## Supplementary materials and methods

### Reagents and antibodies

The following primary antibodies were used in this study: anti-FUNDC1 polyclonal antibody (AVIVA, ARP53280_P050), anti-beta-Actin antibody (Santa Cruz, sc-47778), anti-beta Tubulin monoclonal antibody (Earthox, E021040), anti-ULK1 Antibody (H-240) (Santa Cruz, sc-33182), anti-Atg1/ULK1 antibody (Sigma, A7481), anti-ULK1 (D8H5) Rabbit mAb (Cell Signaling TECHNOLOGY, #8054), anti-LC3B polyclonal antibody (Sigma, L7543) , anti-LC3 polyclonal antibody (MBL, PM036), anti-TIM23 (BD Biosciences, 611222), anti-TOM20 (FL-145) (Santa Cruz, sc-11415), anti-GFP Rabbit Serum (Polyclonal)(Life Technologies, A6455), anti-GFP monoclonal antibody (Santa Cruz, sc-9996), anti-VDAC1 monoclonal antibody (Abcam, ab14734), anti-FLAG M2 monoclonal antibody (Sigma, F1804), anti-FLAG (Sigma, F7425), anti-c-Myc (9E10) (Santa Cruz, sc-40), anti-c-Myc monoclonal antibody (Sigma, C3956), anti-HA.11 Clone 16B12 monoclonal antibody (Covance, MMS-101R). The anti-p-FUNDC1 (Ser-17) polyclonal antibody was generated by immunizing rabbits with purified FUNDC1 phosphopeptides and affinity purified (Epitomics). Secondary antibodies used for western blotting were: HRP affinipure goat anti-mouse IgG (Earthox, E030110), HRP affinipure goat anti-rabbit IgG (Earthox, E030120) and HRP-conjugated goat anti-rabbit IgG Fc (SouthernBiotech, 4041-05). The following fluorescent secondary antibodies were used: Alexa fluor 555-labeled Donkey anti–Mouse IgG antibody (Life Technologies, A31570), Alexa fluor 555-labeled Donkey anti–Rabbit IgG antibody (Life Technologies, A31572), Alexa fluor 488-labeled Donkey anti–Mouse IgG antibody (Life Technologies, A21202), and Alexa fluor 488-labeled Donkey anti–Rabbit IgG antibody (Life Technologies, A21206). Citrate Synthase Activity Colorimetric Assay Kit (BioVision, K318-100). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (C2759), Tetracycline (Tet) (Sigma, T3258) and Bafilomycin A1 (B1793) were purchased from Sigma and used at a final concentration of 20 μM, 10 ng/ml and 50 nM, respectively. Protein A/G plus-agarose immunoprecipitation reagent (Santa Cruz, sc-2003), EBSS (GIBCO, 14155063), and Lipofectamine 2000 (Invitrogen, 11668027) were also used according to the manufacturer's protocol.

### Plasmids

p3×FLAG-CMV14-mULK1 (Deposited by Noboru Mizushima), pcdna6.2-mycULK1wt

(Deposited by Reuben Shaw), pME18S-3HA-mULK1(K46N) (Deposited by Masaaki Muramatsu) and HA-hULK1 (Deposited by Do-Hyung Kim) were obtained from Addgene. The FUNDC1-Myc, FLAG-FUNDC1 and GST-FUNDC1 constructs were described previously. FUNDC1 mutants (FUNDC1S17A-Myc, FUNDC1S17D-Myc and GST-FUNDC1S17A), based on the FUNDC1-Myc and GST-FUNDC1 constructs, were generated using the following primers:

S17D-F: 5'-gaaagtgatgacgacgattatgaagtgttggat-3';

S17D-R: 5'-atccaacacttcataatcgtcgtcatcactttc-3';

S17A-F: 5'-gaaagtgatgacgacgcttatgaagtgttggat-3';

S17A-R: 5'-atccaacacttcataagcgtcgtcatcactttc-3';

The truncated mutants(△50, 1-139, 1-96, and 96-155) were described previously. The construction of 50-138 and 69-138 were based on 1-139, and 50-71/97-155 was based on △50. The construction of the deleted mutants (69-128, 69-118, and 69-108) was based on 69-138. The construction of the point mutants (N118A, R116A, K115A, K114A, I113A, Q112A, R111A, K110A) were based on 69-118. N118A and K110A were also mutated on Full-length (1-155).

The primers:

50-138-F: 5'-gtagctacccagattgtaatgggt-3';

50-138-R: 5'-aatctgggtagctaccgccatggtaccaagcttaagt-3';

50-71/97-155-F: 5'-attgactggaagagagttgaaaa-3';

50-71/97-155-R:tctcttccagtcaataactttctggaacagaaatcctg-3';

69-138-F: 5'-cagaaagttggaaaacttgcagca-3';

69-138-R: 5'-ttttccaactttctgcgccatggtaccaagcttaagt-3';

69-128-F: 5'-ggctcgagtctagagggcccgc-3';

69-128-R: 5'-ctctagactcgagccaattaaattgttgatttcaggtgctgct-3';

69-118-F: 5'-ggctcgagtctagagggcccgc-3';

69-118-R: 5'-ctctagactcgagccgttcgctcgtttcttaatctgtcttt-3';

69-108-F: 5'-ggctcgagtctagagggcccgc-3';

69-108-R: 5'-ctctagactcgagcctttatttacatctttttcaactctcttccagtc-3';

69-118-N118A-F: 5'-attaagaaacgagcggccggctcgagtctagag-3';

69-118-N118A-R: 5'-ctctagactcgagccggccgctcgtttcttaat-3';

69-118-R116A-F: 5'-agacagattaagaaagcagcgaacggctcgagt-3';

69-118-R116A-R: 5’-actcgagccgttcgctgctttcttaatctgtct-3’;

69-118-K115A-F: 5’-aaaagacagattaaggcacgagcgaacggctcg-3’;

69-118-K115A-R: 5’-cgagccgttcgctcgtgccttaatctgtctttt-3’;

69-118-K114A-F: 5’-gcaaaaagacagattgcgaaacgagcgaacggc-3’;

69-118-K114A-R: 5’-gccgttcgctcgtttcgcaatctgtcttttgc-3’;

69-118-I113A-F: 5’-aaagcaaaaagacaggctaagaaacgagcgaa-3’;

69-118-I113A-R: 5’-ttcgctcgtttcttagcctgtcttttgcttt-3’;

69-118-Q112A-F: 5’-aataaagcaaaaagagcgattaagaaacgagc-3’;

69-118-Q112A-R: 5’-gctcgtttcttaatcgctcttttgctttatt-3’;

69-118-R111A-F: 5’-gtaaataaagcaaaagcacagattaagaaacg-3’;

69-118-R111A-R: 5’-cgtttcttaatctgtgcttttgctttatttac-3’;

69-118-K110A-F: 5’-gatgtaaataaagcagcaagacagattaagaa-3’;

69-118-K110A-R: 5’-ttcttaatctgtcttgctgctttatttacatc-3’;

FL-N118A-F: 5’-attaagaaacgagcggccaaagcagcacctgaa-3’;

FL-N118A-R: 5’-ttcaggtgctgctttggccgctcgtttcttaat-3’;

FL-K110A-F: 5’-gatgtaaataaagcagcaagacagattaagaa-3’;

FL-K110A-R: 5’-ttcttaatctgtcttgctgctttatttacatc-3’;

**Cell culture and transfection**

HeLa, *FUNDC1*-inducible HeLa cells, ULK1 (+/+) MEFs and ULK1 (-/-) MEFs (Deposited by Dr. Sharon Tooze) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin at 37 °C under 5% $CO_2$. Hypoxic conditions were achieved with a hypoxic chamber (Billups–Rothenberg) flushed with a pre-analyzed gas mixture of 1% $O_2$, 5% $CO_2$, and 94% $N_2$. The FUNDC1 target sequence for RNA interference was: FUNDC1-mus-160: sense 5’-GACGAAUCAUACGAAGUGUTT-3’; antisense: 5’-ACACUUCGUAUGAUUCGUCTT-3’; FUNDC1-mus-478: sense 5’-GCACCUGAAAUCAACAAUATT-3’; antisense 5’-UAUUGUUGAUUUCAGGUGCTT-3’. The scramble RNA interference sequence was: sense 5’-UUCUCCGAACGUGUCACGUTT-3’; antisense 5’-ACGUGACACGUUCGGAGAATT-3’. RNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

**Mitochondria quantification**

Randomly selected sections from different cell samples with a visible nucleus were analyzed. Mitochondria profiles were manually traced by a blinded investigator using ImageJ software. All cells were checked three times for consistency. Mitochondria cross-sectional area and mitochondria aspect ratio (AR, major axis divided by minor axis, minimum value is 1.0) were calculated. AR was utilized as a shape descriptor. Probability plots were utilized to estimate changes in mitochondria size and shape and statistical differences were tested using Kolmogorov-Smirnov test. Mitochondria coverage = (the total area of mitochondria / the total cellular areas)%.

**SDS-PAGE and western blotting**

Cells were lysed in lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1.5 mM $Na_3VO_4$, 50 mM NaF, 10 mM β-glycerophosphate, 1% triton, phosphatase inhibitor) with 1 mM PMSF and protease inhibitors cocktails (Roche Applied Science). Equivalent protein quantities (30 μg) were subjected to SDS-PAGE, and transferred to PVDF membranes (Millipore). Membranes were probed with the indicated primary antibodies, followed by the appropriate HRP-conjugated secondary antibodies. Immunoreactive bands were visualized with Pro-light HRP (Tiangen) or Immobilon Western Chemiluminescent HRP Substrate (Milllipore).

**Subcellular fractionation**

Cells were collected and resuspended in hypotonic buffer (10 mM KCl, 210 mM sucrose, 70 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 10 mM Hepes (pH adjusted to 7.4 with KOH). After gentle homogenization with a Dounce homogenizer (about 50 times), cell extracts were centrifuged at 3000 g for 10 min at 4°C. The supernatant was removed to an eppendorf tube and 100 μl was set aside in another eppendorf as the post-nuclear supernatant (PNS). The remaining supernatant was centrifuged at 10,000g for 10 min and the new supernatant was removed and set aside as the cytoplasmic fraction (Cyto). The pellet was washed twice with hypotonic buffer, resuspended in 0.5 ml hypotonic buffer, then layered on top of a step Percoll (GE Healthcare Life Science) gradient (2 ml 80%, 4.5 ml 52%, 4.5 ml 26% in a SW40 tube) which was centrifuged at 20000 g for 2 h. A 0.4 ml fraction (the mitochondrial fraction, Mito) was

collected from the interface between the 26% and 52% layers and diluted with 0.8 ml hypotonic buffer. The Mito fraction was centrifuged in an eppendorf tube at 20,000 g for 10 min and washed 3 times with hypotonic buffer. Samples were mixed with 100 μl 1× loading buffer and heated for 10 min at 100℃ before analyzing by western blotting.

***In vitro* kinase assay**

For the ULK1 kinase assay, recombinant Myc-ULK1 was isolated from HeLa cells by immunoprecipitation using anti-Myc antibody (Sigma, C3956). The recombinant substrate proteins GST-FUNDC1 and GST-FUNDC1 S17A mutant were purified from *E. coli* BL21. The kinase reaction was performed in a buffer containing 25 mM MOPS, pH 7.5, 1 mM EGTA, 0.1 mM $Na_3VO_4$, 15 mM $MgCl_2$, 100 μM ATP and 1 μg substrate for 60 min at 37°C. The kinase reactions were stopped by adding 6×SDS loading buffer for 10 min at 100 °C.

**Mass spectrometry**

ULK1 (-/-) cells were co-transfected with Flag-ULK1 and FUNDC1-Myc with Lipofectamine 2000 (Invitrogen). About 24 h post-transfection, cells were lysed and immunoprecipitated with anti-FLAG (Sigma) antibody. The immune complexes were washed by binding buffer and then eluted with sample loading buffer for 10 min at 100 °C and analyzed by 12% SDS-PAGE. Using the PageRuler Plus Prestained Protein Ladder (Fermentas) as a guide, the target band on the PAGE gel was cut. After treatment, the samples were sent to the Institute of Biophysics, Chinese Academy of Science, for analysis by mass spectrometry.