting Video Legends

Supplementary Video. 1| Mitochondrial stress induces dynamic Tollip redistribution in live cells.Time-lapse imaging of wild-type HeLa cells expressing GFP-Tollip (green). Cells were transfected with AcGFP-Tollip for 24hours and treated with either a a combination of 20 μM oligomycin A and 5 μM antimycin A, or B 10 μM CCCP. Images were acquired for 90 min post-treatment. The video demonstrates the active, time-dependent movement and mitochondrial recruitment of cytoplasmic Tollip pools upon induction of chemical stress. Scale bar: 10 μm.

Supplementary Video. 2| Tollip mitochondrial trafficking is impaired in Tom1KO cells under mitochondria stress. Time-lapse imaging of Tom1-knockout (Tom1KO) HeLa cells expressing AcGFP-Tollip (green). Cells were transfected with AcGFP-Tollip for 24hours and then treated with either **a** 20 μM oligomycin A plus 5 μM antimycin A, or **b** 10 μM CCCP, with imaging performed over 90 min. Compared to wild-type cells (Video Supplementary1), Tollip displays markedly restricted movement and reduced mitochondrial recruitment following induction of mitochondrial stress, indicating a requirement for Tom1 in stress-induced Tollip redistribution. Scale bar: 10 μm.

Supplementary Video. 3| Mitochondrial stress induces Parkin perinuclear translocation in live cells.Time-lapse imaging of wild-type HeLa cells expressing mCherry-Parkin(red). Cells were transfected with mCherry-Parkin for 24hours and then treated with either **a** a combination of 20 μM oligomycin A and 5 μM antimycin A, or **b** 10 μM CCCP. Images were acquired for 90 min post-treatment. Scale bar: 10 μm

Supplementary Video. 4| Parkin perinuclear translocation is impaired in Tom1KO cells under mitochondrial stress. Time-lapse imaging of Tom1-knockout (Tom1KO) HeLa cells expressing mCherry-Parkin (red). Cells were transfected with mCherry-Parkin for 24hours and then treated with either a 20 μM oligomycin A plus 5 μM antimycin A, or B 10 μM CCCP. Images were acquired for 90 min post-treatment. Compared with the robust perinuclear accumulation observed in wild-type cells (Video Supplementary3), Parkin translocation is significantly reduced in Tom1KO cells following mitochondrial depolarization, indicating Tom1 is required for efficient stress-induced Parkin recruitment. Scale bar: 10 μm.

Supplementary Video. 5| Depletion of Tom1 impairs mitochondrial trafficking of Tollip. Time-lapse imaging of Tom1-TetON HeLa cells cultured in the absence of doxycycline [Tom1-TetON (-)] to suppress Tom1 expression, AcGFP-Tollip (green) were transefected for 24hours. Cells were treated with 10 μM CCCP, and images were acquired for 90 min post-treatment. Tollip exhibits markedly restricted movement and reduced mitochondrial recruitment following mitochondrial depolarization, consistent with a critical role for Tom1 in stress-induced Tollip redistribution. Scale bar: 10 μm.

Supplementary Video. 6| Restoration of Tom1 expression rescues stress-induced Tollip mitochondrial trafficking. Time-lapse imaging of Tom1-TetON HeLa cells cultured with doxycycline for 12 h to induce Tom1 expression, and expressing AcGFP-Tollip (green). Cells were treated with 10 μM CCCP, and images were acquired for 90 min post-treatment. Re-expression of Tom1 restores the active, time-dependent movement and mitochondrial recruitment of Tollip following mitochondrial depolarization. Scale bar: 10 μm.

Supplementary Table. 1| Statistics of X-ray crystallographic data collection and model refinements

Data set	Tollip(1-27)/Tom1(215-308) complex
Data collection	
Wavelength (Å)	0.97853
Space group	$P4_3$
Unit cell parameters	
a, b, c (Å)	43.27, 43.27, 59.48
α, β, γ(°)	90.00, 90.00, 90.00
Resolution range (Å)	43.27-1.35 (1.37-1.35)
Number of total reflections	139537 (5170)
Number of unique reflections	24279 (1201)
R_{pim} (%)	1.1 (30.9)
I/σI	20.1 (2.4)
Completeness (%)	99.9 (99.9)
Redundancy	5.7 (4.3)
Structure refinement	
Resolution (Å)	24.51-1.35 (1.41-1.35)
R_{work}/R_{free} (%)[a]	17.39 (23.14) / 18.69 (28.96)
Number of reflections	
working set	23089 (2861)
test set	1186 (139)
average *B* factor (Å^2)	29.92
RMSD bonds (Å)	0.015
RMSD angles (°)	1.37
Number of non-hydrogen atoms	
protein	961
ligand	12
water	67
Ramachandran plot (%)	
favored region	100.00
allowed region	0.00

outliers	0.00

[a] $R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are observed and calculated structure factors. $R_{free} = \Sigma_T||F_{obs}| - |F_{calc}||/\Sigma_T|F_{obs}|$, where T is a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.

Numbers in parentheses represent the value for the highest resolution shell.

Supplementary Table. 2| PCR primers and sgRNAs used in this study

Primers for Tom1	**Sequence (5' - 3')**
Tom1-W173A_F	AGCCAGAGACGCTATCGGAAGCGAACCCACCA
Tom1-W173A_R	CGGTTCCGATAGCGTCTCTGGCTGGATCCGGG
Tom1_N230R_F	CGCGTGAGGGTGATGTCGGAGATGCTG
Tom1_N230R_R	CCCACTCACCATCTCCAGCTCACTGCG
Tom1_V233R_F	CGCATGTCGGAGATGCTGACG
Tom1_V233R_R	CCTCACGTTCCCACTCAC
Tom1_T260R_F	CGCTGCCGAGCCATGCAGCAGCGG
Tom1_T260R_R	GCGGTTGAGCTCCTGCAGCAGCTCCAG
Tom1_M264R_F	AGACAGCAGCGGGTCCTGGAGCTCATC
Tom1_M264R_R	GGCTCGGCACGTGCGGTTGAGCTCCTG
Tom1_I275R_F	CGGGCCAATGAGCAGCTGACAGAGGAG
Tom1_I275R_R	CTGAGGGATGAGCTCCAGGACCCGCTG
Tom1_N230R-R267W_F	GAGATGGTGAGTGGGCGCGTGAGGGTGATGTCGGAGA TGCTG
Tom1_N230R-R267W_R	AGGGATGAGCTCCAGGACCCACTGCTGCATGGCTCGGC A
Tom1_G307D_F	GACCAGTGAGAATTCGAGCTCCGT
Tom1_G307D_R	TGTTCGGAACCGTTCAAACCGTTC
Primers for Tollip	**Sequence (5' - 3')**
Tollip 1-27_HR_F	GAGTGCGGCCGCAAGCTTGTCGACTTAGGGCGTGATGC GGAGGAA
Tollip 1-27_HR_R	AGTGCGGCCGCAAGCTTGTCGACTTATGTGGGCGTGAT GCGGAGGAA
Tollip 4-27_HR_F	TTCTGTTCCAGGGGCCCGGATCCACCGTCAGCACTCAG
Tollip 4-27_HR_R	TTCTGTTCCAGGGGCCCGGATCCACCGTCAGCACTCAG
Tollip 8-27_HR_F	TTCTGTTCCAGGGGCCCGGATCCCAGCGCGGGCCGGT GTAC
Tollip 8-27_HR_R	TTCTGTTCCAGGGGCCCGGATCCCAGCGCGGGCCGGT GTAC

Tollip_R9E_F	GAAGGGCCGGTGTACATCGGT
Tollip_R9E_R	CTGAGTGCTGACGGTGGT
Tollip_V12R_F	CGCTACATCGGTGAGCTCCCGCAG
Tollip_V12R_R	CGGCCCGCGCTGAGTG
Tollip_Y13R_F	CGCATCGGTGAGCTCCCGCAG
Tollip_Y13R_R	CACCGGCCCGCGCTGA
Tollip_I14E_F	GAAGGTGAGCTCCCGCAGGAC
Tollip_I14E_R	GTACACCGGCCCGCGCTG
Tollip_L17R_F	CGCCCGCAGGACTTCCTCCGC
Tollip_L17R_R	CTCACCGATGTACACCGG
Tollip_F21R_F	CGCCTCCGCATCACGCCCACA
Tollip_F21R_R	GTCCTGCGGGAGCTCAC
Tollip_L22R_F	CGCCGCATCACGCCCACATAA
Tollip_L22R_R	GAAGTCCTGCGGGAGCTC
Tollip_R23A_F	GCCATCACGCCCACATAAGTC
Tollip_R23A_R	GAGGAAGTCCTGCGGGAG
Resuce_Tollip_L22R_F	CGCCGCATCACGCCCACACA
Rescue_Tollip_L22R_R	GTTGGGTAGGGGTAATGCGCCTGAAGTCCTGCGGGAGCTC
Rescue_Tollip_WT_F	GCATTACCCCTACCCAACAACAGCGGCAGGTCCAGCTG
Rescue_Tollip_WT_R	GTTGGGTAGGGGTAATGCGCAGGAAGTCCTGCGGGAGC
sgRNAs	**sgRNA Sequence (5' - 3')**
Tollip_sgRNA	CACCGGCTGCTGTGTGGGCGTGATG
Tom1_sgRNA	CGCAGTAAAGAAGAGAATCGTTGATG

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Supplementary Table.3| Sequences of siRNAs used in this study

Target Gene	siRNA Name	Sense Strand (5' - 3')	Antisense Strand (5' - 3')
TOM1	siTom1	CCCAGGAGAAAGAUGAUGACAdTdT	UGUCAUCAUCUUUCUCCUGGGdTdT
TOLLIP	siTollip	GCGCCUUCCGAUUCCAUUCAUdTdT	AUGAAUGGAAUCGGAAGGCGCdTdT
Negative Control	siNC	UUCUCCGAACGUGUCACGUdTdT	ACGUGACACGUUCGGAGAAdTdT