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Imaging Supplements

I. Technical parameters of confocal apparatus

Photoconversion of PA-GFP_{mt} to its active (fluorescent) form was achieved by using 2-photon laser (750 nm) to give a 375 nm photon-equivalence at the focal plane. This allowed for selective activation of regions that have submicron thickness and are less than 0.5 μm^2 (Zeiss LSM510) and 1 μm^2 (Leica TCS SP2).

Using the multi-track scanning mode of the LSM-510 microscope, red-emitting TMRE was excited with a 1 mW 543 nm helium / neon laser set at 0.3% and emission was recorded through a BP 650–710 nm filter. Activated PA-GFP_{mt} protein was excited using a 25 mW 488 nm argon laser set at 0.2 %. Emission was recorded through a BP 500–550 nm filter. LysoSensor Blue was excited using 2-photon cameleon laser at 750 nm set at 0.2 %. Emission was recorded through a BP filter set at 400-450 nm.

II. Tracking $\Delta\psi_m$ of a single mitochondrion

Mitochondrial Membrane Potential Analysis

Alterations in TMRE fluorescence intensity (FI) can arise from changes in both mitochondrial membrane potential ($\Delta\psi_m$) and organelle movement-induced changes in the focal plane. We previously showed that calculating the ratio between the FI of TMRE and activated PA-GFP_{mt} can differentiate between the two scenarios. Mitochondrial movement affects the FI of both TMRE and PA-GFP_{mt} in an equivalent manner, leaving the TMRE / PA-GFP_{mt} ratio unaffected. Furthermore, since the FI of the PA-GFP_{mt} molecule is independent of $\Delta\psi_m$, the TMRE / PA-GFP_{mt} remains solely dependent on $\Delta\psi_m$. Therefore, for every time point, the red and green images were used to generate a ratio (TMRE/ PA-GFP_{mt}) image. Ratio data were then used to calculate the change in $\Delta\psi_m$. This analysis was performed on the entire mitochondrial mass or areas therein.

As described by Loew et. al., the $\Delta\psi_m$–dependent component of TMRE accumulates in mitochondria in a Nernstian fashion as reported by the intensity of its fluorescence. The non-$\Delta\psi_m$-dependent component of TMRE, also known as the binding component, can be ignored as it is fixed and voltage-independent (Loew *et al.*, 1993;O'Reilly *et al.*, 2003). A change in mitochondrial membrane potential can be

calculated using the following equation,

$$\Delta\psi_m = 61.5 * \log [(FI_{TMRE\_A} / FI_{TMRE\_B})*(FI_{PA\text{-}GFP\_B} / FI_{PA\text{-}GFP\_A})],$$

at 37 ^{o}C, where FI_{TMRE_A} and FI_{TMRE_B} represent the fluorescence intensities of TMRE at two time points and $FI_{PA\text{-}GFP\_A}$ and $FI_{PA\text{-}GFP\_B}$ represent the respective fluorescence intensities of PA-GFP_{mt}. This is a modified version of the Nernst equation that can be used to determine $\Delta\psi_m$ based on TMRE fluorescence (O'Reilly *et al.*, 2003).

Image processing and thresholding

Image processing and analysis was performed with Metamorph software (Molecular Device CA). The "Integrated Morphometry Analysis" function designed for these experiments extracted PA-GFP_{mt} positive structures that were larger than 10 pixels. These areas were interpreted to be mitochondria, and their TMRE FI were recorded. This procedure enabled the selection of mitochondrial structures using very low threshold levels in the green channel (approximately 10% of the image average intensity) assuring that over 90% of the mitochondrial pixels were included for analysis. To set the threshold level, a test-threshold function first measured the average green FI of the mitochondria. The lower (inclusive) threshold was set at two thirds of this average. Prior to analysis, all images were scanned to verify that all intensity measurements were below saturation. Therefore, an upper threshold was not necessary. To verify that GFP thresholding does not impose an artifact of cutting the mitochondrial objects short, we followed events in which mitochondrial GFP became diluted due to fusion between mitochondria and observed the expected expansion of the object as shown for example in Fig 2a. As the mitochondrial areas were defined by the green pixels (GFP), there was no need to apply threshold in the red channel (TMRE).

Accuracy of $\Delta\psi_m$ measurement using the ratio image

We previously showed that the TMRE/ PA-GFP_{mt} ratio has similar accuracy to multiple scans in the z-stack mode as long the captured mitochondrion size in a given plane is larger than 20% of its maximal cross section area (Twig *et al.*, 2006). Mitochondria that do not meet this criterion are associated with decreased signal-to-noise ratio and increase in $\Delta\psi_m$ fluctuations. Therefore, in experiments where individual

mitochondria were tracked, the maximal size of the mitochondrion was determined in the frame with the largest PA-GFP$_{mt}$ area of the object. Frames with image size smaller than 30% of the maximal mitochondrion's cross section were omitted from analysis.

Controlling for laser phototoxicity

Careful attention was dedicated to determine the dye concentrations and laser parameters that would prevent photodamage-induced artifacts. Laser illumination of TMRE can damage mitochondria and can cause severe $\Delta\psi_m$ fluctuations, destruction and depolarization of mitochondria.

We determined the minimum intensity and duration of laser exposure that initiated changes in $\Delta\psi_{ma}$ and/or mitochondrial morphology in cells treated with TMRE. The parameters utilized in the reported experiments were well below these thresholds.

To determine the safety limits of 2-Photon laser stimulation, in COS-7, INS-1, and beta cells, excitation was delivered over a wide range of intensities and durations. We found that excitation for 600 milliseconds/μm^2 at 1 mW laser intensity at the objective is the threshold dosage for both cell lines above which a reduction in mitochondrial membrane potential can be observed. All subsequent experiments using 2-photon illumination were conducted with duration of 150 ms/μm^2 and an intensity of 1 mW.

Spatial variance in $\Delta\psi_m$ within an individual cell

Non-homogeneous cytoplasmatic distribution of TMRE, oxygen or ATP/ADP may affect the distribution of $\Delta\psi_m$ within a cell. To test this possibility we performed the following experiments: (i) individual mitochondria in INS1 cells (n=11) were tracked over 30 minutes and the level of stability was assessed. During this time frame, significant movement was observed yet the standard deviation of $\Delta\psi_m$ was only 1.6 mV on average with measurements taken every 10 seconds. (ii) TMRE FI of mitochondria located near the nucleus and far from the nucleus were compared (50 marginal mitochondria and 50 peri-nuclear mitochondria in each cell for a total of 14 cells). We found no statistical difference between the two groups (P=0.78).

Spatial variance in the excitation strength and emission detection sensitivity

Spatial variability in excitation strength and/or detection can bias $\Delta\Psi_m$ measurements making mitochondria in one part of the field appear different from those on the opposite side. To test this possibility, 10.2 μm fluorescent beads (Bangs Laboratories, Fishers, IN) were embedded in 1 % agarose gel on a glass bottom dish. Confocal images where recorded using 488 nm and 561 nm excitation lasers and analyzed similar to the live cells. The ratio of green and red fluorescence emission was quantified and the same mathematical approach used to calculate $\Delta\Psi$ differences was applied. The maximal differences in FI measured from the different beads was 4 % (n=10), which would be equivalent to 1 mV if these were measurements obtained from mitochondria having activated PA-GFP_{mt} and stained with TMRE.

III. Mitochondrial dynamics analysis and protocols

Mitochondrial fusion assay (whole cell)

We used the diffusion of PA-GFP_{mt} through the mitochondrial web as an index for mitochondrial fusion (Karbowski *et al.*, 2004). A region of interest (ROI) occupying ~10 % of the cell in one confocal section was activated using the 2-photon laser. The diffusion of activated PA-GFP_{mt} within the mitochondrial web was assessed by repeatedly scanning the entire cell along its z-axis (6 confocal sections) at 3 min time intervals. Quantification of fusion was performed by measuring the average intensity of the mitochondria that became PA-GFP_{mt} positive based on a fixed threshold. Diffusion of PA-GFP_{mt} reached a steady state within 40-50 min after photoactivation. At this point in time, mitochondria that retained non-diluted GFP FI (intensity that was identical to the measured GFP FI immediately after photoactivation) were defined as non-fusing mitochondria.

Cumulative probability analysis for fusion-fission clusters

The cumulative probability curve for a fusion or a fission event was fitted to the following hyperbolic function:

$$P=Pmax * t_i /(t_i+t_{50\%}),$$

where P is the cumulative probability for either a fusion or a fission event at a time point t_i. Pmax reflects the cumulative probability and was set as 1, $t_{50\%}$ denotes the time it took for 50% of a given type of event (fusion or fission) to occur. All fitting procedures and statistical tests were conducted using KaleidaGraph software (Synergy Software, Reading, PA). We predicted that if fusion and fission occur independently from each other, the cumulative probability for either event will follow a linear increase over time, rising from 0 to 100% at the calculated time interval between two consequent events (total imaging time/total number of fusion [or fission] events).

Mitochondrial Morphometry.

FIS1 knockdown and empty vector control cells were exposed to 10mM H_2O_2 for one hour prior to imaging of mitochondria with TMRE. The geometric properties of mitochondrial webs were measured using the 'particle analysis' feature on ImageJ (NIH). A single web is defined as a continuous cluster of (at least 10) TMRE-positive pixels, regardless of matrix continuity. For each web, the perimeter, area maximal and minimal aspect length were measured and the aspect ratio (AR; maximal aspect divided by minimal aspect) was derived.

Measuring organelle-specific autophagy*:* To study the organnells in the APs, proteolytic activity was inhibited using E64d (10 μg/ml) and pepstatin A (10 μg/ml) or Bafilomycin 0.2μM for a duration of 1-2 hrs (as stated per experiment). Total AP content was assessed by measuring clusters of LC3:GFP within 4 confocal sections for a given cell. An LC3:GFP cluster was identified by size and intensity using a morphometry filter in Metamorph analysis program. mtDsRed expression, Mitotracker red (MTR, 50 nM) and ER-Tracker (100 nM), both from Invitrogen, USA, were used to label mitochondria or ER respectively. The number of AP containing mitochondria or ER was assessed by the colocalization of the two signals.

OTHER TECHNIQUES

I. XF24 Oxygen consumption Assay (DRP1-DN cells and C2C12 cells).

XF Assays were performed as described in detail(Wu *et al.*, 2007). Briefly, cells seeded in XF plate were incubated in 600 µl of low buffered RMPI assay media at 37°C for 45 minutes to allow media temperature and pH to reach equilibrium before first measurement. Baseline oxygen consumption rate (OCR) was measured with the oxygen sensor cartridge in the XF24 Analyzer (Seahorse Bioscience, Billerica, MA). After the assay, cells were removed by trypsin and counted with ViCell (Beckman-Coulter, Fullerton, CA).

Basal OCR of mutant and control INS1 cells were normalized to basal OCR per million cells.

To determine maximal respiration capacity, mitochondrial uncoupler 2,4-DNP (Sigma, St. Louis, MO. USA) at increasing dosages was loaded into reagent ports in the XF24 sensor cartridge and injected into each well following baseline rate measurement. 3 additional responses OCR were measured immediate following injection.

Oxygen consumption in FIS1 RNAi and in ATG5 cells

Oxygen consumption in FIS1 RNAi and in ATG5 cells was measured as previously described (Corkey *et al.*, 1986). For additional details, see supplemental information.

Reactions were carried out in KRP buffer. O_2 consumption was measured at 25 $^{\circ}$C using a Clark-type O_2-sensitive electrode with amplifier in a stirred, water jacketed, closed, silicon-coated chamber. After recording the basal O_2 consumption, fuel substrates and other chemicals (FCCP) were added by injection through a pinhole while the O_2 consumption was monitored continuously.

II. Electron Microscopy

The cultured INS1 cells, FIS1 *RNAi* and Control, were trypsinized, spun and formed into a pellet. These were immediately fixed in Trump's solution (4% glutaraldehyde and 1%

formaldehyde), post-fixed in 1% osmium tetroxide in sodium cacodylate buffer for 3 hr at 20 degree C, and then stained with 5% aqueous uranyl acetate. Specimens were dehydrated with graded ethanol solutions and embedded in E-812. Thick sections (1um) were stained with toluidine blue (TB) for light microscopy. Thin sections (50-70 nm) were cut with a LKB8801 ultramicrotome, stained with uranyl acetate and lead citrate, and imaged with a Philip EM201 electron microscope.

III. Insulin secretion from INS-1 Cells

INS-1 832/13 cells were cultured in RPMI media supplemented with 10% FBS, 10 mM HEPES buffer, 1 mM pyruvate, 50 μM 2-ß-mercaptoethanol, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were used between passage 60 and 75. Prior to glucose-induced insulin secretion, cells were cultured for two hrs in RPMI containing 2 mM glucose without serum. Cells were then washed and preincubated for 30 min in modified Krebs-Ringer bicarbonate buffer (KRB) containing (in mM) 119 NaCl, 4.6 KCl, 5 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$, 0.15 Na_2HPO_4, 0.4 KH_2PO_4, 20 HEPES, 2 glucose, 0.05% BSA, pH 7.4. This was followed by a 30 min incubation in media containing either 2 mM or 8 mM glucose. Media was collected and stored at –20˚C for insulin measurement. Insulin was measured by radioimmunoassay (LINCO RESEARCH INC., St. Charles, MO.).

IV. Protein Oxidation Detection

Oxyblot™ protein oxidation detection kit (Chemicon International) was used according to manufacturer's manual. The technique is based on detection of carbonyl groups which are introduced into protein side chains when proteins are exposed to oxidative stress. Briefly, FIS1 siRNA and EV lentivirus - transduced INS1 cells were grown in 10cm tissue culture plates until confluence into incubator (37^oC, 5%CO2). Both adherent (alive) and floating (apoptotic or death) cells were harvested and pooled to proceed with mitochondrial isolation using the Pierce's Mitochondrial Isolation kit for tissue and following manufacturer's instructions. Once mitochondrial pellet was obtained, it was resuspended in 30ul of PBS of which 10ul were used for FIS1 and Porin (Complex III) immunoblot detection, 10ul for protein concentration determination (Bradford Reagent,

Biorad) and 10ul for Oxyblot. For the Oxyblot, 5ul of sample were used for positive derivatization on and the other 5ul for the negative one (control). Then, the derivatized samples were fractionated by PAGE followed by Western blotting. Western blots were quantitatively analyzed by densitometry with the ImageJ software.

V. Plasmid and viral construction

Construct	*Source of transgene*	*Used for*	*Mode of delivery*	*Vector plasmid*	*Ref*
Fis1 RNAi (shRNA)		Fis 1 knockdown	Lentivirus	pLVCTH	
OPA1 OE	cDNA from Dr. Scorrano	OPA1 over - expression	Adenovirus	Adenoeasy	
RNAi control	Tronolab	Control shRNA	Lentivirus	pLVCTH	
PWPI	Tronolab	Control for OE	Lentivirus	pWPI (Trono)	
DRP1-DN	cDNA from Dr. Van Der Bliek	expression of a dominant negative form of DRP1	Lentivirus	pWPI (Trono)	(Smirnova *et al.*, 2001)
mtDsRed	cDNA from Dr. Hajnoczky	Red fluorescent protein	Lentivirus	pWPI (Trono)	
mtPA-GFP lenti	cDNA from Dr. Lippincot-Shwartz	Expression of mtPAGFP in INS1 and COS7 cells	Lentivirus	pWPI (Trono)	
mtPA-GFP adeno	cDNA from Dr. Lippincot-Shwartz	Expression of mtPAGFP in beta cells	Adenovirus	Adenoeasy	
hFis1 OE	ATCC	Rescue of Fis1 KD effect	Lentivirus	pWPI (Trono)	
LC3:GFP	cDNA from Dr. Mizushima	Detection of autophagosomes	Lentivirus	pWPI (Trono)	

The preparation of a DNA plasmid containing mitochondrial matrix-targeted photo-activatable GFP (PA-GFP_{mt}) was previously reported by us in detail (Twig *et al.*, 2006).

The plasmid was delivered to the cells (~80% confluent) by a lentivirus carrying the PA-GFP_{mt} sequence under CMV-actin promoter. mtDsRed and DRP1-DN (DRP1-K38A), gifts from Gyorgy Hajnoczky, Thomas Gefferson University, Philladelphia, were similarly cloned into a lentiviral vector.

OPA1 overexpression adenoviral vector

The OPA1 cDNA was a gift from Luca Scorrano (Dulbecco Telethon Institute, Padova, Italy) and was cloned into Adenoviral pShuttle backbone vector. This was similarly cloned into the pShuttle backbone. Adenovirus was produced using the Adeasy system (Molecular Genetics Laboratory, Johns Hopkins Oncology Center, Baltimore, MA. USA).

RNAi and Cloning Procedures

FIS1 RNAi: For RNA interference studies, two complementary oligonucleotides were designed specific for mouse *FIS1* silencing. They harbor both MluI and ClaI sites at 5' and 3' ends respectively and a 5' phosphate modification. Sense: 5' cgc gtg ccc agg cat cgt gct gct gga gtt caa gag act cca gca gca cga tgc ctt ttt tgg aaa t 3', Antisense: 5' cga ttt cca aaa aag gca tcg tgc tgc tgg agt ctc ttg aac tcc agc agc acg atg cct ggg ga. The primers were annealed (heated up to 95^{o}C into water bath and then cooled down at room temperature in annealing buffer – 100 mM potassium acetate, 30 mM HEPES pH 7.4, 2 mM magnesium acetate) and cloned into pLVCTH lentiviral transfer vector at MluI and ClaI sites. The positive sh*FIS1*– pLVCTH clone was verified by DNA sequencing.

BECLIN1 RNAi: DNA fragments covering part of human Beclin-1 cDNA, 5 - tgaggatgacagtgaacag-3 were cloned in pSRP vectors in order to produce double-stranded RNAs as already described (Shibata *et al.*, 2006). Each pSRP vector was co-transfected in 293T cells with gag-pol-env constructs derived from Moloney leukemia virus, and virus-rich supernatants were used to infect human neuroglioma H4 cells. Stable cell lines with knock-down expression of beclin-1 and their respective control were established through puromycin selection (2-11 μg/ml).

An siRNA-resistant hFIS-1 was generated in order to rescue the Fis knocking down effect using PCR site directed mutagenesis. The commercial plasmid pOTB7 carrying on the CDS of human FIS1 (ATCC, MGC-10803) was amplified in a three step PCR

reaction. PmeI restriction site at both ends and three silent mutations (underlined) in addition to the one coming from the human FIS gene were introduced in the region that is recognized by DICER: Reaction 1: PmeI-Forward: 5'-GTT TAA ACA TGG AGG CCG TGC TGA ACG A-3' and Mutated Reverse: 5'-CGA GCA GTA CAA TTC CTT TAC GGA TGT CAT CAT TGT ACT TGC-3' ; Rection 2: Mutated Forward: 5'-CCG TAA AGG AAT TGT ACT GCT CGA GGA GCT GCT GC-3' and PmeI-Reverse: 5'-GTT TAA ACT CAG GAT TTG GAC TTG GAC ACA GCA AG-3'. The third reaction was done utilizing as a template both amplicons coming from reaction 1 and 2 and the two Pme I-Forward and Pme I-Reverse primers. This last PCR product was cloned into p-GEM-T easy vector (Promega) and then into pWPI over-expression vector (Tronolab) in the PmeI restriction site.

Lentivirus production

Lentiviral vectors were generated by transient transfection in 293T cells using the fugene6 transfection reagent (Roche) according to manufacturer's instructions and the three-plasmid system according to Tronolab's protocols (www.tronolab.com). This system is composed of the packaging plasmid pCMV-dR8.91, the envelope coding plasmid pMD2.G and either the transfer vector plasmid pLVCTH or the overexpression vector plasmid pWPI. In order to test lentivirus silencing efficiency, FIS1 expression was assessed at mRNA and protein levels by both qPCR (forward primer: 5' gcc tgg ttc gaa gca aat aca 3', reverse primer: 5' ttc ctc ttt gct ccc ttt gg 3') and Western blot respectively.

Gene delivery

INS1 cells, COS7 Cells and C2C12 cells were infected with lentiviruses for either gene silencing or over expression. In these three cell lines we found that exposure of the cells for 4 hours to lentivirus preparation which is diluted 1:6 in the cell specific culture media is sufficient to result in over 95% of cells expressing a reporter gene. Under these conditions we experienced little or no cytotoxicity as revealed by oxygen consumption, cell division and viability. A single transduction of a reporter gene (DsRed or PAGFP)

resulted in expression of the transgene for months without compromising insulin secretion or respiration.

Beta cells were adenovirus transduced prior to islet dispersion and used for experimentation 48 hours after transduction.

Figures and Tables

	Total experiments	Total imaging Time [hr]	Total Fusion Events (out of number of experiments)	Total Fission Events (out of number of experiments)	f_{fusion} [events/hr/ mitochondrion]	$f_{fission}$ [events/hr/ mitochondrion]	Fusion-fission cluster
INS1	94	7.9	21 (17)	22 (18)	2.51	2.74	14/17
COS	25	4.6	14 (11)	12 (10)	3.07	2.63	10/11

Table S1: Summary for fusion and fission events in INS1 cells and COS7 cells. A fusion event was determined by the transfer of PA-GFP$_{mt}$ from one mitochondrion to its neighbor, while a fission event was determined by a physical separation or an onset of voltage gradient larger than 2 mV between the two mitochondrial poles. The calculated frequency (*f*) of fusion and fission was derived by dividing the total imaging time in the total events of a given type (fusion or fission). A cluster of fusion-fission events was defined as the occurrence of fusion and then fission in the same experiment. Values represent the number of clusters out of all fusion events in a given cell type. The length of each experiment ranged between 5 min to 60 min.

Figure S1: Stability of membrane potential of mitochondria during the solitary period. This histogram reflects cumulative data from 300 minutes of membrane potential tracking of 17 mitochondria from 17 different cells. For each mitochondrion the average membrane potential over time was determined and the probability for deviations from the average is presented. Note that mitochondria reside close to their own average. A deviation of more than 2.7mV is a rare event.

Figure S2: Localization of mitochondria in autophagosomes and autolysosomes is rarely seen under normal conditions. (A) Triple staining with TMRE, MTG and

LysoSensor Blue was performed to observe colocalization of autophagolysosomes and depolarized mitochondria. It is evident that in the control conditions (two left columns), depolarized mitochondria are rarely engulfed by lysosomes. However, 30 min incubation with pepstatin A and E64D (two right columns) which inhibit autolysosome formation, led to accumulation of depolarized mitochondria within lysosomes. (B) Summary of autophagosomes (AP)-containing mitochondria (colocalization of LC3 and Mitotracker red) and APL with and without pepstatin A (n=6). These results indicate that under control conditions depolarized mitochondria are rarely seen within autophagosomes or autolysosomes and that once the process of autophagy was initiated, it was a rapid process. (C) LC3:GFP and TMRE staining in a living INS1 cell show that polarized mitochondria cannot be seen in APs as identified by LC3:GFP.

Figure S3: Tracking a depolarized mitochondrion over time. A single mitochondrion tagged with PA-GFP_{mt} and tracked over time. This mitochondrion had reduced TMRE staining, and compared to average mitochondria in this cell, was 10-15 mV more positive. It was tracked for a period of 45 minutes. During that period this mitochondrion did not engage in a fusion or fission event and retained its' GFP FI during the recording period (GFP $FI_{(t=0)}$ = 233 ; GFP $FI_{(t=1\ hr)}$= 221). This experiment also shows that the depolarized level of the mitochondrion was independent of its location within cell, suggesting that limitations in diffusion of TMRE and oxygen concentration had little effect on $\Delta\psi_m$.

Figure S4: Inhibition of autophagy results in increased subcellular heterogeneity in mitochondrial membrane potential (a) **Knockdown of the autophagy protein, Beclin 1, in liver H4 cell line** results in the accumulation of small depolarized mitochondria. Depolarized mitochondria appear green. Mitotracker Green and TMRE (red). (b) Western blot analysis of Beclin 1 in shRNA treated cells and control. (c) ATG5 -/- MEF cells are characterized by depolarized mitochondria. Images are showing both merge of MTG and TMRE as well as a ratio image which is presented in FI coded color where blue color represents depolarized mitochondria.

Figure S5: *Fis1* shRNA treatment and DRP1-DN expression. (a) INS1 cells were transduced with a vector carrying a previously described *Fis1* RNAi. After 2 weeks of cell culture, the level of FIS1 was measured using Western blot analysis. RT-PCR analysis demonstrated a reduction of *Fis1* mRNA levels by 87%± 7%. A single lentiviral infection (exposure of cells to virus for 4 hours) resulted in stable reduction of Fis1 transcript for 3 weeks. Reduction in gene expression was apparent 3 days after infection. (b) Expression of DRP1 dominant negative lentiviral transduction. DRP1-DN lentivirus had DRP1-DN clonedupstream of an IRES- EGFP sequence, which enabled the monitoring of the expression rate of the transgene. As a control cells were infected with the same virus expressing only GFP. (c) Morphometry analysis of mitochondria in cells infected with Fis1 KD lentivirus as compared to control shRNA virus. The geometric properties of mitochondrial webs were measured using the 'particle analysis' feature on ImageJ (NIH). A single web is defined as a continuous cluster of (at least 10) TMRE-positive pixels, regardless of matrix continuity. For each web, the perimeter, area maximal and minimal aspect length were measured and the aspect ratio (AR; maximal aspect divided by minimal aspect) was derived.

Figure S6: *Fis1* RNAi treatment does not reduce the total number of lysosomes. Lysosomes in INS1 cells were stained using lysosensor blue. Dye was loaded for 30 minutes and cells were imaged using 2-Photon confocal microscopy (Excitation/Emission 750/500-550 at 37C). Metamorph analysis was used to quantify the number of lysosomes based on fluoresce intensity and size (See Figure 6).

Figure S7: *Fis1* RNAi cells have increased resistance to apoptotic stimuli. INS1 cells expressing *Fis1* and control RNAi were stained with activated caspase 3 antibody following 6 hr treatment with staurosporine (1μM). Combined bright field and fluorescent images are shown. Red: activated caspase 3 positive cells are more abundant in control cells (left panel). This experiment supports the anti-apoptotic effect reported after *Fis1* silencing and indicates that the altered metabolic state seen in *Fis1* RNAi cells does not emerge from technical factors related to the RNAi construct or to the infection procedure.

Figure S8: Electron Microscopic analysis of autophagosome maturation in Fis1KD and control.

FIS KD and vector control INS-1 cells were treated with autophagosomes inhibitors Bafilomycin or Pepstatin+E64D, which block autophagy in early and late stages of autophagosome vacuole maturation respectively. Knocking Down of Fis-1 protein does not alter autophagosome maturation nor their morphology as revealed by the presence of AVi (Early/Initial autophagic vacuole), AVd (Late/Degradative autophagic vacuole) and AVd/Lys (Autolysosome)(Eskelinen, 2005).

Figure S9: ***INS1 expressing DRP1-DN have increased level of oxidized MnSOD.***

COS7 cells co-expressing DRP1-DN and DsRed were stained with an antibody against the nitrotyrosinated form of MnSOD (SOD2) (Xu *et al.*, 2006). Control cells were infected with mtPAGFP lentivirus. One week post infection, cells were fixed, permeabilized and stained. Note that similar to the staining shown in figure 7, mitochondria in DRP1-DN accumulate oxidized protein in the thick, distal ending of each mitochondrion. Antibody is a gift of Dr. Richard Cohen, Boston University.

Figure S10: ***3D projection of INS1 cells expressing LC3GFP and stained with mitotracker red for the detection of mitochondrial autophagy as presented in Figure 4b.*** We used LC3:GFP, which translocates from the cytosol to the isolation membrane, to label early stages of AP assembly. Mitotracker red (MTR), a membrane potential dye that stains mitochondria irreversibly and is retained during depolarization, was used to pulse label mitochondria in INS1 cells (14 minutes, 50nM) at 1hour prior to detection of APs content. For detection cells were treated with pepstatin A (10μM) and E64d (10μM) for 30 minutes to arrest digestion inside the APs, then subjected to confocal microscopy (see schematic illustration of experiment protocol at Fig. 4b). While mitochondria outside APs showed bright MTR fluorescence, those localized in APs had reduced MTR, indicating that at the time of MTR pulse they were already depolarized.

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