Moleculuar Cell, Vol *XX*

Supplemental Information

Fine-tuning of Drp1/Fis1 availability by AKAP121/Siah2 regulates mitochondrial adaptation to hypoxia

Hyungsoo Kim, Maria C. Scimia, Deepti Wilkinson, Ramon D. Trelles, Malcolm R. Wood, Andrew Dillin, David Bowtell, Mark Mercola & Ze'ev A. Ronai

Inventory of Supplemental Information

Figure S1, related to Figure 1

Hypoxia induces mitochondria fragmentation

Figure S2, related to Figure 2

Siah-dependent regulation of hypoxia-mediated mitochondria fragmenta□on in primary MEFs

Figure S3, related to Figure 2

HIF-1α is dispensable for hypoxia-mediated mitochondria fission

Figure S4, related to Figure 4

PKA-dependent regulation of mitochondria dynamics by AKAP121

Figure S5, related to Figure 5

Central domain of AKAP121 regulates Drp1-Fis1 interaction and contains Siah-degron motif.

Figure S6, related to Figure 6

Siah2-mediated mitochondria dynamics is associated with ischemia- induced cell death of cardiomyocytes

Figure S7, related to Figure 7

Depletion of *siah-1* or *drp-1* from adulthood shows less influence on the life span of *C.elegans*

Movie S1, related to Figure 6

Echocardiography_*Siah1aWT_Siah2WT*_24 h_IS

Movie S2, related to Figure 6

Echocardiography_*Siah1aHT_Siah2KO*_24 h_IS

Figure S1

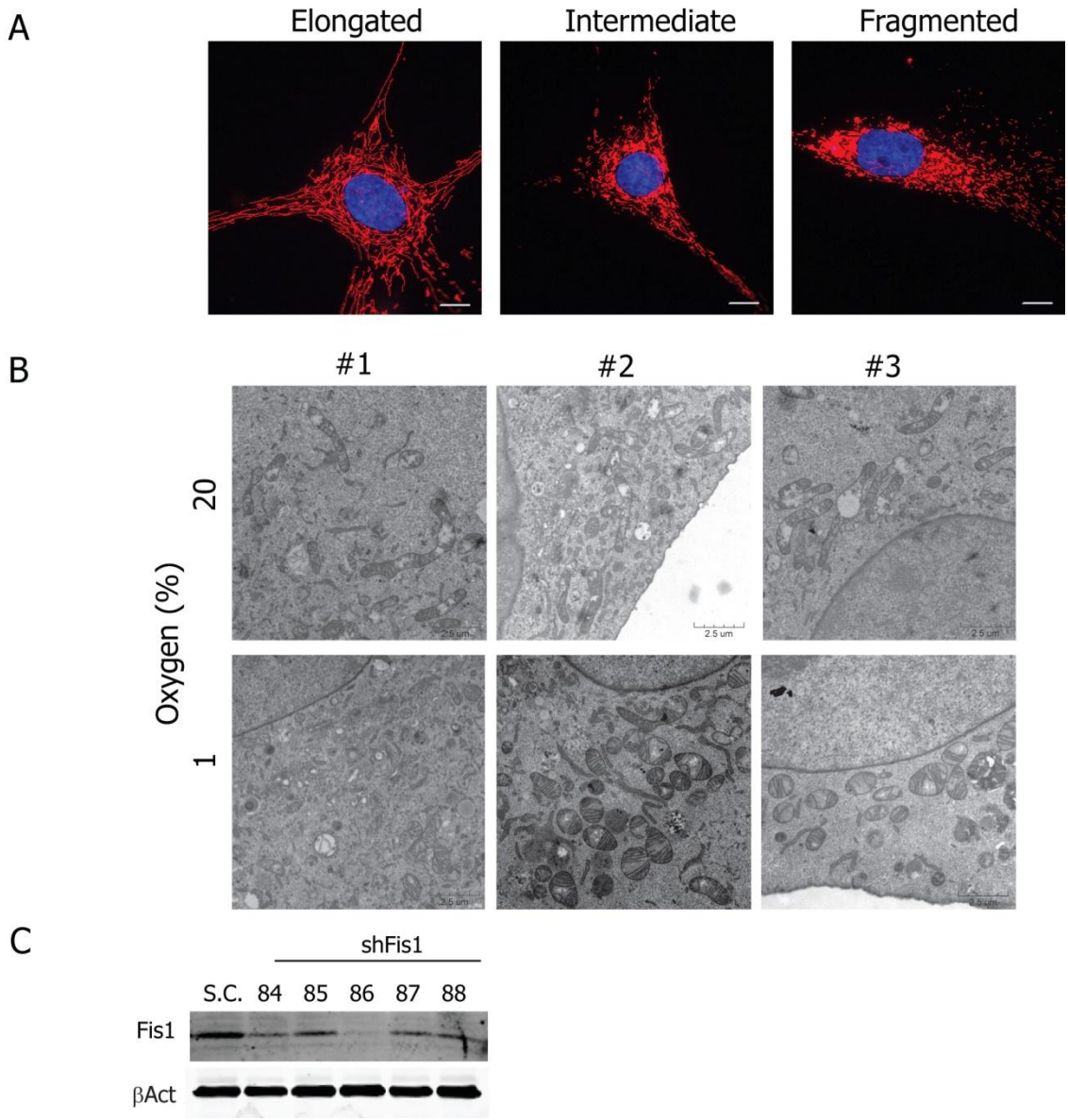

Figure S1. **Hypoxia induces mitochondria fragmentation, Related to Figure 1**. (A) Representative mitochondrial morphologies. Human skin fibroblast cells expressing mito-RFP were cultured under normoxia or hypoxia for 24 h. Cells with less than 25%, between 25–75%, and more than 75% of mitochondrial puncta were classified as elongated, intermediate and fragmented, respectively. Scale bars indicate 10 μm. (B) Mitochondrial ultrastructure in NIH3T3 cells cultured under indicated oxygen concentrations. Scale bars indicate 2.5 μm. (C) MEFs (WT) were transduced with scrambled shRNA or 5 different shRNAs against Fis1. Lysates were obtained and analyzed using anti-Fis1 antibody.

Figure S2

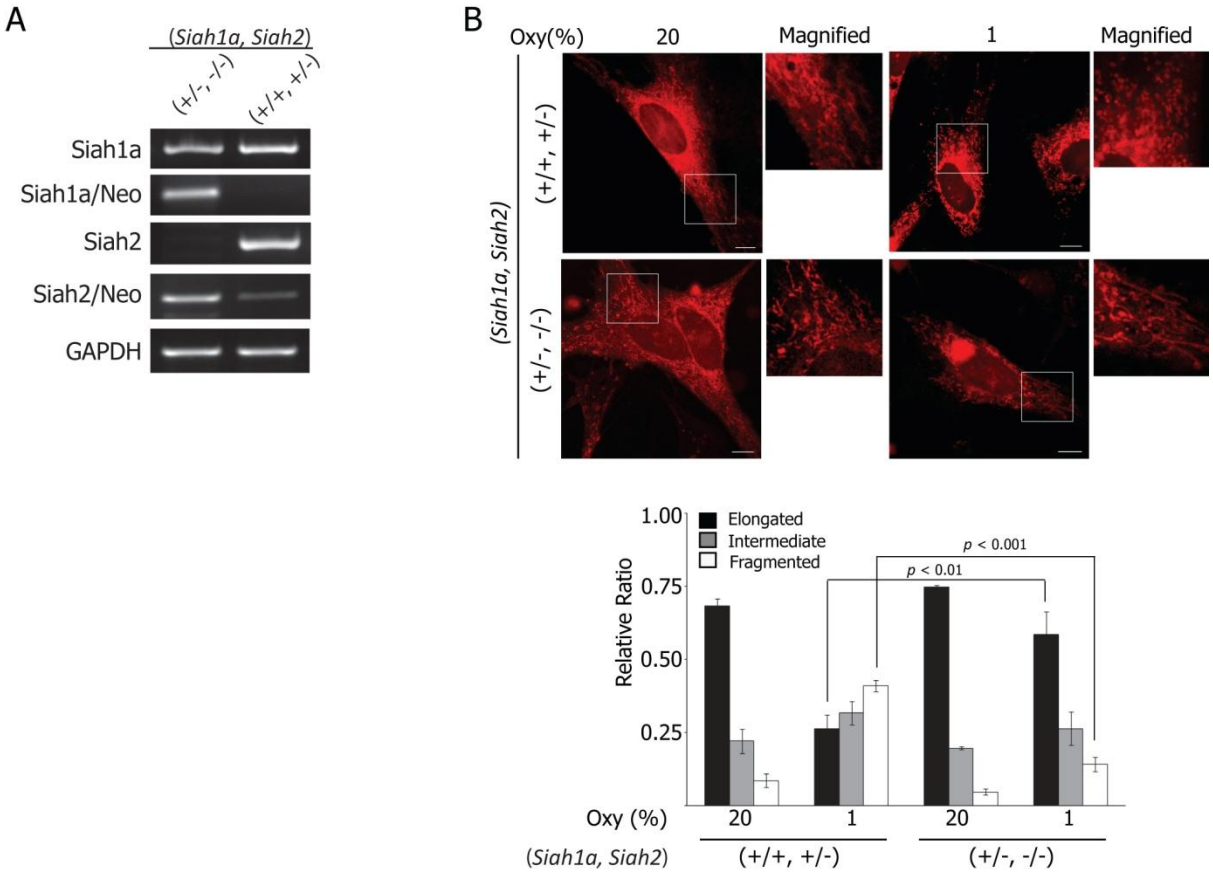

Figure S2. **Siah-dependent regulation of hypoxia-mediated mitochondria fragmentation in primary MEFs, Related to Figure 2.** (A) Confirmation of genotype of primary MEFs isolated. (B) Primary MEFs (*Siah1a*^{WT}*/Siah2*$^{+/-}$ or *Siah1a*$^{+/-}$*/Siah2*$^{-/-}$) were grown under normoxic (20%) or hypoxic (1%) conditions for 24 h. Mitochondria were visualized with mitoTracker (Invitrogen) and quantified. Data were presented as mean ±SD. Scale bars indicate 10 μm.

Figure S3

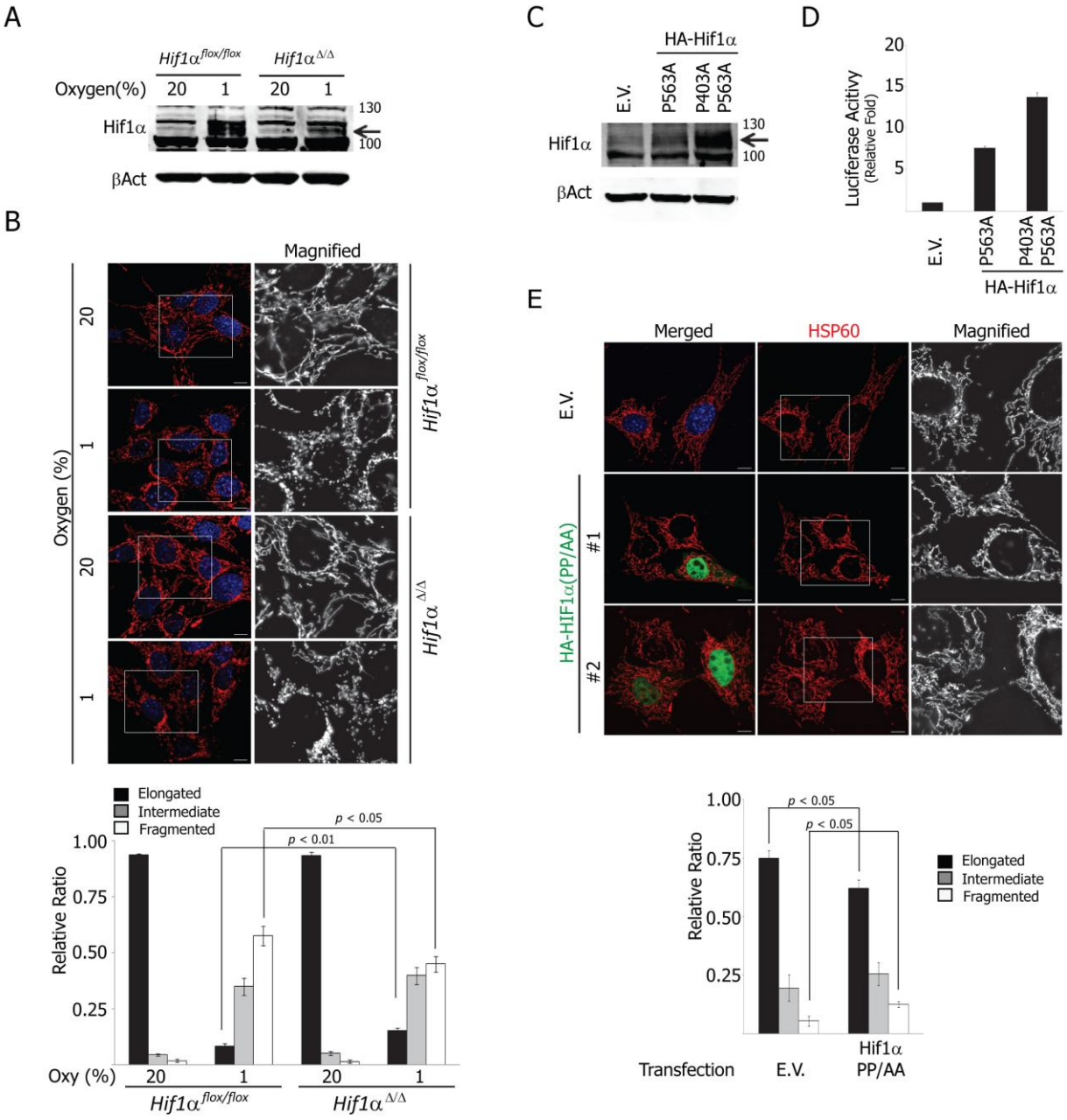

Figure S3. **HIF-1α is dispensable for hypoxia-mediated mitochondria fission, Related to Figure 2**. (A) Double floxed HIF1α MEFs (*HIF1α*$^{flox/flox}$) and HIF1α null MEFs (*HIF1α*$^{Δ/Δ}$) were cultured under normoxia or hypoxia for 5 h. Cell lysates were obtained and analyzed with anti-HIF1α antibody. The arrow indicates HIF1α. (B) *HIF1α*$^{flox/flox}$ and *HIF1α*$^{Δ/Δ}$ MEFs were maintained under indicated oxygen concentration for 24 h. Mitochondria were visualized with anti-Tom20 antibody and quantified from 300–400 cells from three independent slides. Data is presented as mean ±SD. Scale bars indicate 10 μm.

(C) HEK293T cells were transfected with control vector, HA-tagged HIF1α (P563A) or HIF1α (P402A/P563A) mutant. Cell lysates were obtained and analyzed with anti- HIF1α antibody. The arrow indicates HIF1α. (D) NIH3T3 cells were transfected with control plasmid, HA-tagged HA-tagged HIF1α (P563A) or HIF1α (P402A/P563A) mutant along with 3XHRE-luciferase plasmid. Lysates were obtained and subjected to assess luciferase activity. The luciferase activity was normalized with β-galatosidase activity. Relative fold of luciferase activity was calculated by setting a value from the control group as 1. Data were presented as mean ±SD. (E) NIH3T3 cells transfected with control plasmid or HA-HIF1α (P402A/P563A) mutant. After 24 h of culture, cells were stained with anti-HA antibody (HA-HIF1α, green). Mitochondria were visualized with anti-HSP60 (red) and nucleus with DAPI (blue). Images were representative of transfectants. Mitochondria morphology was quantified from 300–400 cells from three independent transfections. Data were presented as mean ±SD. Scale bars indicate10 μm.

Figure S4

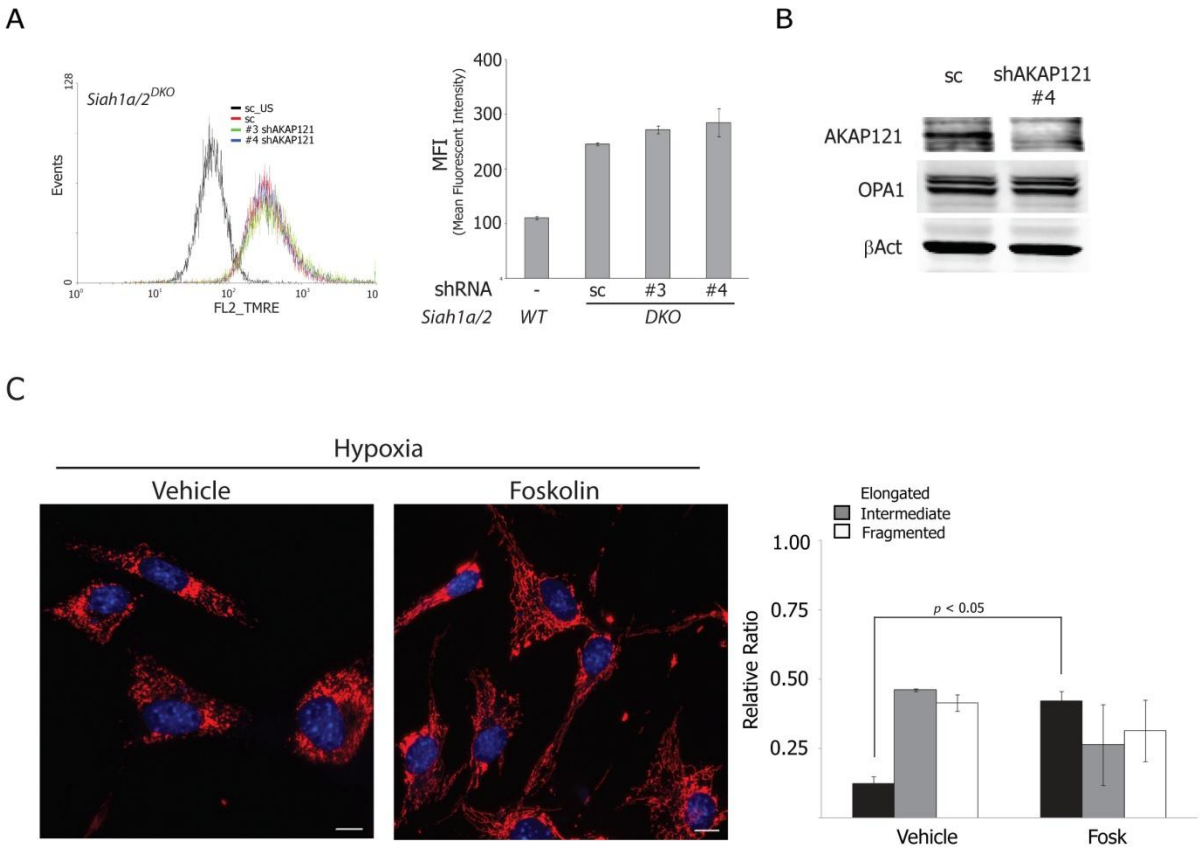

Figure S4. PKA-dependent regulation of mitochondria dynamics by AKAP121, Related to Figure 4. (A) Mitochondrial membrane potential of *Siah1a/2*^{DKO} MEFs transduced with scrambled (S.C.) or two different shRNAs against AKAP121 was measured as described in Materials and Methods. Unstained cells (S.C._US) served as a control. Mean fluorescent intensity (MFI) values from three independent experiments were calculated and presented as mean ±SD. (B) Lysates obtained from *Siah1a/2*^{DKO} MEFs transduced with scrambled shRNA or shAKAP121 were analyzed with indicated antibodies. (C) NIH3T3 cells expressing mito-RFP were grown for 24 h under hypoxia in the presence of vehicle (DMSO) or forskolin (10 μM). Mitochondrial morphology in 200 cells was analyzed and quantified according to established criteria (Figure S1A). Data were presented as mean ±SD.

Figure S5

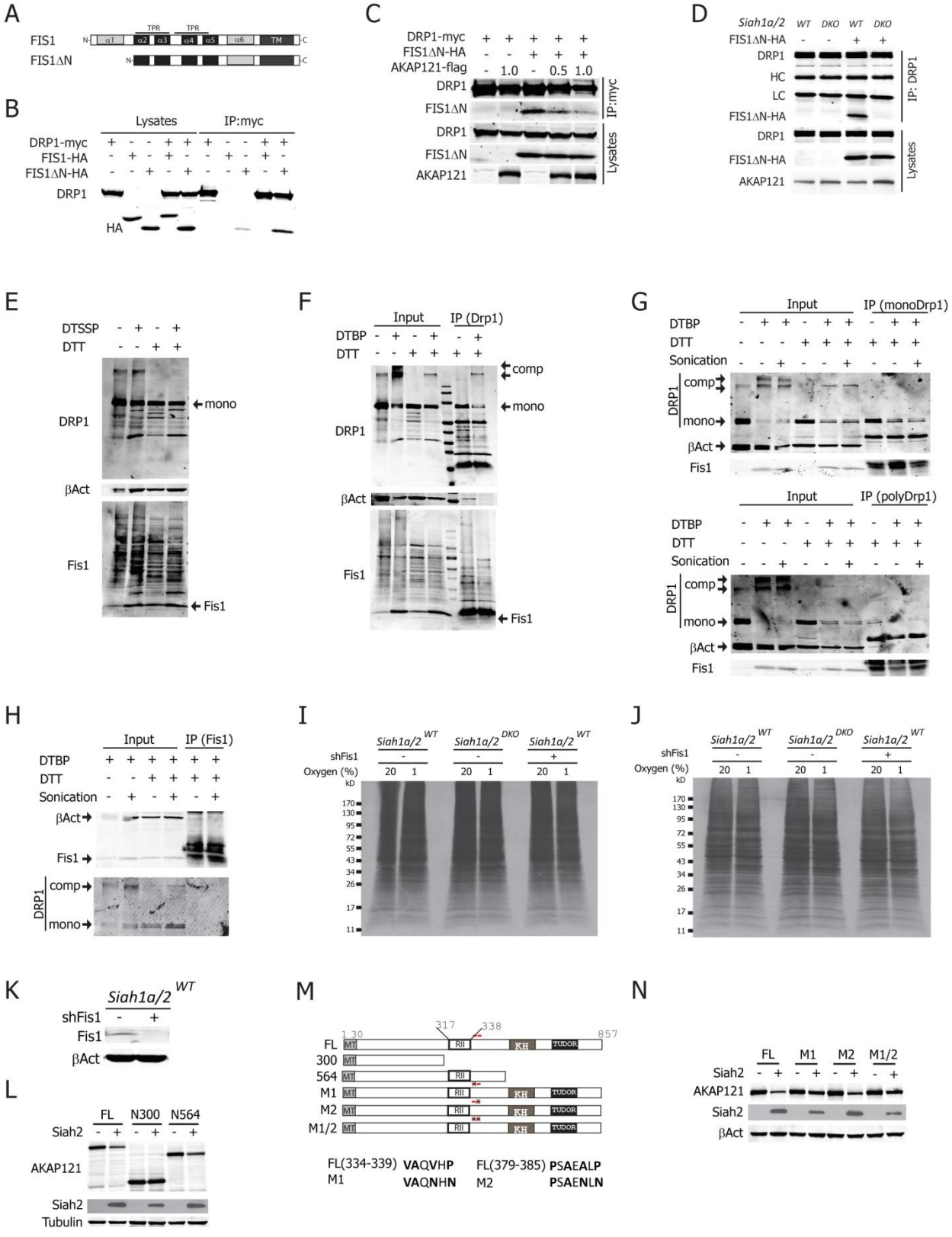
A
FIS1
FIS1ΔN
TPR
TPR
α1
α2
α3
α4
α5
α6
TM
B
Lysates
IP:myc
DRP1-myc
FIS1-HA
FIS1ΔN-HA
DRP1
HA
C
DRP1-myc
FIS1ΔN-HA
AKAP121-flag
DRP1
FIS1ΔN
AKAP121
IP:myc
Lysates
D
Siah1a/2
WT
DKO
WT
DKO
FIS1ΔN-HA
DRP1
HC
LC
FIS1ΔN-HA
DRP1
FIS1ΔN-HA
AKAP121
IP: DRP1
Lysates
E
DTSSP
DTT
DRP1
mono
βAct
Fis1
Fis1
F
Input
IP (Drp1)
DTBP
DTT
comp
mono
DRP1
βAct
Fis1
Fis1
G
Input
IP (monoDrp1)
DTBP
DTT
Sonication
DRP1
comp
mono
βAct
Fis1
Input
IP (polyDrp1)
DTBP
DTT
Sonication
DRP1
comp
mono
βAct
Fis1
H
Input
IP (Fis1)
DTBP
DTT
Sonication
βAct
Fis1
DRP1
comp
mono
I
Siah1a/2 WT
Siah1a/2 DKO
Siah1a/2 WT
shFis1
Oxygen (%)
20
1
20
1
20
1
kD
170
130
95
72
55
43
34
26
17
11
J
Siah1a/2 WT
Siah1a/2 DKO
Siah1a/2 WT
shFis1
Oxygen (%)
20
1
20
1
20
1
kD
170
130
95
72
55
43
34
26
17
11
K
Siah1a/2 WT
shFis1
Fis1
βAct
L
FL
N300
N564
Siah2
AKAP121
Siah2
Tubulin
M
1
30
317
338
857
FL
300
564
M1
M2
M1/2
MT
RII
KH
TUDOR
FL(334-339) VAQVHP
M1 VAQNHN
FL(379-385) PSAEALP
M2 PSAENLN
N
FL
M1
M2
M1/2
Siah2
AKAP121
Siah2
βAct

Figure S5. Central domain of AKAP121 regulates Drp1-Fis1 interaction and contains Siah-degron motif, Related to Figure 5. (A) Human Fis1 is composed of six alpha helixes and one transmembrane domain at carboxy-terminus. The central four helixes constitute two tandem repeat TPR (tricopeptide repeats) domains. The N-terminal arm (31 amino acids) of Fis1 was deleted. (B) HEK293T cells were transfected with AKAP121-flag and full-length wild-type hFis1 or N-truncated hFis1. Lysates were immunoprecipitated and blotted with indicated antibodies. (C) Cells were transfected with Drp1 and/or Fis1ΔN and increasing amount of AKAP121. Lysates were obtained and analyzed by immunoprecipitation with anti-myc (Drp1), followed by immunoblotting with anti-myc (Drp1), HA (Fis1ΔN) and flag (AKAP121) antibodies. (D) *Siah1a/2*^{WT} and *Siah1a/2*^{DKO} MEFs transfected with empty vector or Fis1ΔN were grown for 24 h under normoxia. Cell lysates were obtained, immunoprecipitated with anti-Drp1 antibody and immunoblotted with anti-HA (Fis1ΔN) and indicated antibodies. HC and LC indicate immunoglobulin heavy and light chain, respectively. (E) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTSSP. Cell lysates treated with or without DTT were analyzed using indicated antibodies. (F) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTBP. Cell lysates were treated with DTT as described or immuoprecipitated with anti-Drp1 antibody. The blot was analyzed with indicated antibodies. (G) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTBP. Cell lysates for input were prepared as described with or without sonication for 30 min. Lysates were immunoprecipitated with monoclonal anti-Drp1 (monoDrp1, BD Bioscience) or polyclonal anti-Drp1 (polyDrp1, Santa Cruz). The blots were analyzed with indicated antibodies. “mono” and “comp” represent monomer and oligomer or complex of Drp1, respectively. (H) Cell lysates from DTBP-treated *Siah1a/2*^{WT} MEFs were immunoprecipitated with anti-Fis1 (Santa Cruz). (I, J) The indicated MEFs were cultured under normoxia (20%) or hypoxia (1%) for 24 h with 4 h of labeling during last 4 h. After cross-linking whole cells, lysates were prepared, treated with vehicle (I) or DTT (J) and subjected to SDS-PAGE. (K) Cell lysates from *Siah1a/2*^{WT} MEFs that were subjected to knockdown of Fis1 with corresponding shRNA were analyzed with indicated antibodies. (L) HEK293T cells were transfected with indicated truncated constructs of AKAP121 and/or Siah2. Lysates were immunblotted using indicated antibodies. (M) Two candidate sequences of Siah-degron in central domain of AKAP121. (N) HEK293T cells were transfected with Siah2 and wild-type (FL) construct or mutant constructs (M1 or M2, M1/2). Lysates were analyzed using indicated antibodies.

Figure S6

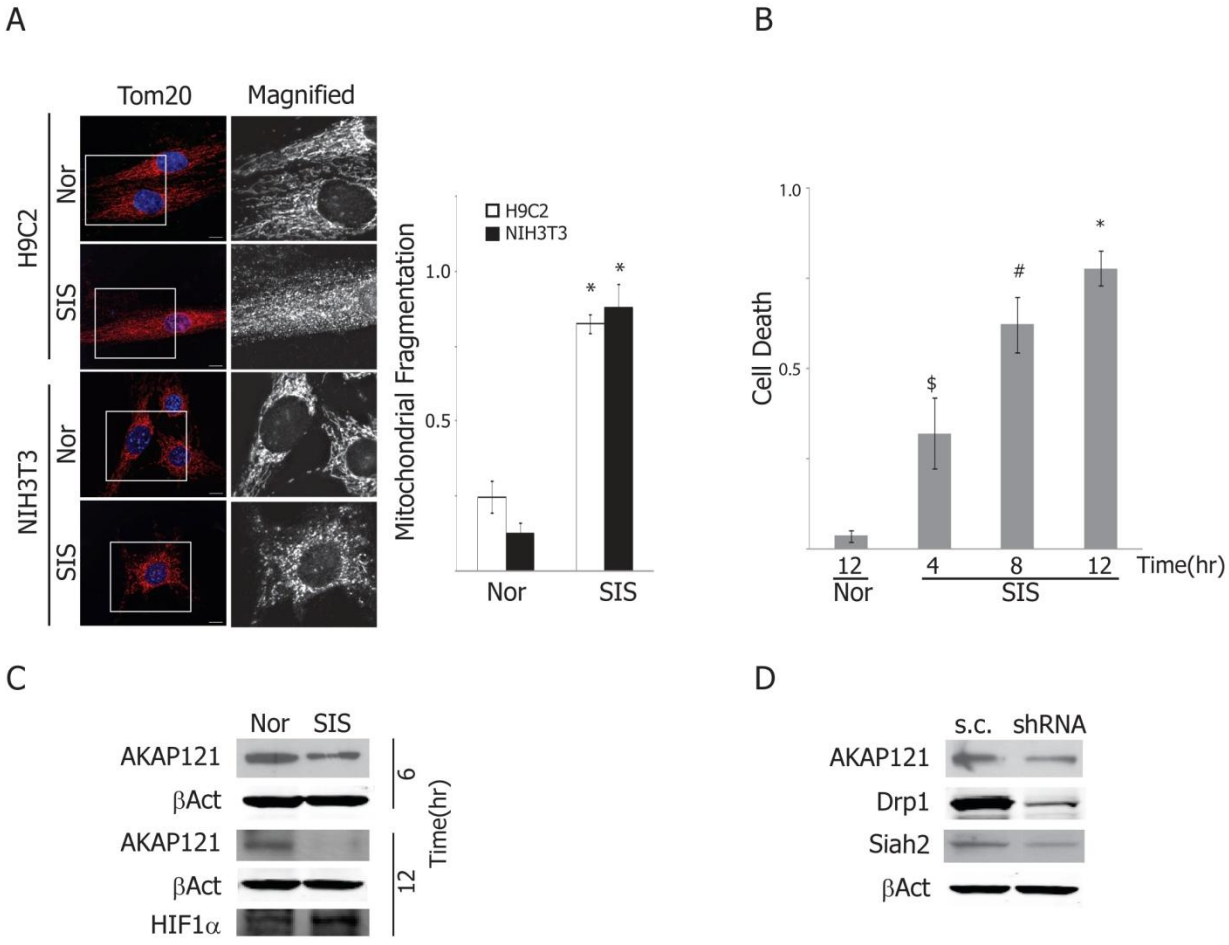

Figure S6. Siah2-mediated mitochondria dynamics is associated with ischemia-induced cell death of cardiomyocytes, Related to Figure 6. (A) H9C2 and NIH3T3 were cultured under normoxic (20% oxygen, presence of serum and glucose) or anoxic (0%, absence of serum and glucose) condition for 12 h. The morphology of mitochondria was visualized with anti-Tom20 antibody and quantified by counting cells with fragmented mitochondria. Data were presented as mean ±SD. The asterisk indicates $p < 0.001$. (B) H9C2 cells were cultured under the ischemic condition (as in (A)) for indicated time. The cell-death rate was quantified by counting cells stained with propidium iodide. Data were presented as mean ±SD. *, # and $ indicate $p < 0.001$, $p < 0.005$ and $p < 0.05$, respectively. (C) H9C2 cells were cultured as in (A) for indicated time. Cell lysates were analyzed using anti-AKAP121, HIF-1α, and β-actin antibodies. (D) H9C2 cells were transduced with lentivirus expressing indicated shRNA. Cell lysates were obtained and analyzed with indicated antibodies.

Figure S7

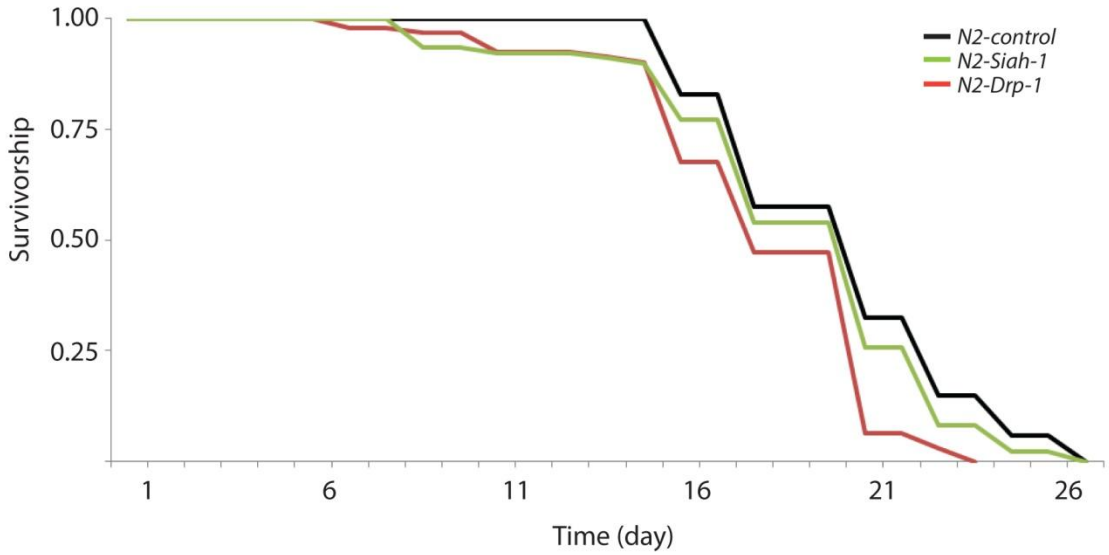

Figure S7. Depletion of *Siah-1* or *Drp-1* from adulthood shows much weaker influence on the life span of *C. elegans*, Related to Figure 7. RNAi-mediated depletion of C.elegans *drp-1* and *siah-1* resulted in reduction of mean lifespan *N2-control* (black line, mean 20.3 ± 0.3 days*), N2-drp-1* (red line, 18.5 ± 0.4 days), *N2-siah-1* (green line, 19.4 ± 0.4 days). RNAi depletion was initiated from adulthood.

Movie S1

Movie S1. Echocardiography_*Siah1aWT_Siah2WT*_24 h_IS, related to Figure 6.

Movie S2

Movie S2. Echocardiography_*Siah1aHT_Siah2KO*_24 h_IS, related to Figure 6

Supplemental Experimental Procedures

MEF preparation, Cell culture and Hypoxia Treatment

MEFs were prepared from embryos at embryonic day (E11 or E12) and maintained in DMEM supplemented with 15% fetal bovine serum, penicillin/streptomycin and 0.2 mM 2-mercaptoethanol (Sigma). Genotypes of MEFs were analyzed with primer sets described previously (Frew et al., 2003). Primary MEFs with less than seven passages were used for experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum, penicillin/streptomycin and 25 mM HEPES (pH 7.4). For hypoxic treatment (1% oxygen), cells were incubated in hypoxia work station (In vivo2 400; Ruskin Corp).

Reagents and Antibodies

MitoTracker Red CMXRos was purchased from Molecular Probes. Foskolin and cyclosporine were purchased from Sigma. Antibodies used were obtained as follows; antibodies to flag-tag and Siah2 from Sigma; antibodies to HA-tag from Covance or Cell Signaling; antibodies to myc-tag, AKAP149 (specific to mouse AKAP121), Fis1, Tom20, Mfn1 and Mfn2 from Santa Cruz; antibodies to Drp1, OPA1, and Hsp60 from BD bioscience; antibody to phopho-Drp1 (S637) was kindly provided by Dr. Blackstone (Chang and Blackstone, 2007); antibody to β-actin, tubulin and Complex 1 (NDUFB8) were from Invitrogen.

DNA Constructs, Transfection and Transduction

Constructs encoding HA or flag-tagged Siah1a and Siah2 were previously described (Nakayama et al., 2004). PCR products of Siah2 and Siah2 ring domain mutant (RM) were subcloned into pEGFP-N1.

Plasmids encoding mouse AKAP121 (NM_001042541) and mouse Fis1 (NM_25562) were constructed by subcloning of PCR product from MEF's cDNA into pcDNA3.0-C-flag and pCMV-N-HA. The series of truncated mutant of AKAP121, Fis1ΔN and HA-tagged Drp1ΔGTPase (deletion of 256 amino acids from N-terminus) were generated by subcloning of corresponding PCR products in to pcDNA3.0-C-flag or pCMV-N-HA plasmid. Deletion mutants (ΔRIIs), Siah-degron mutants of AKAP121 and phosphorylation site mutants (S637A and S637D) of Drp1 were generated by site-directed mutagenesis (Staratagene). AKAP121(MT-351/446) plasmid was generated by sequential subcloning of mitochondria targeting sequences (1 to 34) into HindIII/BamH1 site and central domain (351 to 446) into BamH1/EcoRI site of pcDNA3.0-C-flag vector. Plasmids encoding Drp1 and Drp1 (K38A) mutant were kindly provided by Dr. Blackstone (National Institute of Health), mito-RFP from Dr. Chan (California Institute of Technology). HIF1α mutants [P563A or P402A/P563A] were generated by subcloning to pcDNA plasmid from pBMN-HIF1 α (kindly provided by Dr. Ratcliffe, University of Oxford) and following site-directed mutagenesis (Stratagene). HEK293T, NIH3T3 and MEFs cells were transfected using calcium phosphate, Lipofectamine 2000 (invitrogen) or JetPrime (Polyplus-transfection) according to manufacturer's direction. Retro- or lenti virus expressing appropriate gene were spin-infected to cells [1600×g for 30 min at room temperature].

Gene Silencing

pLKO-scrambled and pLKO-mSiah2 were kindly provided by Olexander Korchynsky and pRetroSuper-Siah2 was constructed using previously reported sequence (Hara et al., NCB 2005). pLKO-shAKAP121, pLKO-shFis1, and pLKO-shDrp1 were purchased from Openbiosystems. The efficiency of silencing was validated from cell lysates of stable clones using appropriate antibodies or RT-PCR analysis of mRNA.

For RT-PCR analysis of Siah2 mRNA, the following primer set was used; 5′-GCTGAGAACTTTGCCTACAG-3′ and 5′- GCTATGCCCAAATAACTTCC-3′.

Immunocytochemistry

Mitochondria were stained as follows; cells expressing mito-RFP were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS, pH 7.4) for 15 min; cells were stained with MitoTracker red (20–40nM; Invitrogen) for 30 min and subsequently fixed as described; cells were fixed as described and then permeabilized with 0.2% TritonX-100 in PBS (pH 7.4) for 10 min at room temperature. After blocking with 3% bovine serum albumin (BSA) in PBS, mitochondria were stained with anti-Tom20 (1:300) or anti-HSP60 (1:500) antibody for 1 h at room temperature or overnight at 4°C. The fixation of cells cultured under hypoxia was performed in interlock (anaerobic and room temperature) of hypoxia work-station. For staining of ectopically expressed protein, cells were fixed, permeabilized and blocked as described. Cells were stained with appropriate antibodies [flagM2 (1:750) (Sigma), HA (1:750) (Cell Signaling) and Myc (1:500) (SantaCruz)] for 1 h at room temperature or overnight at 4°C. After rinsing with PBS, coverslips were incubated with Alexa350, Alexa488, or Alexa568-conjugated antibodies (Invitrogen). Image data were obtained using Olympus TH4–100 microscope and deconvolved through constrained iterative algorithm using Slidebook 4.1 digital microscopy software. Z projection images were built using NIH Image J software. For TUNEL-staining we used TUNEL-Biotin-Avidin-HRP kit (Promega). Images were taken using Aperio System and Tunel positive cells were manually counted using Imaje J software (cell counter plugin).

Immunoprecipitation and Western Blotting

Cell lysates were obtained from HEK293T cells expressing appropriate proteins using lysis buffer containing 1% TritonX-100. Pre-cleared cell lysates were incubated with control mouse IgG (Santa Cruz), anti-flag (M2, Sigma), anti-myc (9E10, Santa Cruz) or anti-HA (Covance) antibody at 4°C overnight. Lysates were added with protein A/G agarose (Santa Cruz) and further incubated for 2 h at 4°C. After

extensive washing with lysis buffer, samples were added with Laemmli buffer, boiled and subjected to SDS-PAGE. For detecting Drp1-Fis1 interaction in MEFs, cell lysates were prepared from cells and incubated with anti-Drp1 (1 ug/mg of protein; BD bioscience) antibody. Precipitation and analysis were performed as described.

Subcellular Fractionation

Cells were washed with PBS (pH 7.4) and rinsed with ice-cold isolation buffer [5 mM HEPES, 4.3 mM $MgCl_2$, 1 mM EGTA, 250 mM Sucrose, protease inhibitor cocktail (Sigma), phosphotase inhibitor cocktail I and 2 (Sigma)]. Suspended cells in Buffer A were homogenized with 30 - 40 strokes and centrifuged at 700×g for 10 min at 4°C. The post-nuclear supernatant (PN fraction) was further centrifuged at 10,000×g for 10 min at 4°C. The post-heavy membrane supernatant (PHM fraction) was collected and mitochondria-enriched heavy membrane pellet (HM fraction) was rinsed twice with isolation buffer. HM pellet was lysed with buffer containing 1% TritonX-100. To get pure mitochondria for analysis of Siah2 translocation, HM fraction was layered over sucrose gradient (1.5 M/1 M) and centrifuge at 200,000×g for 45 min. Hazy phase was collected and lysed as described.

Cross-Linking

For cross-linking intracellular proteins, cell were washed with PBS and incubated with cross-linking solution [2.5 mM DTBP (Dimethyl 3,3'-dithio-*bis*-propionimidate, Sigma) in PBS] for 30 min. Reaction was quenched by adding Tris-HCl (50 mM, pH 7.5) for 15 min. Lysates were obtained by lysing cells with lysis buffer (50 mM Tris-HCl, pH 7.4, 1% triton-X 100, 150 mM NaCl, protease inhibitor cocktail, phosphatase inhibitor cocktail) and subjected to immunoprecipitation with anti-Drp1 (BD Bioscience).

Samples were washed, incubated at 37°C for 30 min in the presence of 150 mM DTT and boiled with Laemmli sample buffer. Samples are subjected to SDS-PAGE, electrotransfer and finally visualized by autoradiography. Membrane–impermeable cross-linker, DTSSP (3,3-Dithio-bis-(sulfosuccinimidyl) propionate; Sulfo-DSP), was used as control cross-linker to verify the efficient cross-linking by DTBP (Figure S5E). In brief, cells were treated with 1 mM DTSSP for 30 min. Cell lysates were obtained as described and treated with DTT (50 mM) at 37°C for 30 min.

In Vitro, Simulated-Ischemia Model

Medium was changed with ischemic media (IS) [DMEM base (Cellgro) without glucose and serum, pre-flushed with N_2/ CO_2(95% /5%) gas mixture] or normoxic media [IS media supplemented with 4.5 g/l glucose, 4 mM L-glutamin and 10% fetal bovine serum]. Cells were further incubated in air-tight modular hypoxia chamber which was flushed with N_2/CO_2 gas mixture. For analysis of cell death, cells were stained with propidium iodide (1 μg/ml) for 3 min. After twice washing of cells with PBS, PI positive cells were counted in, at least, 10 different fields.

In Vivo, Myocardial Infarction Model

Myocardial infarction was performed as described (Denzel et al., 2010). 8- to 10-week-old mice with genotypes, *Siah1a*^{WT}*/Siah2*^{WT} and *Siah1a*^{HT}*/Siah2*^{KO} mice were anesthetized with ketamine (100 mg/Kg)-xylazine (2.5 mg/Kg). The chest cavity was entered by blunt dissection of the fourth intercostal space, 2 mm away from the left sternal border. Opening the pericardial sac exposed the heart. The left anterior descending artery was ligated with a 6-0 silk suture about 1–2 mm beneath the tip of the left auricleatrium. The lungs were reinflated with a small catheter left in the thorax to evacuate air and

fluids. The chest cavity was closed, and the mouse was extubated and maintained on a warm pad. 24 h after myocardial infarction, echocardiography was performed to assess cardiac function. Animals were anesthetized with ketamine (100 mg/Kg)-xylazine (2.5 mg/Kg) and 0.5 ml of a 1% Evans blue (Sigma-Aldrich) solution was injected at the apex of the left ventricle (LV) to identify the non-ischemic tissue (perfused area). The heart was then excised, washed in PBS, and cut into 5 transverse slices, which were subsequently stained in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 15 min at room temperature to aid visualization of the infarcted area (IA). Digital images of heart sections were analyzed for AAR (Evans blue negative) , and infarcted area (TTC negative) LV area (Evans blue positive and negative) using Image J software.

Echocardiographic analysis

Cardiac function and heart dimensions were evaluated by two-dimensional echocardiography using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics, Canada) and a 13-MHz linear array transducer. M-mode tracings were used to measure anterior and posterior wall thicknesses and LV internal diameter at end diastole and end systole at the level of the papillary muscles. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = [(LVIDd - LVIDs)/LVIDd] $\times$ 100. Four short axis images were taken in parasternal short axis view at different levels, from the mitral valve to the apex to trace the endocardial border in systole and in diastole, and in parasternal long axis view the left ventricle length from the aortic annulus to the apex level in diastole and systole were measured. EF% was then automatically calculated as summation of the areas of the 4 sections using the Simpson's rule, $V = (A_1 + A_2 + A_3 + A_4/2)h+(\pi/6)h$, for which V = volume, A = LV chamber cross-sectional area of the individual sections, h = LV length/4. The data were analyzed by a single observer blinded to mouse genotype.

Electron microscopy

Cells grown under normoxia or hypoxia were prepared for electron microscopy using previous method (Gilula et al., 1978). Briefly this consisted of fixing the cells in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and after a brief buffer wash, postfixing in 1% osmium tetroxide. Following a further buffer wash, the cells were treated with tannic acid, sodium sulfate and dehydrated in a graded ethanol series. The cells were cleared in 2-hydroxypropyl methacrylate (HPMA) and embedded in LX112 (Ladd Research, Williston, VT). Thin sections were prepared, mounted on copper mesh grids coated with parlodion and stained with uranyl acetate and lead citrate. Sections were observed in the Electron Microscope Hitachi H-600A, at 75kV and at different instrumental magnifications. Digital images were acquired with 11.2 megapixel cooled monochromatic CCD camera L9C (SIA, Atlanta) controlled by the MaxIm DL software (Diffraction Limited, Ottawa, Ontario, Canada). For mitochondria analysis, the length of mitochondria of cells in 4 different slices was measured using NIH Image J software.

The electron microscopic analysis of hearts was performed as followings: In preparation of heart sections for EM, mice were initially perfused with saline followed 4% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer all at room temperature. The heart was removed and the left ventricle sliced into thin strips to continue fixation in the above fixative for 6 h at 4°C before being transferred to cacodylate buffered 3% glutaraldehyde overnight at 4°C. The tissues were then buffer washed, post fixed in buffered 1% osmium tetroxide and subsequently dehydrated in graded ethanol series, transitioned in propylene oxide and embedded in EMbed 812 / Araldite (Electron Microscopy Sciences, Hatfield PA). Thick sections (2 μm) from the block with the ligature were cut close to the injury site, mounted on glass slides and stained in toluidine blue for general injury assessment in the light microscope. Regions of the left ventricle distant from the ligation site were also examined. Subsequently, 70 nm thin sections were cut using a diamond knife (Diatome, Hatfield PA) mounted on

copper slot grids coated with parlodion and then stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR). Images were documented with a Megaview III CCD camera (Olympus Soft Imaging Solutions, Lakewood CO).

Analysis of life span in C. elegans

Lifespan assays were performed as previously described (Mair et al., 2011). Briefly, 110 wild-type (N2 Bristol strain) animals were used for each RNAi condition and scored every day or every other day for survivorship. Animals that died prematurely (exploded at their vulva, internally hatched their embryos, crawled off the plate) were censored. The animals were maintained at 20°C during the entire duration of life-span analysis. Statistical analysis was performed using JMP IN 8 software. In all cases, P-values were calculated using the log-rank (Mantel–Cox) method.

Assessment of Luciferase Activity

For confirming activities of HIF1α mutants, 3XHRE_luciferase and β-galactosidase plasmids were transfected with HIF1α mutants (P563A or P402A/P563A) to NIH3T3 cells. After 24 h of culture, cell lysates were obtained and subjected to analysis of luciferase activity using luciferase assay system (Promega). β-galactosidase activities in the lysates were used for normalization of luciferase activity.

Measurement of mitochondrial activities

For measuring mitochondrial membrane potential, TMRE (150 nM, Invitrogen) in culture medium was pre-conditioned under hypoxia (1% O_2). For depolarized control, CCCP (50 μM, Sigma) were pre-treated

for 1 h before adding TMRE. After addition of TMRE solution, cells were further incubated for 30 min, subsequently rinsed with warmed PBS and trypsinized. Cells were washed with warmed PBS supplemented with 5% FBS and analyzed using FACSCanto. Data obtained were analyzed using WinMDI (ver 2.9; TSRI).

Quantitation and Statistical Analysis

For evaluation of morphological difference of mitochondria, 100–300 cells of coverslips from three independent experiments were examined. Unless specified, data were presented as mean ±SD and significance of differences were analyzed with two-tailed, unpaired t-Test. Difference with *p* values less than 0.05 was considered as significant.

Supplemental References

Denzel, M.S., Scimia, M.C., Zumstein, P.M., Walsh, K., Ruiz-Lozano, P., and Ranscht, B. (2010). T-cadherin is critical for adiponectin-mediated cardioprotection in mice. J Clin Invest 120, 4342-4352.

Gilula, N.B., Epstein, M.L., and Beers, W.H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J Cell Biol *7*, 58-75.

Mair, W., Morantte, I., Rodrigues, A. P., Manning, G., Montminy, M., Shaw, R. J., and Dillin, A. (2011). Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. Nature 470, 404-408.

Figure S1

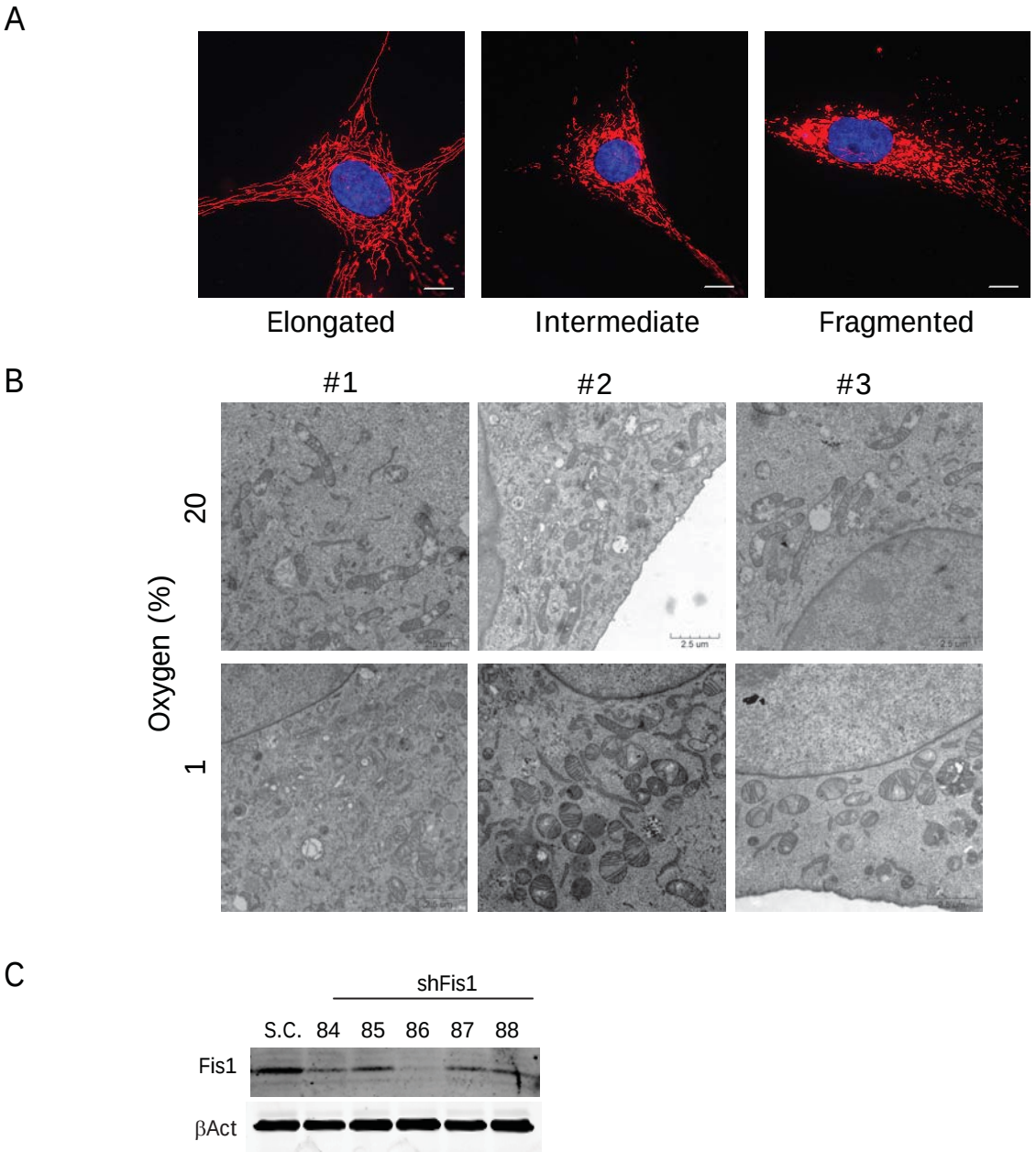

Figure S1. **Hypoxia induces mitochondria fragmentation. Related to Figure 1**. (A) Representative mitochondrial morphologies. Human skin fibroblast cells expressing mito-RFP were cultured under normoxia or hypoxia for 24hr. Cells with less than 25%, between 25 – 75%, and more than 75% of mitochondrial puncta were classified as elongated, intermediate and fragmented, respectively. Scale bars indicate 10μm. (B) Mitochondrial ultrastructure in NIH3T3 cells cultured under indicated oxygen concentrations. Scale bars indicate 2.5μm. (C) MEFs were transduced with scrambled shRNA or 5 different shRNAs against Fis1. Lysates were obtained and analyzed using anti-Fis1 antibody.

Figure S2

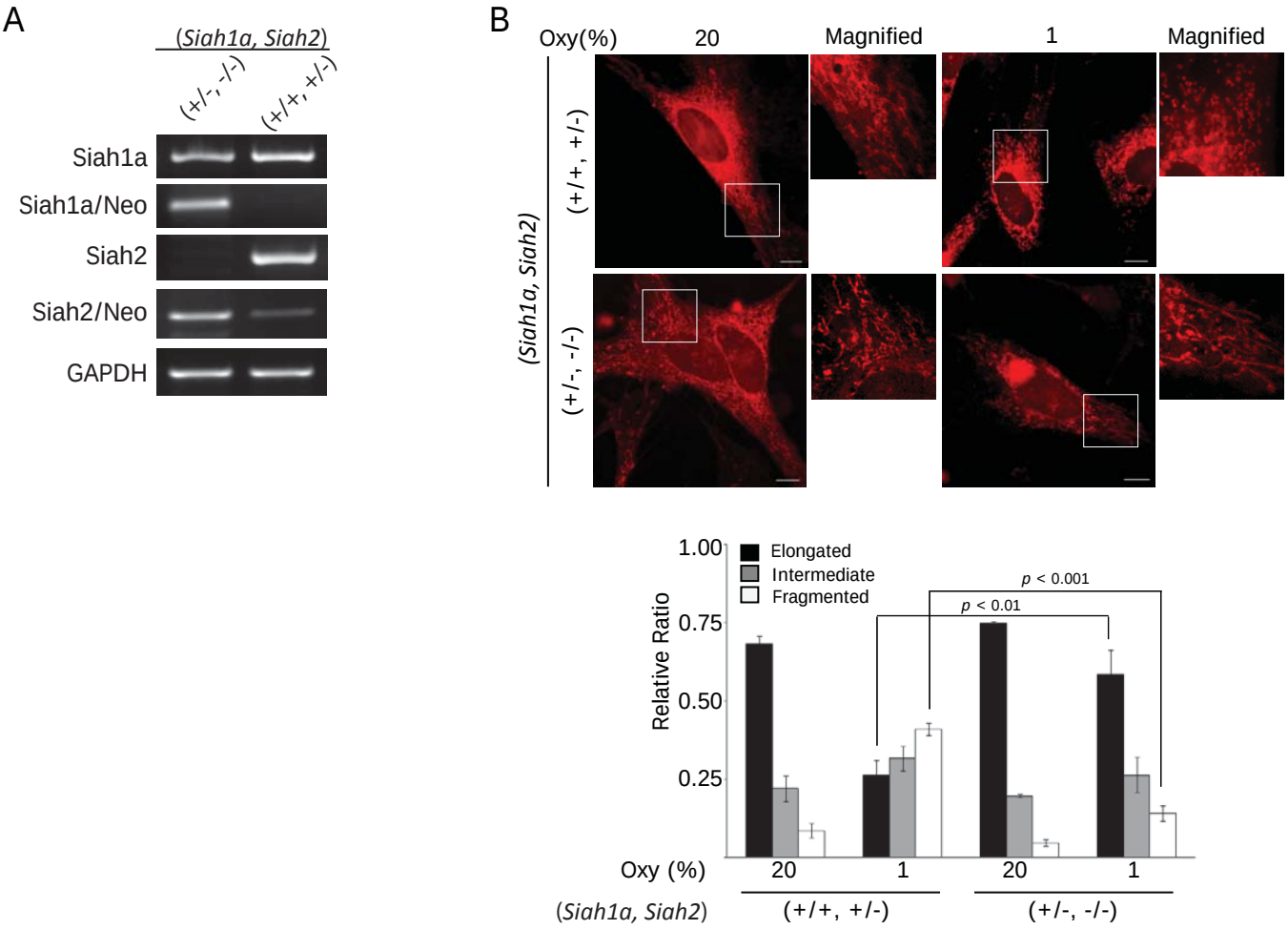

Figure S2. **Siah-dependent regulation of hypoxia-mediated mitochondria fragmentation in primary MEFs. Related to Figure 2.** (A) Confirmation of genotype of primary MEFs isolated. (B) Primary MEFs (*Siah1a*$^{+/+}$*/Siah2*$^{+/-}$ or *Siah1a*$^{+/-}$*/Siah2*$^{-/-}$) were grown under normoxic (20%) or hypoxic (1%) conditions for 24hr. Mitochondria were visualized with mitoTracker (Invitrogen) and quantified. Data were presented as mean ±SD. Scale bars indicate 10μm.

Figure S3

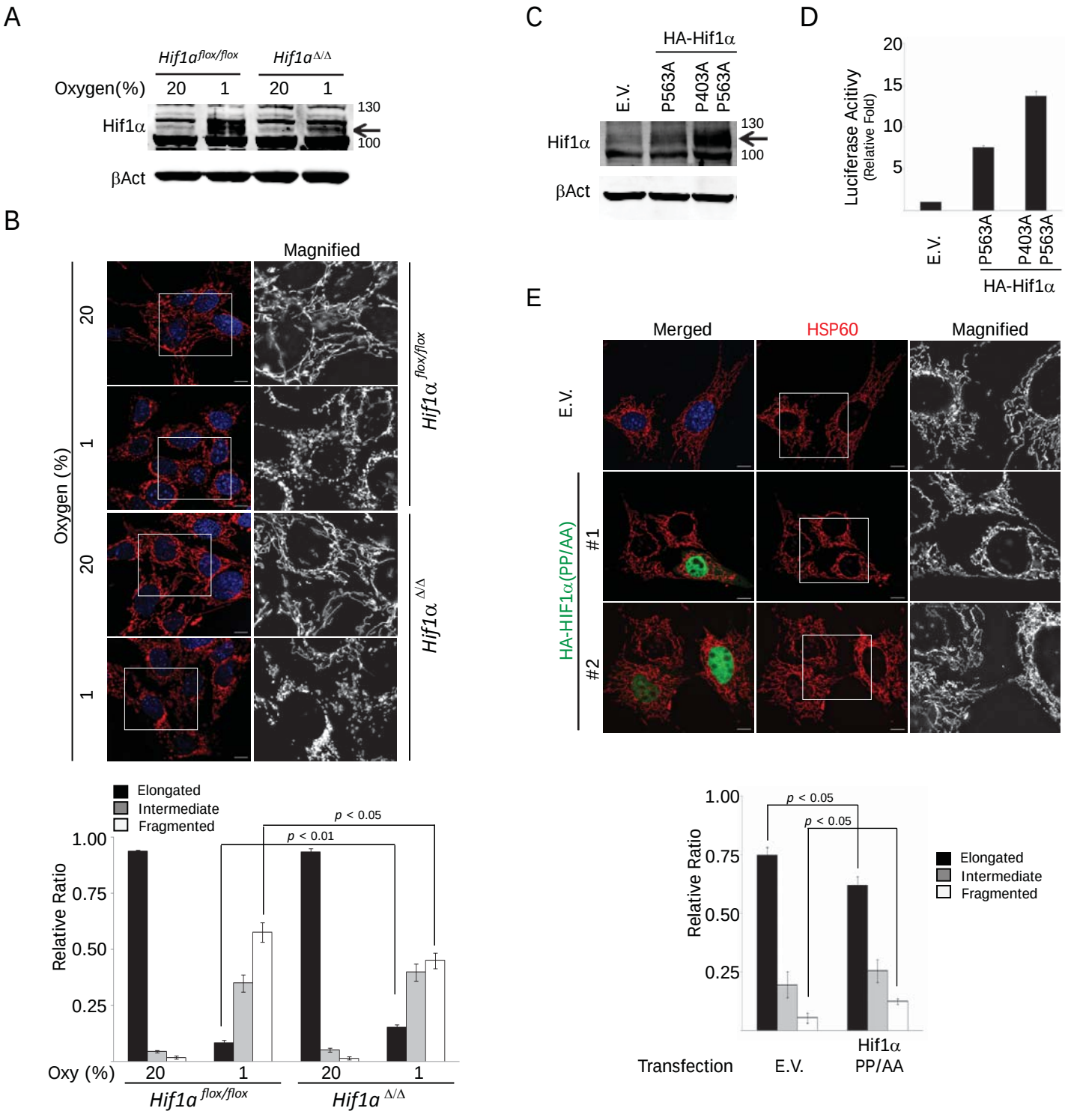
A
Hif1α flox/flox
Hif1α Δ/Δ
Oxygen(%)
20
1
20
1
Hif1α
130
100
βAct
C
HA-Hif1α
E.V.
P563A
P403A
P563A
Hif1α
130
100
βAct
D
Luciferase Acitivy
(Relative Fold)
20
15
10
5
E.V.
P563A
P403A
P563A
HA-Hif1α
B
Magnified
Oxygen (%)
20
1
20
1
Hif1α flox/flox
Hif1α Δ/Δ
E
Merged
HSP60
Magnified
E.V.
HA-HIF1α(PP/AA)
#1
#2
Elongated
Intermediate
Fragmented
p < 0.05
p < 0.01
Relative Ratio
1.00
0.75
0.50
0.25
Oxy (%)
20
1
20
1
Hif1α flox/flox
Hif1α Δ/Δ
p < 0.05
p < 0.05
Relative Ratio
1.00
0.75
0.50
0.25
Elongated
Intermediate
Fragmented
Transfection
E.V.
Hif1α
PP/AA

Figure S3. **HIF-1α is dispensable for hypoxia-mediated mitochondria fission. Related to Figure 2**. (A) Double floxed HIF1α MEFs (*HIF1α*$^{flox/flox}$) and HIF1a null MEFs (*HIF1α*$^{\Delta/\Delta}$) were cultured under normoxia or hypoxia for 5hr. Cell lysates were obtained and analyzed with anti-HIF1α antibody. The arrow indicates HIF1α. (B) *HIF1α*$^{flox/flox}$ and *HIF1α*$^{\Delta/\Delta}$ MEFs were maintained under indicated oxygen concentration for 24hr. Mitochondria were visualized with anti-Tom20 antibody and quantified from 300-400 cells from three independent slides. Data were presented as mean ±SD. Scale bars indicate 10μm. (C) HEK293T cells were transfected with empty vector (E.V.), HA-tagged HIF1α (P563A) or HIF1α (P402A/P563A) mutant. Cell lysates were obtained and analyzed with anti-HIF1α antibody. The arrow indicates HIF1α. (D) NIH3T3 cells were transfected with empty vector, HA-tagged HIF1α (P563A) or HIF1α (P402A/P563A) mutant along with 3XHRE-luciferase plasmid. Lysates were obtained and subjected to assess luciferase activity. The luciferase activity was normalized with β-galatosidase activity. Relative fold of luciferase activity was calculated by setting a value from the control group as 1. Data were presented as mean ±SD. (E) NIH3T3 cells transfected with empty vector or HA-HIF1α (P402A/P563A) mutant. After 24hr of culture, cells were stained with anti-HA antibody (HA-HIF1α, green). Mitochondria were visualized with anti-Hsp60 (red) and nucleus with DAPI (blue). Images were representative of transfectants. Mitochondria morphology was quantified from 300-400 cells from three independent transfections. Data were presented as mean ±SD. Scale bars indicate10μm.

Figure S4

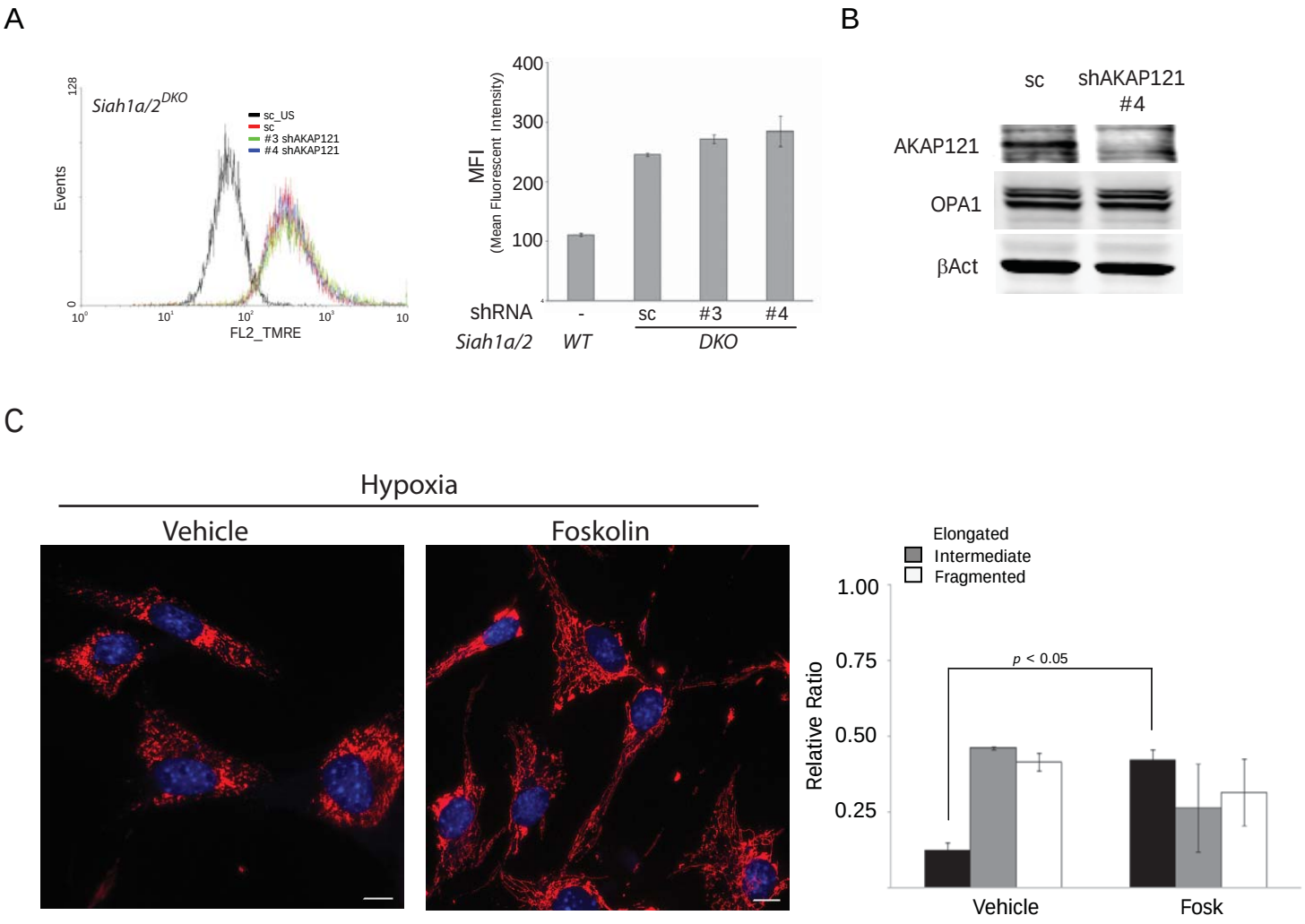

Figure S4. PKA-dependent regulation of mitochondria dynamics by AKAP121. Related to Figure 4. (A) Mitochondrial membrane potential of *Siah1a/2*^{DKO} MEFs transduced with scrambled (S.C.) or 2 different shRNAs against AKAP121 was measured as described in experimental procedures. Unstained cells (S.C._US) served as a control. Mean fluorescent intensity (MFI) values from 3 independent experiments were calculated and presented as mean ±SD. (B) Lysates obtained from *Siah1a/2*^{DKO} MEFs transduced with scrambled shRNA or shAKAP121 were analyzed with indicated antibodies. (C) NIH3T3 cells expressing mito-RFP were grown for 24hr under hypoxia in the presence of vehicle (DMSO) or forskolin (10μM). Mitochondrial morphology in 200 cells was analyzed and quantified according to established criteria (Figure S1A). Data were presented as mean ±SD.

Figure S5

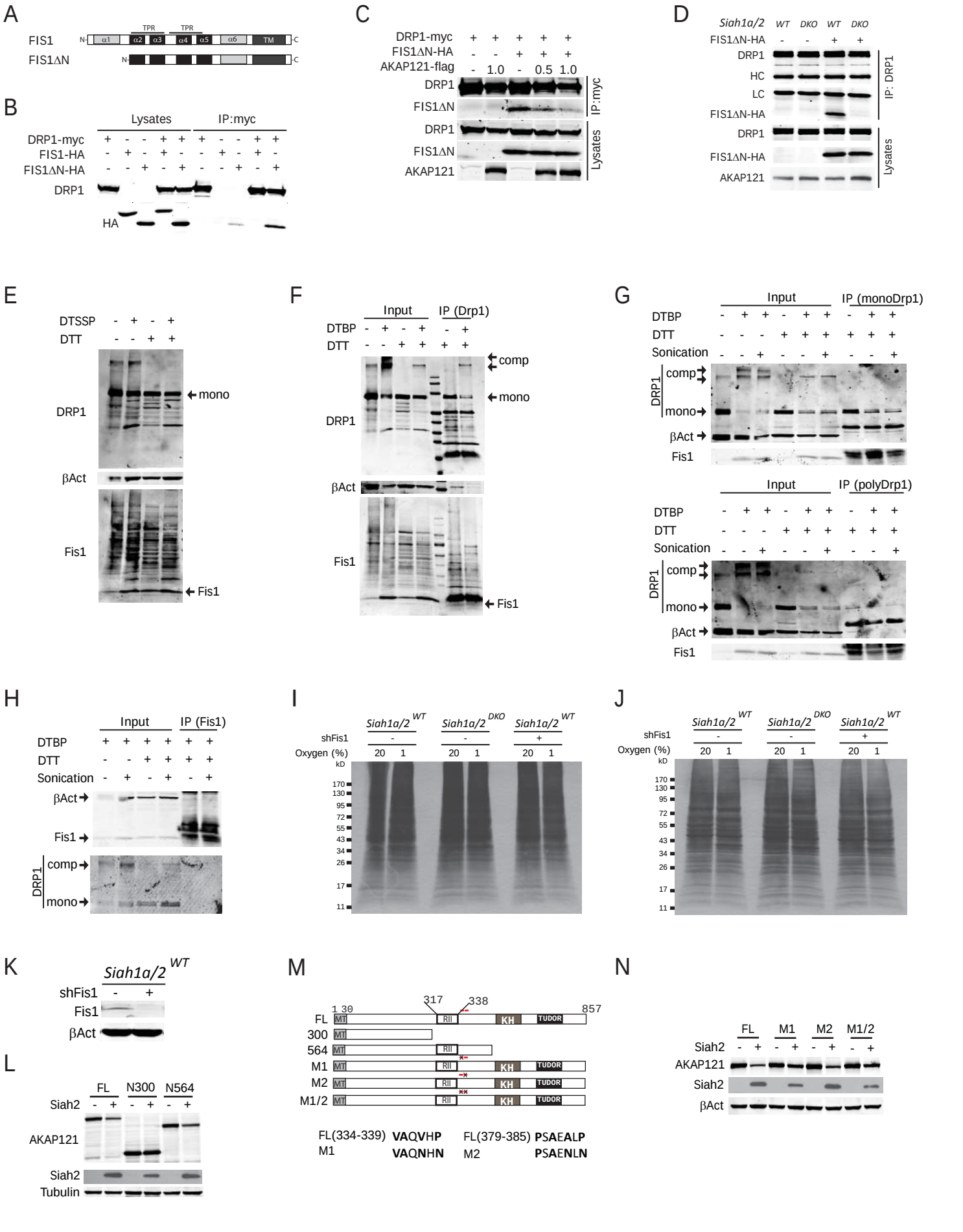
A
FIS1
FIS1ΔN
TPR
TPR
α1
α2
α3
α4
α5
α6
TM
B
Lysates
IP:myc
DRP1-myc
FIS1-HA
FIS1ΔN-HA
DRP1
HA
C
DRP1-myc
FIS1ΔN-HA
AKAP121-flag
DRP1
FIS1ΔN
AKAP121
IP:myc
Lysates
D
Siah1a/2
WT
DKO
WT
DKO
FIS1ΔN-HA
DRP1
HC
LC
FIS1ΔN-HA
DRP1
FIS1ΔN-HA
AKAP121
IP: DRP1
Lysates
E
DTSSP
DTT
DRP1
mono
βAct
Fis1
Fis1
F
Input
IP (Drp1)
DTBP
DTT
comp
mono
DRP1
βAct
Fis1
Fis1
G
Input
IP (monoDrp1)
DTBP
DTT
Sonication
DRP1
comp
mono
βAct
Fis1
Input
IP (polyDrp1)
DTBP
DTT
Sonication
DRP1
comp
mono
βAct
Fis1
H
Input
IP (Fis1)
DTBP
DTT
Sonication
βAct
Fis1
DRP1
comp
mono
I
Siah1a/2 WT
Siah1a/2 DKO
Siah1a/2 WT
shFis1
Oxygen (%)
20 1 20 1 20 1
kD
170
130
95
72
55
43
34
26
17
11
J
Siah1a/2 WT
Siah1a/2 DKO
Siah1a/2 WT
shFis1
Oxygen (%)
20 1 20 1 20 1
kD
170
130
95
72
55
43
34
26
17
11
K
Siah1a/2 WT
shFis1
Fis1
βAct
L
FL
N300
N564
Siah2
AKAP121
Siah2
Tubulin
M
1 30
317
338
857
FL
300
564
M1
M2
M1/2
MT
RII
KH
TUDOR
FL(334-339) VAQVHP
M1 VAQNHN
FL(379-385) PSAEALP
M2 PSAENLN
N
FL
M1
M2
M1/2
Siah2
AKAP121
Siah2
βAct

Figure S5. Central domain of AKAP121 regulates Drp1-Fis1 interaction and contains Siah-degron motif. Related to Figure 5. (A) Human Fis1 is composed of 6 alpha helixes and one trnsmembrane domain at Carboxy-terminus. The central four helixes constitute two tandem repeat TPR (tricopeptide repeats) domains. The N-terminal arm (31 amino acids) of Fis1 was deleted. (B) HEK293T cells were transfected with AKAP121-flag and full length wildtype hFis1 or N-truncated hFis1. Lysates were immunoprecipitated and blotted with indicated antibodies. (C) Cells were transfected with Drp1 and/or Fis1ΔN and increasing amount of AKAP121. lysates were obtained and analyzed by immunoprecipitation with anti-myc (Drp1), followed by immunobloting with anti-myc (Drp1), HA(Fis1ΔN) and flag (AKAP121) antibodies. (D) *Siah1a/2*^{WT} and *Siah1a/2*^{DKO} MEFs transfected with empty vector or Fis1ΔN were grown for 24hr under normoxia. Cell lysates were obtained, immunoprecipitated with anti-Drp1 antibody and immunoblotted with anti-HA (Fis1ΔN) and indicated antibodies. HC and LC indicate immunoglobulin heavy and light chain, respectively. (E) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTSSP. Cell lysates treated with or without DTT were analyzed using indicated antibodies. (F) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTBP. Cell lysates were treated with DTT as described or immuoprecipitated with anti-Drp1 antibody. The blot was analyzed with indicated antibodies. (G) *Siah1a/2*^{WT} MEFs were treated with vehicle or DTBP. Cell lysates for input were prepared as described with or without sonication for 30 min. Lysates were immunoprecipitated with monoclonal anti-Drp1 (monoDrp1, BD Bioscience) or polyclonal anti-Drp1 (polyDrp1, Santa Cruz). The blots were analyzed with indicated antibodies. “mono” and “comp” represent monomer and oligomer or complex of Drp1, respectively. (H) Cell lysates from DTBP-treated *Siah1a/2*^{WT} MEFs were immunoprecipitated with anti-Fis1 (Santa Cruz). (I, J) The indicated MEFs were cultured under normoxia (20%) or hypoxia (1%) for 24hr with 4hr of labeling during last 4hr. After cross-linking whole cells, lysates were prepared, treated with vehicle (I) or DTT(J) and subjected to SDS-PAGE. (K) Cell lysates from *Siah1a/2*^{WT} MEFs that were subjected to knockdown of Fis1 with corresponding shRNA were analyzed with indicated antibodies. (L) HEK293T cells were transfected with indicated truncated constructs of AKAP121 and/or Siah2. Lysates were immunblotted using indicated antibodies. (M) Two candidate sequences of Siah-degron in central domain of AKAP121. (N) HEK293T cells were transfected with Siah2 and wildtype (FL) construct or mutant constructs (M1 or M2, M1/2). Lysates were analyzed using indicated antibodies.

Figure S6

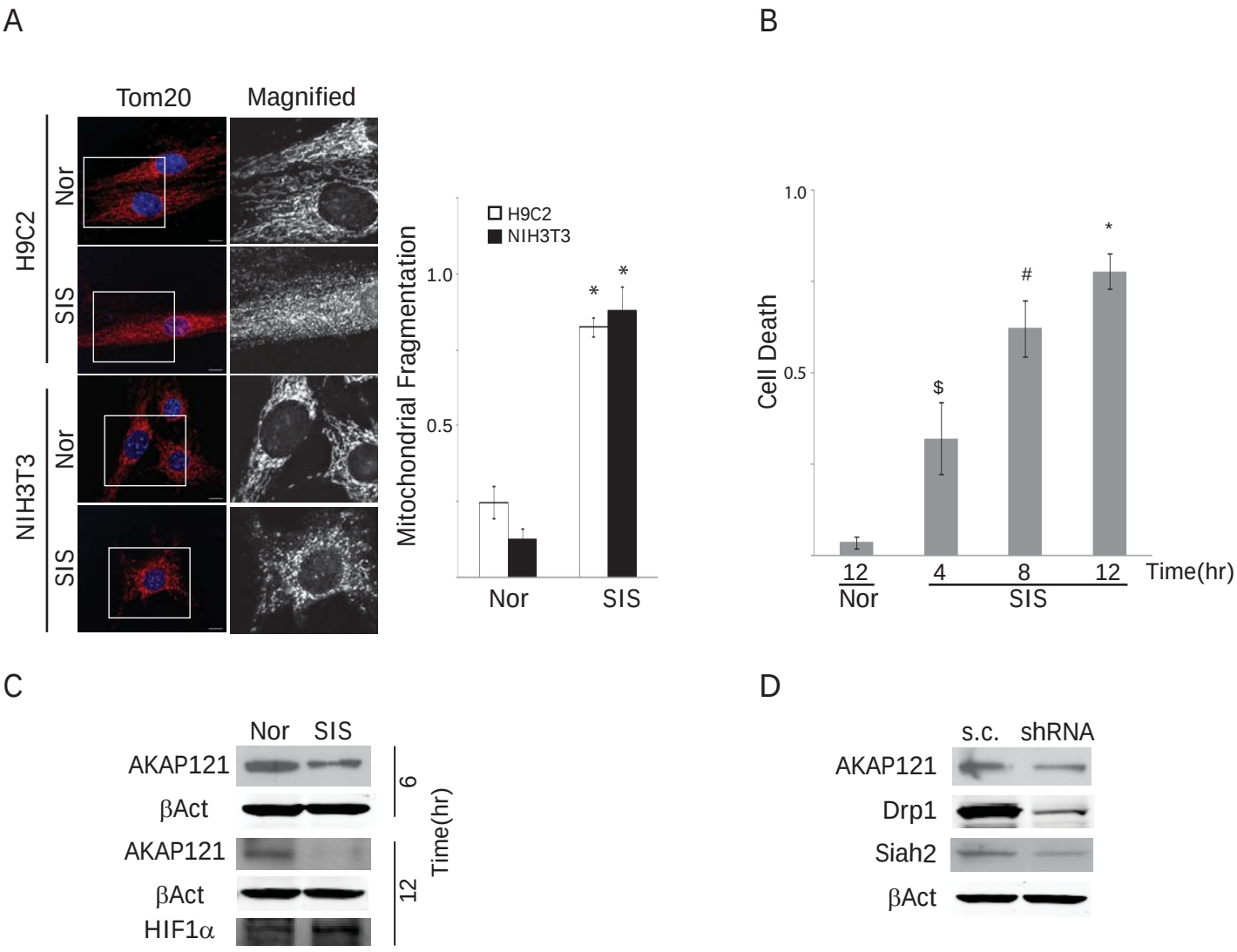

Figure S6. Siah2-mediated mitochondria dynamics is associated with ischemia- induced cell death of cardiomyocytes. Related to Figure 6. (A) H9C2 and NIH3T3 cells were cultured under Nor (Normoxia; 20% oxygen, presence of serum and glucose) or SIS (simulated ischemic in vitro; 0% oxygen, absence of serum and glucose) condition for 12hr. The morphology of mitochondria was visualized with anti-Tom20 antibody and quantified by counting cells with fragmented mitochondria. Data were presented as mean ±SD. The asterisk indicates $p<0.001$. (B) H9C2 cells were cultured under the ischemic condition (as in (A)) for indicated time. The cell death rate was quantified by counting cells stained with propidium iodide. Data were presented as mean ±SD. *, # and $ indicate $p<0.001$, $p<0.005$ and $p<0.05$, respectively. (C) H9C2 cells were cultured as in (A) for indicated time. Cell lysates were analyzed using anti-AKAP121, HIF-1α antibodies. (D) H9C2 cells were transduced with lentivirus expressing indicated shRNA. Cell lysates were obtained and analyzed with indicated antibodies.

Figure S7

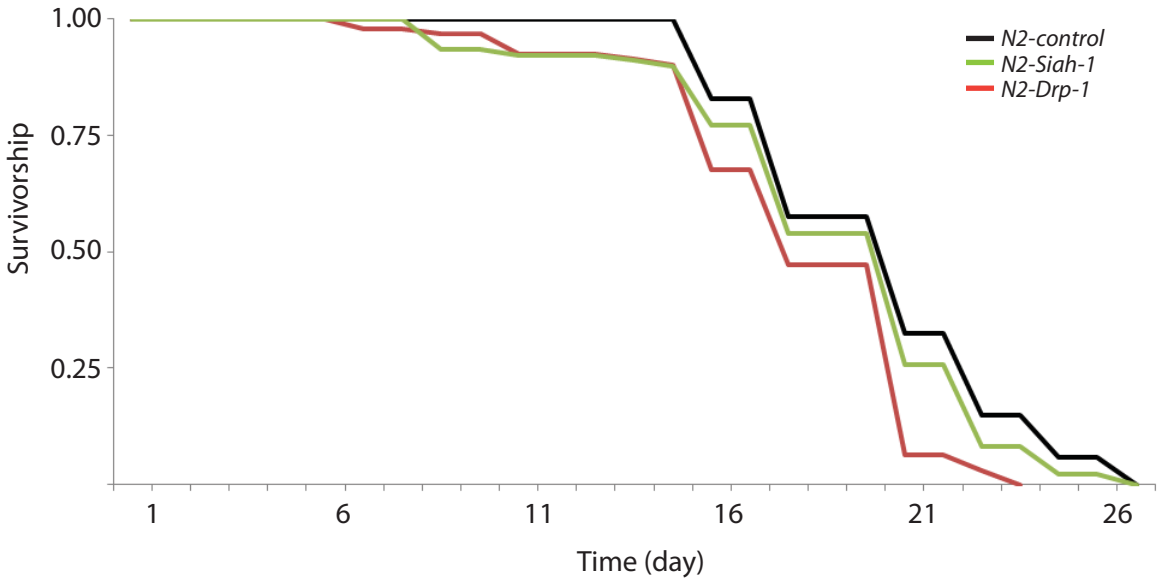

Figure S7. Depletion of *siah-1* or *drp-1* from adulthood shows less influence on the life span of C. elegans. Related to Figure 7. RNAi-mediated depletion of *C.elegans drp-1* and *siah-1* resulted in reduction of mean lifespan *N2-control* (black line, mean 20.3±0.3 days*), N2-drp-1* (red line, 18.5±0.4 days), *N2-siah-1* (green line, 19.4±0.4 days). RNAi depletion was initiated from adulthood.

Fine-tuning of Drp1/Fis1 availability by AKAP121/Siah2 regulates mitochondrial adaptation to hypoxia

Hyungsoo Kim, Maria C. Scimia, Deepti Wilkinson, Ramon D. Trelles, Malcolm R. Wood, Andrew Dillin, David Bowtell, Mark Mercola & Ze'ev A. Ronai

Supplemental Experimental Procedures.

MEF preparation, Cell culture and Hypoxia Treatment

MEFs were prepared from embryos at embryonic day (E11 or E12) and maintained in DMEM supplemented with 15% fetal bovine serum, penicillin/streptomycin and 0.2mM 2-mercaptoethanol (Sigma). Genotypes of MEFs were analyzed with primer sets described previously (Frew et al., 2003). Primary MEFs with less than 7 passages were used for experiments. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum, penicillin/streptomycin and 25mM HEPES (pH7.4). For hypoxic treatment (1% oxygen), cells were incubated in hypoxia work station (In vivo2 400; Ruskin Corp).

Reagents and Antibodies

MitoTracker Red CMXRos was purchased from Molecular Probes. Foskolin and cyclosporine were purchased from Sigma. Antibodies used were obtained as follows; antibodies to flag-tag and Siah2 from Sigma; antibodies to HA-tag from Covance or Cell Signaling; antibodies to myc-tag, AKAP149 (specific to mouse AKAP121), Fis1, Tom20, Mfn1 and Mfn2 from Santa Cruz; antibodies to Drp1, OPA1, and Hsp60 from BD bioscience; antibody to phopho-Drp1 (S637) was kindly provided by Dr. Blackstone (Chang and Blackstone, 2007); antibody to β-actin, tubulin and Complex 1 (NDUFB8) were from Invitrogen.

DNA Constructs, Transfection and Transduction

Constructs encoding HA or flag-tagged Siah1a and Siah2 were previously described (Nakayama et al., 2004). PCR products of Siah2 and Siah2 ring domain mutant (RM) were subcloned into pEGFP-N1. Plasmids encoding mouse AKAP121 (NM_001042541) and mouse Fis1 (NM_25562) were constructed by subcloning of PCR product from MEF's cDNA into pcDNA3.0-C-flag and pCMV-N-HA. The series of truncated mutant of AKAP121, Fis1ΔN and HA-tagged Drp1ΔGTPase (deletion of 256 amino acids from N-terminus) were generated by subcloning of corresponding PCR products in to pcDNA3.0-C-flag or

pCMV-N-HA plasmid. Deletion mutants (ΔRIIs), Siah-degron mutants of AKAP121 and phosphorylation site mutants (S637A and S637D) of Drp1 were generated by site-directed mutagenesis (Staratagene). AKAP121(MT-351/446) plasmid was generated by sequential subcloning of mitochondria targeting sequences (1 to 34) into HindIII/BamH1 site and central domain (351 to 446) into BamH1/EcoRI site of pcDNA3.0-C-flag vector. Plasmids encoding Drp1 and Drp1 (K38A) mutant were kindly provided by Dr. Blackstone (Nantional Institute of Health), mito-RFP from Dr. Chan (California Institute of Technology). HIF1α mutants [P563A or P402A/P563A] were generated by subcloning to pcDNA plasmid from pBMN-HIF1 α (kindly provided by Dr. Ratcliffe, University of Oxford) and following site-directed mutagenesis (Stratagene). HEK293T, NIH3T3 and MEFs cells were transfected using calcium phosphate, Lipofectamine 2000 (invitrogen) or JetPrime (Polyplus-transfection) according to manufacturer's direction. Retro- or lenti virus expressing appropriate gene were spin-infected to cells [1600xg for 30min at room temperature].

Gene Silencing

pLKO-scrambled and pLKO-mSiah2 were kindly provided by Olexander Korchynsky and pRetroSuper-Siah2 was constructed using previously reported sequence (Hara et al., NCB 2005). pLKO-shAKAP121, pLKO-shFis1, and pLKO-shDrp1 were purchased from Openbiosystems. The efficiency of silencing was validated from cell lysates of stable clones using appropriate antibodies or RT-PCR analysis of mRNA.
For RT-PCR analysis of Siah2 mRNA, the following primer set was used; 5'-GCTGAGAACTTTGCCTACAG-3' and 5'- GCTATGCCCAAATAACTTCC-3'.

Immunocytochemistry

Mitochondria were stained as follows; cells expressing mito-RFP were fixed with 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS, pH7.4) for 15 min; cells were stained with MitoTracker red

(20 – 40nM; Invitrogen) for 30min and subsequently fixed as described; cells were fixed as described and then permeabilized with 0.2% TritonX-100 in PBS (pH7.4) for 10 min at room temperature. After blocking with 3% bovine serum albumin (BSA) in PBS, mitochondria were stained with anti-Tom20 (1:300) or anti-HSP60 (1:500) antibody for 1hr at room temperature or overnight at 4°C. The fixation of cells cultured under hypoxia was performed in interlock (anaerobic and room temperature) of hypoxia work-station. For staining of ectopically expressed protein, cells were fixed, permeabilized and blocked as described. Cells were stained with appropriate antibodies [flagM2 (1:750)(Sigma), HA(1:750)(Cell Signaling) and Myc(1:500) (SantaCruz)] for 1hr at room temperature or overnight at 4°C. After rinsing with PBS, coverslips were incubated with Alexa350, Alexa488, or Alexa568-conjugated antibodies (Invitrogen). Image data were obtained using Olympus TH4–100 microscope and deconvolved through constrained iterative algorithm using Slidebook 4.1 digital microscopy software. Z projection images were built using NIH Image J software. For TUNEL-staining we used TUNEL-Biotin-Avidin-HRP kit (Promega). Images were taken using Aperio System and Tunel positive cells were manually counted using Imaje J software (cell counter plugin).

Immunoprecipitation and Western Blotting

Cell lysates were obtained from HEK293T cells expressing appropriate proteins using lysis buffer containing 1% TritonX-100. Pre-cleared cell lysates were incubated with control mouse IgG (Santa Cruz), anti-flag (M2, Sigma), anti-myc (9E10, Santa Cruz) or anti-HA (Covance) antibody at 4°C overnight. Lysates were added with protein A/G agarose (Santa Cruz) and further incubated for 2hr at 4°C. After extensive washing with lysis buffer, samples were added with Laemmli buffer, boiled and subjected to SDS-PAGE. For detecting Drp1-Fis1 interaction in MEFs, cell lysates were prepared from cells and incubated with anti-Drp1 (1ug/mg of protein; BD bioscience) antibody. Precipitation and analysis were performed as described.

Subcellular Fractionation

Cells were washed with PBS (pH7.4) and rinsed with ice-cold isolation buffer [5mM HEPES, 4.3mM MgCl2, 1mM EGTA, 250mM Sucrose, protease inhibitor cocktail (Sigma), phosphotase inhibitor cocktail I and 2 (Sigma)]. Suspended cells in Buffer A were homogenized with 30-40 strokes and centrifuged at 700Xg for 10min at 4°C. The post-nuclear supernatants (PN fraction) were further centrifuged at 10,000Xg for 10min at 4°C. The post-heavy membrane supernatant (PHM fraction) were collected and mitochondria-enriched heavy membrane pellet (HM fraction) were rinsed twice with isolation buffer. HM pellets were lysed with buffer containing 1% TritonX-100. To get pure mitochondria for analysis of Siah2 translocation, HM fractions were layered over sucrose gradient (1.5M/1M) and centrifuge at 200,000Xg for 45min. Hazy phase was collected and lysed as described.

Metabolic Labeling and Cross-Linking

MEFs were cultured under normoxia or hypoxia (1% oxygen) for 20hr. After washing with labeling media (DMEM-Met/Cys-free, Invitrogen), cells were labeled by incubation for 4hr in labeling media [DMEM-Met/Cys-free with of Expre^{35}S^{35}S protein labeling mix (50μCi/ml), PerkinElmer]. For cross-linking intracellular proteins, cell were washed with PBS and incubated with cross-linking solution [2.5mM DTBP (Dimethyl 3,3'-dithio-*bis*-propionimidate, Sigma) in PBS] for 30min. Reaction was quenched by adding Tris-HCl (50mM, pH7.5) for 15min. Lysates were obtained by lysing cells with lysis buffer (50mM Tris-HCl, pH7.4, 1% triton-X 100, 150mM NaCl, protease inhibitor cocktail, phosphatase inhibitor cocktail) and subjected to immunoprecipitation with anti-Drp1 (BD Bioscience). Samples were washed, incubated at 37°C for 30min in the presence of 150mM DTT and boiled with Laemmli sample buffer. Samples are subjected to SDS-PAGE, electrotransfer and finally visualized by autoradiography. Membrane –impermeable cross-linker, DTSSP (3,3-Dithio-bis-(sulfosuccinimidyl) propionate; Sulfo-DSP),

was used as control cross-linker to verify the efficient cross-linking by DTBP (Figure S5E). In brief, cells were treated with 1mM DTSSP for 30 min. Cell lysates were obtained as described and treated with DTT (50mM) at 37°C for 30 min.

In Vitro, Simulated-Ischemia Model

Medium was changed with ischemic media (IS) [DMEM base (Cellgro) without glucose and serum, pre-flushed with N_2/ CO_2(95% /5%) gas mixture] or normoxic media [IS media supplemented with 4.5 g/l glucose, 4mM L-glutamin and 10% fetal bovine serum]. Cells were further incubated in air-tight modular hypoxia chamber which was flushed with N_2/ CO_2 gas mixture. For analysis of cell death, cells were stained with propidium iodide (1 μg/ml) for 3 min. After twice washing of cells with PBS, PI positive cells were counted in, at least, 10 different fields.

In Vivo, Myocardial Infarction Model

Myocardial infarction was performed as described (Denzel et al., 2010). 8- to 10-week-old mice with genotypes, $Siah1a^{WT}/Siah2^{WT}$ and $Siah1a^{HT}/Siah2^{KO}$ mice were anesthetized with ketamine (100mg/Kg)-xylazine (2.5mg/Kg). The chest cavity was entered by blunt dissection of the fourth intercostal space, 2 mm away from the left sternal border. Opening the pericardial sac exposed the heart. The left anterior descending artery was ligated with a 6-0 silk suture about 1–2 mm beneath the tip of the left auricleatrium. The lungs were reinflated with a small catheter left in the thorax to evacuate air and fluids. The chest cavity was closed, and the mouse was extubated and maintained on a warm pad. 24 hours after myocardial infarction, echocardiography was performed to assess cardiac function. Animals were anesthetized with ketamine (100mg/Kg)-xylazine (2.5mg/Kg) and 0.5 ml of a 1% Evans blue (Sigma-Aldrich) solution was injected at the apex of the left ventricle (LV) to identify the non ischemic tissue (perfused area). The heart was then excised, washed in PBS, and cut into 5 transverse slices, which were

subsequently stained in 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 15 minutes at room temperature to aid visualization of the infarcted area (IA). Digital images of heart sections were analyzed for AAR (Evans blue negative) , and infarcted area (TTC negative) LV area (Evans blue positive and negative) using Image J software.

Echocardiographic analysis

Cardiac function and heart dimensions were evaluated by two-dimensional echocardiography using a Visual Sonics Vevo 770 Ultrasound (Visual Sonics, Canada) and a 13-MHz linear array transducer. M-mode tracings were used to measure anterior and posterior wall thicknesses and LV internal diameter at end diastole and end systole at the level of the papillary muscles. LV fractional shortening (FS) was calculated according to the following formula: FS (%) = [(LVIDd - LVIDs)/LVIDd] x 100. Four short axis images were taken in parasternal short axis view at different levels, from the mitral valve to the apex to trace the endocardial border in systole and in diastole, and in parasternal long axis view the left ventricle length from the aortic annulus to the apex level in diastole and systole were measured. EF% was then automatically calculated as summation of the areas of the 4 sections using the Simpson's rule, $V=(A_1+A_2+A_3+A_4/2)h+(\pi/6)h$, for which V=volume, A=LV chamber cross-sectional area of the individual sections, h=LV length/4. The data were analyzed by a single observer blinded to mouse genotype.

Electron microscopy

Cells grown under normoxia or hypoxia were prepared for electron microscopy using previous method (Gilula et al., 1978). Briefly this consisted of fixing the cells in 2.5% glutaraldehyde in 0.1M cacodylate buffer and after a brief buffer wash, postfixing in 1% osmium tetroxide. Following a further buffer wash, the cells were treated with tannic acid, sodium sulfate and dehydrated in a graded ethanol series. The cells were cleared in 2-hydroxypropyl methacrylate (HPMA) and embedded in LX112 (Ladd Research,

Williston, VT). Thin sections were prepared, mounted on copper mesh grids coated with parlodion and stained with uranyl acetate and lead citrate. Sections were observed in the Electron Microscope Hitachi H-600A, at 75kV and at different instrumental magnifications. Digital images were acquired with 11.2 megapixel cooled monochromatic CCD camera L9C (SIA, Atlanta) controlled by the MaxIm DL software (Diffraction Limited, Ottawa, Ontario, Canada). For mitochondria analysis, the length of mitochondria of cells in 4 different slices was measured using NIH Image J software.

The electron microscopic analysis of hearts was performed as followings: In preparation of heart sections for EM, mice were initially perfused with saline followed 4% paraformaldehyde, 1.5% glutaraldehyde in 0.1M Na cacodylate buffer all at room temperature. The heart was removed and the left ventricle sliced into thin strips to continue fixation in the above fixative for 6 hours at 4˚C before being transferred to cacodylate buffered 3% glutaraldehyde overnight at 4˚C. The tissues were then buffer washed, post fixed in buffered 1% osmium tetroxide and subsequently dehydrated in graded ethanol series, transitioned in propylene oxide and embedded in EMbed 812 / Araldite (Electron Microscopy Sciences, Hatfield PA). Thick sections (2µm) from the block with the ligature were cut close to the injury site, mounted on glass slides and stained in toluidine blue for general injury assessment in the light microscope. Regions of the left ventricle distant from the ligation site were also examined. Subsequently, 70nm thin sections were cut using a diamond knife (Diatome, Hatfield PA) mounted on copper slot grids coated with parlodion and then stained with uranyl acetate and lead citrate for examination on a Philips CM100 electron microscope (FEI, Hillsbrough OR). Images were documented with a Megaview III CCD camera (Olympus Soft Imaging Solutions, Lakewood CO).

Analysis of life span in C. elegans

Lifespan assays were performed as previously described (Mair et al., 2011). Briefly, 110 wild-type (N2 Bristol strain) animals were used for each RNAi condition and scored every day or every other day for

survivorship. Animals that died prematurely (exploded at their vulva, internally hatched their embryos, crawled off the plate) were censored. The animals were maintained at 20°C during the entire duration of life-span analysis. Statistical analysis was performed using JMP IN 8 software. In all cases, P-values were calculated using the log-rank (Mantel–Cox) method.

Assessment of Luciferase Activity

For confirming activities of HIF1α mutants, 3XHRE_luciferase and β-galactosidase plasmids were transfected with HIF1α mutants (P563A or P402A/P563A) to NIH3T3 cells. After 24hr of culture, cell lysates were obtained and subjected to analysis of luciferase activity using luciferase assay system (Promega). β-galactosidase activities in the lysates were used for normalization of luciferase activity.

Measurement of mitochondrial activities

For measuring mitochondrial membrane potential, TMRE (150nM, Invitrogen) in culture medium was pre-conditioned under hypoxia (1% O_2). For depolarized control, CCCP (50μM, Sigma) were pre-treated for 1hr before adding TMRE. After addition of TMRE solution, cells were further incubated for 30min, subsequently rinsed with warmed PBS and trypsinized. Cells were washed with warmed PBS supplemented with 5% FBS and analyzed using FACSCanto. Data obtained were analyzed using WinMDI (ver 2.9; TSRI).

Quantitation and Statistical Analysis

For evaluation of morphological difference of mitochondria, 100-300 cells of coverslips from three independent experiments were examined. Unless specified, data were presented as mean ±SD and significance of differences were analyzed with two-tailed, unpaired t-Test. Difference with *p* values less than 0.05 was considered as significant.

Supplementary References

Denzel, M.S., Scimia, M.C., Zumstein, P.M., Walsh, K., Ruiz-Lozano, P., and Ranscht, B. (2010). T-cadherin is critical for adiponectin-mediated cardioprotection in mice. J Clin Invest 120, 4342-4352.

Gilula, N.B., Epstein, M.L., and Beers, W.H. (1978). Cell-to-cell communication and ovulation. A study of the cumulus-oocyte complex. J Cell Biol *7*, 58-75.

Mair, W., Morantte, I., Rodrigues, A. P., Manning, G., Montminy, M., Shaw, R. J., and Dillin, A. (2011). Lifespan extension induced by AMPK and calcineurin is mediated by CRTC-1 and CREB. Nature 470, 404-408.