

## **ADDITIONAL METHODS**

### **cDNA and shRNA plasmids**

To replace endogenous with mutant Drp1 three shRNAs were selected (Reynolds et al., 2004) and tested for knockdown of co-transfected Drp1 by expression from an H1 promoter-driven plasmid (pSUPER (Brummelkamp et al., 2002)). The most effective H1-promoter::shRNA cassette was subcloned into the PciI site of pEGFP-C1::Drp1, a plasmid expressing splice variant 1 of rat Drp1 with an N-terminal GFP tag (Pitts et al., 1999). The Drp1 coding sequence was rendered RNAi-resistant by introducing 5 silent base changes into the shRNA target sequence (GAAGAGTGTAAGTCA), and S656A and S656D mutations were introduced similarly by site-directed mutagenesis (QuikChange protocol). Plasmids with three hemagglutinin (HA) epitope tags instead of GFP (3HA-Drp1) were constructed similarly.

Transient transfections were performed with LipoFECTAMINE 2000 (Gibco). To generate stable PC12 cell lines, shRNA/cDNA plasmids were linearized prior to transfection. Cells were selected in 500 µg/ml G418 and several GFP-positive clones were expanded and characterized separately.

### **Antibodies**

To generate phospho Ser656-specific monoclonal antibodies, the peptide acetyl-Arg-Lys-Leu-[phospho-Ser]-Ala-Arg-Glu-Gln-Arg-Asp-Cys was conjugated via the C-terminal Cys to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL). Mice were immunized with antigen and monoclonal antibodies were generated by the University of Iowa Hybridoma Facility according to standard methods (Galfre and Milstein, 1981). Hybridoma supernatants were screened for specific detection of PKA-phosphorylated GST-Drp1<sub>643-755</sub> compared to non-phosphorylated and Ser656-mutated fusion proteins.

Commercial antibody sources were: Drp1 (BD Biosciences, Franklin Lakes, NJ), GFP (Abcam, Cambridge, UK), cytochrome C oxidase (MTCO2, Lab Vision, Fremont, CA), ERK1/2 (Santa Cruz Biotechnologies, Santa Cruz, CA), phospho-Ser40 tyrosine hydroxylase (Cell Signaling Technology, Danvers, MA).

### **Biochemical characterization of Drp1 mutants**

Chemical crosslinking of Drp1 with bis[sulfosuccinimidyl]-suberate was carried out as described (Zhu et al., 2004), while GTP-agarose (Innova Biosciences, Cambridge, UK) affinity precipitations were performed essentially as described for immunoprecipitations above. For GTP hydrolysis experiments, human Drp1 with an N-terminal calmodulin-binding peptide tag was purified from bacteria via calmodulin-agarose essentially as described (Zhu et al., 2004). GTP hydrolysis reactions were carried out at 30°C and contained ~10 µg/ml recombinant Drp1, 10-20 µCi/ml [ $\gamma$ -<sup>32</sup>P]GTP (Perkin Elmer, Boston, MA), 25 µM GTP, 25 mM HEPES, pH 7.5, 50 mM KCl, 19 mM NaCl, 2 mM MgCl<sub>2</sub>, 12.5% glycerol. Reactions were terminated by addition of activated charcoal slurry, and percent GTP hydrolysis was determined by liquid scintillation counting of the supernatant (Natochin et al., 2005).

### **Mitochondrial morphometry**

To assess mitochondrial morphology in CV1 fibroblasts, cells were fixed 72 h post-transfection with 4% paraformaldehyde, blocked with 2% normal goat serum in Tris-buffered saline containing 0.1% Triton X-100, and incubated (16 h, 4°C) with 1:500 dilutions of antibodies directed against GFP and mitochondrial cytochrome oxidase, followed by Alexa Fluor-488 and -543 conjugated secondary antibodies. The mitochondria channel of epifluorescence micrographs was analyzed by a custom macro written for ImageJ image analysis software. The macro performs background subtraction and binary thresholding of the image, followed by particle analysis and computation of several metrics involving area, perimeter, major and minor axis. For the area<sup>2</sup> metric, the area of individual particles is squared to de-emphasize noise particles before averaging for the entire cell. For validation, this metric reliably detected mitochondrial fusion as a consequence of Drp1/Fis1-directed RNAi and mitochondrial fragmentation following Fis1 overexpression.

For morphometric analysis of mitochondria by transmission electron microscopy, stable PC12 cell cultures were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in Eponate-12. 90 nm sections were counterstained with 5% uranyl acetate/2.66% lead citrate (Hayat, 2000), and digital images were captured on a JEOL 1230 transmission electron microscope. Length (major axis) and area of mitochondrial cross-sections were quantified using ImageJ software.

### ***In vivo* analysis of Ser656 phosphorylation**

All animal procedures were approved and carried out according to the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa. Adult (3-5 month old) male C57BL/6 mice received intraperitoneal injections of 20 mg/kg isoproterenol or saline vehicle and were returned to their cages for 45 to 60 min. Other mice were forced to swim for 15 min in 25°C water. Hearts were rapidly dissected, snap-frozen, and stored at -70°C. To prepare extracts, frozen tissue was pulverized and homogenized in lysis buffer (see above). The cleared extracts were immunoprecipitated with total Drp1 antibody, followed by immunoblotting for phospho-Ser656 and total Drp1.

### **Viability and caspase activity assay**

PC12 cell were seeded in collagen-coated 96-well plates and treated with toxins 48 h later. Caspase-3/7 activity was assayed based on cleavage of the fluorogenic substrate acetyl-Asp-Glu-Val-Asp-aminoethyl-coumarin (Ac-DEVD-AMC, 20 µM; Biomol, Hamburg, Germany) as described (Strack et al., 2004). Cell viability was determined by two assays, which gave essentially identical results. Colorimetric assays based on reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) to formazan (CellTiter 96™ Aqueous nonradioactive cell proliferation assay, Promega) (Strack et al., 2004) and fluorometric assays based on the reduction of resazurin to resorufin (CellTiter Blue™ cell viability assay, Promega, Madison, WI) were performed according to the manufacturer's instructions.

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