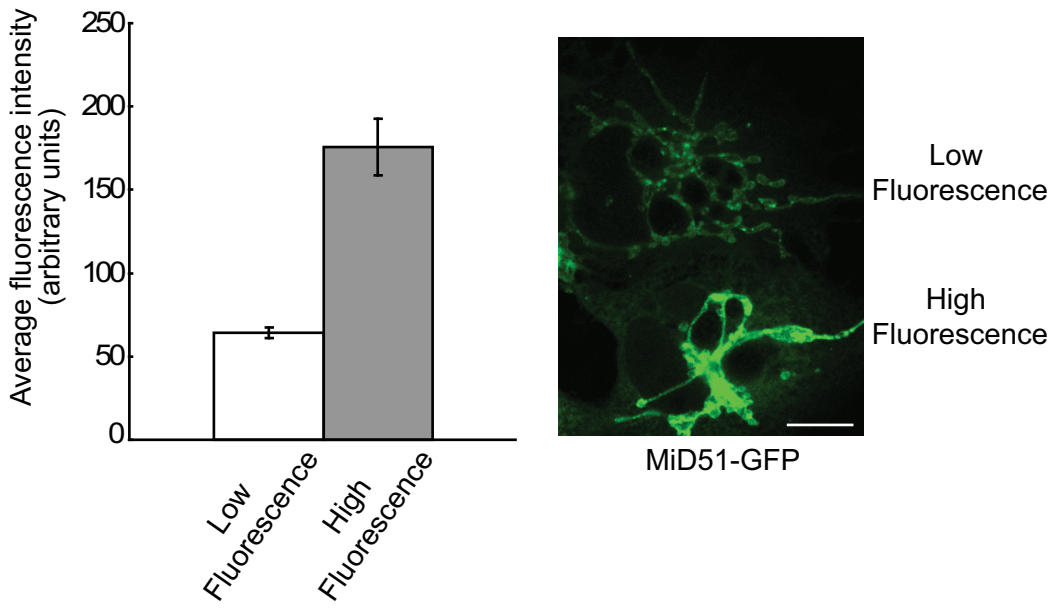
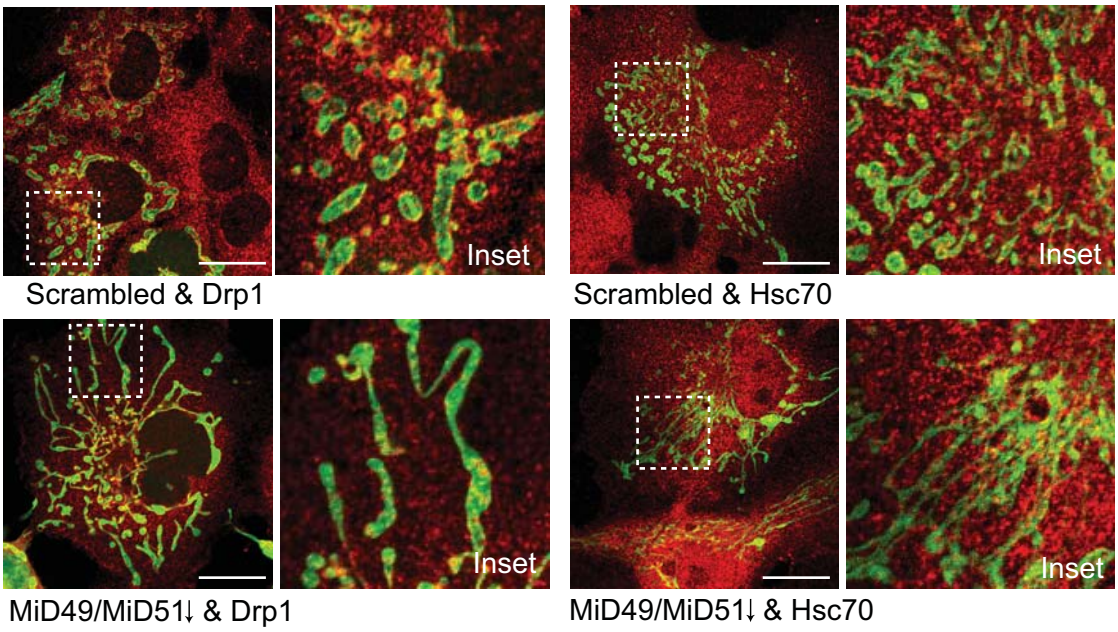
SUPPLEMENTAL FIGURES



Supplemental Fig S1. Representative linescans of low and high MiD51-GFP expressing COS-7 cells were analyzed for GFP fluorescence intensity using Metamorph software. The relative fluorescence intensity of 10 linescans from each cell was then determined (mean ± s.e.m.). Scale bars: 5 μm.



Supplemental Fig S2. Confocal imaging of COS-7 cells cotransfected with either scrambled or MiD49/MiD51 specific RNAi constructs along with mt-Dendra2 to visualize mitochondria (green). Cells were fixed and immunostained for Drp1 or Hsc70 (red) as control**.** Scale bars: 5 μm.

SUPPLEMENTAL INFORMATION

MOVIE LEGENDS

Movie 1 MiD induced mitochondrial movement. The movement of mitochondria in COS-7 cells transfected with MiD51-GFP (green) was analyzed via fluorescent confocal time-lapse microscopy (Leica TCS SP2). Images were captured every 2 minutes for 60 minutes. Movie frame rate: 2.5 frames/second.

Movie 2 MiD51-GFP induced mitochondrial tubules are dependent on F-actin. MiD51-GFP (green) and MAP4-Katushka (red) cotransfected COS-7 cells were analyzed via fluorescent confocal time-lapse microscopy following cytochalasin D treatment (LSM 510, Carl Zeiss). Depolymerization of F-actin by cytochalasin D results in retraction of mitochondrial tubules. Cell volume was visualized by MAP4-Katushka labeling of microtubules. Images were captured every 15 minutes for 45 minutes. Movie frame rate: 3 frames/second.

Movie 3 MiD49-GFP forms foci on the mitochondrial outer membrane. The mitochondrial localization of MiD49-GFP (green) in COS-7 cells was analyzed via confocal microscopy (LSM 510, Carl Zeiss). 3D reconstruction and z-stacks obtained using Metamorph software. Movie frame rate: 10 frames/second.

Movie 4 MiD51-GFP foci form independent of Drp1. MiD51-GFP (green) foci formation in Drp1 depleted HeLa cells were analyzed via confocal microscopy (LSM 510, Carl Zeiss). Mitochondria were visualized with MitoTracker Far Red (red). 3D reconstruction and z-stacks obtained using Metamorph software. Movie frame rate: 3 frames/second.

Movie 5 GFP-Drp1 is recruited following induction of MiD51 expression**.** MiD51 inducible MEFs were transfected with GFP-Drp1 (green) and analyzed via time-lapse confocal microscopy at 37^{o}C (LSM 510, Carl Zeiss). Mitochondria were visualized with MitoTracker Far Red (red). Following MiD51 induction with the addition of 4 hydroxy-tamoxifen (4HT), GFP-Drp1 is recruited to mitochondria (Z-stacks captured every 30 minutes for 17 hours). Movie frame rate: 3 frames/second.

Movie 6 GFP-Drp1 is not recruited in the absence of MiD51 induction. MiD51 inducible MEFs were transfected with GFP-Drp1 (green) and analyzed via time-lapse confocal microscopy at 37^{o}C (LSM 510, Carl Zeiss). Mitochondria were visualized with MitoTracker Far Red (red). In the absence of 4HT, GFP-Drp1 is not recruited to mitochondria (Z-stacks captured every 30 minutes for 12 hours). Movie frame rate: 3 frames/second.

Movie 7 GFP-Drp1 is not recruited in wild type MEFs. Wild type MEFs were transfected with GFP-Drp1 (green) and analyzed via time-lapse confocal microscopy at 37^{o}C (LSM 510, Carl Zeiss). Mitochondria were visualized with Mitotracker Far Red (red). Following the addition of 4 hydroxy-tamoxifen (4HT), GFP-Drp1 is not recruited to

mitochondria (Z-stacks captured every 30 minutes for 13 hours). Movie frame rate: 3 frames/second.

METHODS

Cloning Procedures. The random cellular localization screen conducted by Simpson et al. (2000) can be found at (http://www.inet.dkfz-heidelberg.de/LIFEdb). cDNAs encoding human MiD49 (SMCR7) and MiD51 (SMCR7L) were obtained from MRC Geneservice (Nottingham, UK). MiD49 (Accession: Q96C03), MiD51 (Accession: Q9NQG6), MiD49$^{1\text{-}50}$ (transmembrane domain, residues 1-50) and MiD49$^{50\text{-}454}$ (ΔTMD, residues 50-454) were cloned into the pE-GFP-N1 (Clontech, CA, USA) vector at *Hin*dIII and *Bam*HI restriction sites to generate in-frame C-terminal GFP fusion proteins. Untagged constructs were generated by cloning MiD49 and MiD51 into pE-GFP at *Hin*dIII and *Bam*HI, with the inclusion of a stop codon. Human Drp1 and Drp1^{K38A} were cloned into a vector downstream of GFP at a *Bam*HI restriction site (Johnston *et al*, 2002). The Drp1 mutants S600D, A395D and G363D were generated using PCR to introduce the mutation and then cloned downstream of GFP at *Bam*HI in the same vector. Different residue numbers for S600D (instead of S637D) reflects the use of a different Drp1 isoform (Cereghetti *et al*, 2008).
For RNAi studies, oligonucleotides for MiD49 (sense 5'-GATCC GGCAGACAGCTGGAAGGAATTCAAGAGATTCCTTCCAGCTGTCTGCCTTTTTT GGAAA-3', antisense, 5'-AGCTTTTCCAAAAAAGGCAGACAGCTGGAAG-3') and MiD51 (sense 5' GATCCGTCACGACTCCGCATGTCCTTCAAGAGAGGACATGCGGAGTCGTGAC TTTTTTGGAAA-3', antisense 5'-GCAGTGCTGAGGCGTACAGGAAGTTCTCTCCTGTACGCCTCAGCACTGAAAA AACCTT TTCGA- 3') were annealed and cloned into the p*Silencer* 3.0-H1 (Ambion, TX, USA) vector at *Bam*HI and *Hin*dIII restriction sites. For bacterial expression, full length MiD51 was cloned into the pQE-30 vector (QIAGEN, MA, USA) at *Bam*HI and *Hin*dIII restriction sites. Plasmids encoding mt-GFP (pOTC-GFP), Tom7-GFP, GFP-Miro1, mt-Dendra2 and huMilt1 have been reported previously (Johnston *et al*, 2002; Koutsopoulos *et al*, 2010; Stojanovski *et al*, 2004; Yano *et al*, 2000; Yano *et al*, 1998).
Cell Culture, Transient Transfections and Live Cell Imaging. COS-7 cells were cultured under standard conditions (Stojanovski *et al*, 2004). Generation, selection and induction of stable MEF cell lines was performed as previously reported (Dunning *et al*, 2007). Transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. For RNAi studies, p*Silencer* 3.0-H1-MiD49 and p*Silencer* 3.0-H1-MiD51, or a scrambled sequence control (Ambion, TX, USA), were cotransfected into COS-7 cells with mt-Dendra2 at a 9:1 ratio. Following transfection, cells were incubated with 50 nM MitoTracker Red CMX*Ros* (Molecular Probes, CA, USA) and 10μg/mL Hoechst 33258 (Sigma, MO, USA).
Immunofluorescence Assays. For immunofluorescence studies, cells were fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, for 10 minutes at room temperature, or 100% ice-cold acetone for 3 minutes at -20°C. Paraformaldehyde fixed cells were permeabilized by the addition of 0.2% Triton X-100. For nuclear staining, cells were incubated with 10μg/mL Hoechst 33258 in PBS containing 0.2% Triton X-100. Anti-Drp1 (BD Biosciences, MD, USA), anti-Tom20 (monoclonal) (Santa

Cruz, CA, USA) and anti-β-tubulin (Sigma, MO, USA) were visualized using Alexa Fluor 568 or Alexa Fluor 647 (Molecular Probes, CA, USA) secondary antibodies. F-actin was visualized using Phalloidin Alexa Fluor 568 (Molecular Probes, CA, USA).

Microscopy. Epifluorescent microscopy was conducted using either an Olympus BX-50 microscope equipped with a SPOT RT 3CCD camera (Diagnostic Instruments) and processed used SPOT Advanced software, or an Olympus IX8I microscope equipped with an F-view2 camera and processed using Soft System SIS (Olympus). Confocal microscopy was performed with a Leica TC2 SP2 confocal microscope or a Zeiss Confocal equipped with a Confocor 3 system containing an Avalanche PhotoDiode (APD) detector. Samples were maintained at 37°C on a heated stage in a humidified chamber with 5% CO_2. A Coolsnap-HQ camera (Photometrics) was used to acquire images. For z-sectioning, the number of slices collected was determined by the software (slices 0.1 µm apart). Images obtained using the Leica Confocal: green fluorescence was detected using an Argon laser (100 mW), while red fluorescence was detected using the HeNe laser. Images obtained using the Zeiss Confocal: green fluorescence was detected using an Argon laser (30 mW), red fluorescence was detected using a DPSS laser (15 mW), and far red fluorescence was detected using a HeNe laser (5.0 mW). All images were processed using Image J (http://rsbweb.nih.gov/ij/index.html), Leica, Zeiss and/or MetaMorph (Visitron Systems) software.

Colocalization analysis of MiD-GFP transfected COS-7 cells with endogenous Drp1 was conducted following single plane image acquisition on the Zeiss Confocal. Images in all experimental groups were obtained with the same settings, except for detector gain adjustments that were performed to normalize saturation levels. Images were analyzed using Metamorph software. Colocalization statistics were determined from 30-35µm linescans intersecting mitochondria and presented as Pearson's Correlation values that vary between -1 to 1 (Karbowski *et al*, 2007).

Antibodies and Reagents. Antibodies against MiD49 were raised in rabbits using a peptide antigen directed against the C-terminus of MiD49 conjugated to KLH (IGYALYSGLQEPEGLL-KLH) (GL Biochem, Shanghai). Antibodies specific for MiD51 were raised in rabbits using recombinant insoluble His-tagged MiD51 as an antigen. Western blots were probed with in-house antibodies against MiD49, MiD51, Mitofusin-2, Hsc70 and B17.2L (Lazarou et al, 2010). Commercial antibodies used were cytochrome *c* (BD Biosciences, MD, USA), OPA1 (BD Biosciences, MD, USA) and β-actin (Sigma, MO, USA). Antibodies against rabbit mt-Hsp70 and Tom40 have been reported elsewhere (Johnston *et al*, 2002). Nocodazole (Sigma, MO, USA) was used at a final concentration of 5 µM. Cytochalasin D (Sigma, MO, USA) was used at a final concentration of 10 µM. CCCP (carbonyl cyanide m-chlorophenylhydrozone) (Sigma, MO, USA) was used at a final concentration of 20 µM.

Mitochondrial Treatments and Western Blotting. Mitochondrial isolation, fractionation, proteinase K digests and sodium carbonate extractions were performed using standard methods (Johnston *et al*, 2002; Ryan *et al*, 2001). Tris-tricine sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described (Schagger & von Jagow, 1987). ECL chemiluminescent substrate (Amersham) was used to detect immunoreactive proteins in blots.

Protein-Protein interaction assay.
The pGBKT7 vector (Clontech, CA, USA) containing the GAL4 binding domain (BD) was used to create a GAL4 binding domain-MiD49$^{\Delta N120}$ (BD- MiD49$^{\Delta N120}$) fusion. The soluble domain of MiD49 (amino acids 121-463) was PCR amplified and cloned into the pGBKT7 vector. The pGADT7 vector (Clontech, CA, USA) that contains the GAL4 activating domain (AD) was used to create a GAL4 activating domain-Drp1 (AD-Drp1) fusion. The soluble domain of Fis1 was cloned into the pGADT7 vector (Clontech, CA, USA) (AD-Fis1). All other vectors and the yeast strain AH109 were kindly provided by Dr. Quan Zhao (Walter and Eliza Hall Institute, Melbourne). All methods employed were undertaken according to the manufacturer's instructions (Clontech, CA, USA), unless stated otherwise. Constructs were cotransformed into the yeast strain AH109. Small scale AH109 yeast transformations were conducted using the Lithium Acetate (LiAc) mediated transformation protocol. Cotransformants were selected on SD/-Leu/-Trp agar and incubated at 30°C for 3 days. Following selection cotransformants were grown in SD/-Leu/-Trp minimal media at 30°C overnight and spotted onto SD/-Leu/-Trp/-His/-Ade + X-α-Gal (20μg/ml) (Clontech, CA, USA) and incubated at 30°C for 3 days.
Co-immunoprecipitation. HeLa cells were transiently transfected with GFP-Fis1, MiD49-GFP or GFP-Miro1 constructs. Whole cells were chemically crosslinked with 20 μM BMH (bis(maleimido)hexane; Thermo Scientific, IL, USA) then transfected cells were immunoprecipitated using GFP-Trap beads (Chromotek, Martinsried, Germany) according to manufacturer's instructions. Whole cell extracts were solubilized in RIPA buffer and incubated with GFP-Trap beads for 1 hr at 4 degrees. Samples were eluted in SDS PAGE Lamelli buffer.

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