Article

Dephosphorylation by calcineurin regulates translocation of Drp1 to mitochondria

CEREGHETTI, G M., *et al*.

Abstract

Changes in mitochondrial morphology that occur during cell cycle, differentiation, and death are tightly regulated by the balance between fusion and fission processes. Excessive fragmentation can be caused by inhibition of the fusion machinery and is a common consequence of dysfunction of the organelle. Here, we show a role for calcineurin-dependent translocation of the profission dynamin related protein 1 (Drp1) to mitochondria in dysfunction-induced fragmentation. When mitochondrial depolarization is associated with sustained cytosolic Ca(2+) rise, it activates the cytosolic phosphatase calcineurin that normally interacts with Drp1. Calcineurin-dependent dephosphorylation of Drp1, and in particular of its conserved serine 637, regulates its translocation to mitochondria as substantiated by site directed mutagenesis. Thus, fragmentation of depolarized mitochondria depends on a loop involving sustained Ca(2+) rise, activation of calcineurin, and dephosphorylation of Drp1 and its translocation to the organelle.

Reference

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Supporting Information

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SI Experimental Procedures

Molecular Biology. Mitochondrially targeted dsRED (mtRFP) was previously described (1). Drp-1^{K38A}, was from Dr. A. van der Bliek (University of California, Los Angeles, CA) (2); pDCR-HA-ΔCnA and pDCR-CnB were from Dr. S. Schiaffino (Venetian Institute of Molecular Medicine, Padova, Italy) (3, 4); CnA^{H151Q} was subcloned by PCR from pT7–7-CnA^{H151Q}, (provided by Dr. B. Sikkink, Mayo Clinic, Rochester, NY) into the NheI and XhoI sites of pcDNA3.1/Zeo (+). The truncated form ΔCnA^{H151Q} was obtained by external PCR of pcDNA3.1/Zeo (+)-CnA^{H151Q} by inserting a stop codon at position 392. Drp1-YFP mutants were prepared from wt (provided by Dr. R. Youle, National Institute of Neurological Disorders and Stroke, Bethesda, MD) by site directed point mutagenesis. Constructs were verified by sequencing.

To generate Drp1-YFP mutants site directed mutagenesis was performed using the following primer sequences: S616A (FW: 5′-CCC ATT CCA ATT ATG CCA GCC GCT CCA CAA AAA GGT CAT GCC GTG-3′; RV: 5′-CAC GGC ATG ACC TTT TTG TGG AGC GGC TGG CAT AAT TGG AAT GGG-3′), S637A (FW: 5′-CCA GTT CCT GTT GCA CGA AAA CTA GCT GCT CGG GAA CAG CGA GAT TGT GAG-3′; RV: 5′-CTC ACA ATC TCG CTG TTC CCG AGC AGC TAG TTT TCG TGC AAC AGG AAC TGG-3′), S616D (FW: 5′-CCC ATT CCA ATT ATG CCA GCC GAT CCA CAA AAA GGT CAT GCC GTG-3′; RV: 5′-CAC GGC ATG ACC TTT TTG TGG ATC GGC TGG CAT AAT TGG AAT GGG-3′), S637D (FW: 5′-CCA GTT CCT GTT GCA CGA AAA CTA GAT GCT CGG GAA CAG CGA GAT TGT GAG-3′; RV: 5′-CTC ACA ATC TCG CTG TTC CCG AGC ATC TAG TTT TCG TGC AAC AGG AAC TGG-3′).

Cell Culture and Transfection. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FBS (FBS) (Gibco), 50 U/ml Penicillin, 50 μg/ml Streptomycin, 100 μM non essential aminoacids (MEM, Gibco) and 2 mM glutamine (Gibco). Transfection was performed using Lipofectamine 2000 Reagent (Invitrogen) following manufacturer instructions. In cotransfection experiments, plasmids were always cotransfected in equimolar ratios.

Imaging. For PTP and TMRM imaging, HeLa cells grown on 24 mm round coverslips were loaded with calcein [Molecular Probes, 2 μM, 45 min, 37 °C, dissolved in Hank's Balanced Salt Solution (HBSS)], to analyze PTP opening (5), or TMRM (Molecular Probes, 20 nM, 45 min, 37 °C, dissolved in HBSS), to analyze mitochondrial depolarization in the presence of cyclosporine H (CsH, 2 μM), an inhibitor of the MDR pump, added to avoid extrusion of TMRM from the cell. After 45 min cells were placed on a stage of an inverted epifluorescence microscope (Olympus, CellR), and imaged every 60 s using a 40X, 1.4 NA objective (Olympus) and exposure times of 30 msec. Images were background-corrected, acquired, and stored for analysis using the CellR software. Mean fluorescence intensity over 20 mitochondria-rich regions per experiment was calculated and normalized.

For confocal *z*-axis stacks, 24 h after transfection cells were excited with exposure times of 50–100 msec using a 60X, 1.4 NA Plan Apo objective, and stacks of 20 images separated by 0.3 μm along the *z*-axis were acquired. The 3D reconstruction and volume rendering of the stacks were performed with the appropriate plug-in of ImageJ package (NIH, Bethesda, MD).

Drp1-YFP Crosslinking Studies. HeLa cells transfected with Drp1-YFP wt and point mutation variants were lysed in CSPB buffer 24 h after transfection; 100 μg protein from each lysate was treated with 1 mM crosslinker *Bis*[sulfosuccinimidyl]suberate (BS^3, Pierce) 2 h on ice. The crosslink reaction was stopped by 15 min incubation with 100 mM Tris/HCl buffer (pH 7.4) at RT. After addition of SDS/PAGE loading buffer (NuPAGE), samples from crosslinked and non-crosslinked lysates were boiled and 60 μg proteins were loaded on 3–8% Tris-Acetate gels (NuPAGE). Separated proteins were transferred onto polyvinylidene fluoride (Millipore) membranes and probed with the indicated antibodies. The following antibodies were used: anti-GFP (Invitrogen, 1:1000), anti-actine (Millipore, 1:8000). Isotype-matched, horseradish peroxidase-conjugated secondary antibodies (Amersham) were used, followed by detection by chemiluminescence (Amersham).

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2. Smirnova E, Griparic L, Shurland DL, van der Bliek AM (2001) Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Mol Biol Cell* 12:2245–2256.
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5. Petronilli V, *et al.* (2001) The mitochondrial permeability transition, release of cytochrome c and cell death Correlation with the duration of pore openings in situ. *J Biol Chem* 276:12030–12034.

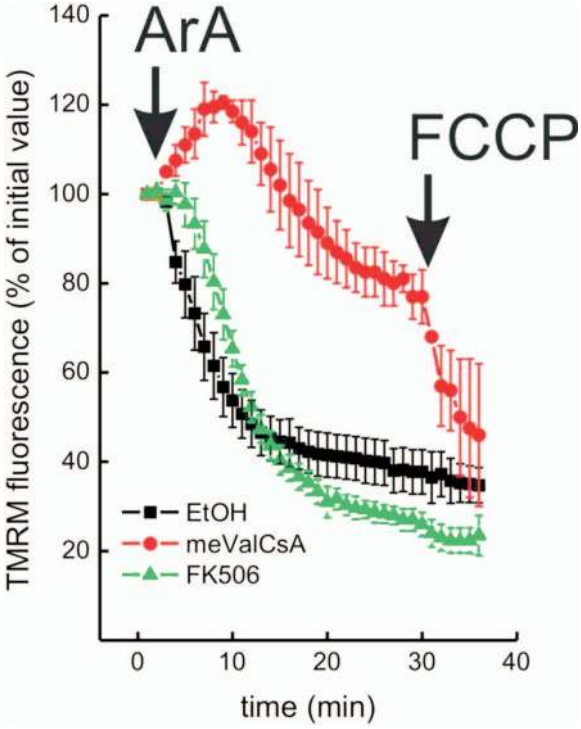

Fig. S1. MeValCsA but not FK506 and PPD1 prevent mitochondrial depolarization. HeLa cells loaded with TMRM were treated where indicated (arrows) with 20 μM ArA or 2 μM FCCP after 30 min incubation with 0.6 μM FK506, 2 μM meValCsA or 24 h transfection with PPD1 as indicated. Fluorescence intensities are normalized to the initial value for comparative reasons and data represent mean $\pm$ SE of 4 independent experiments.

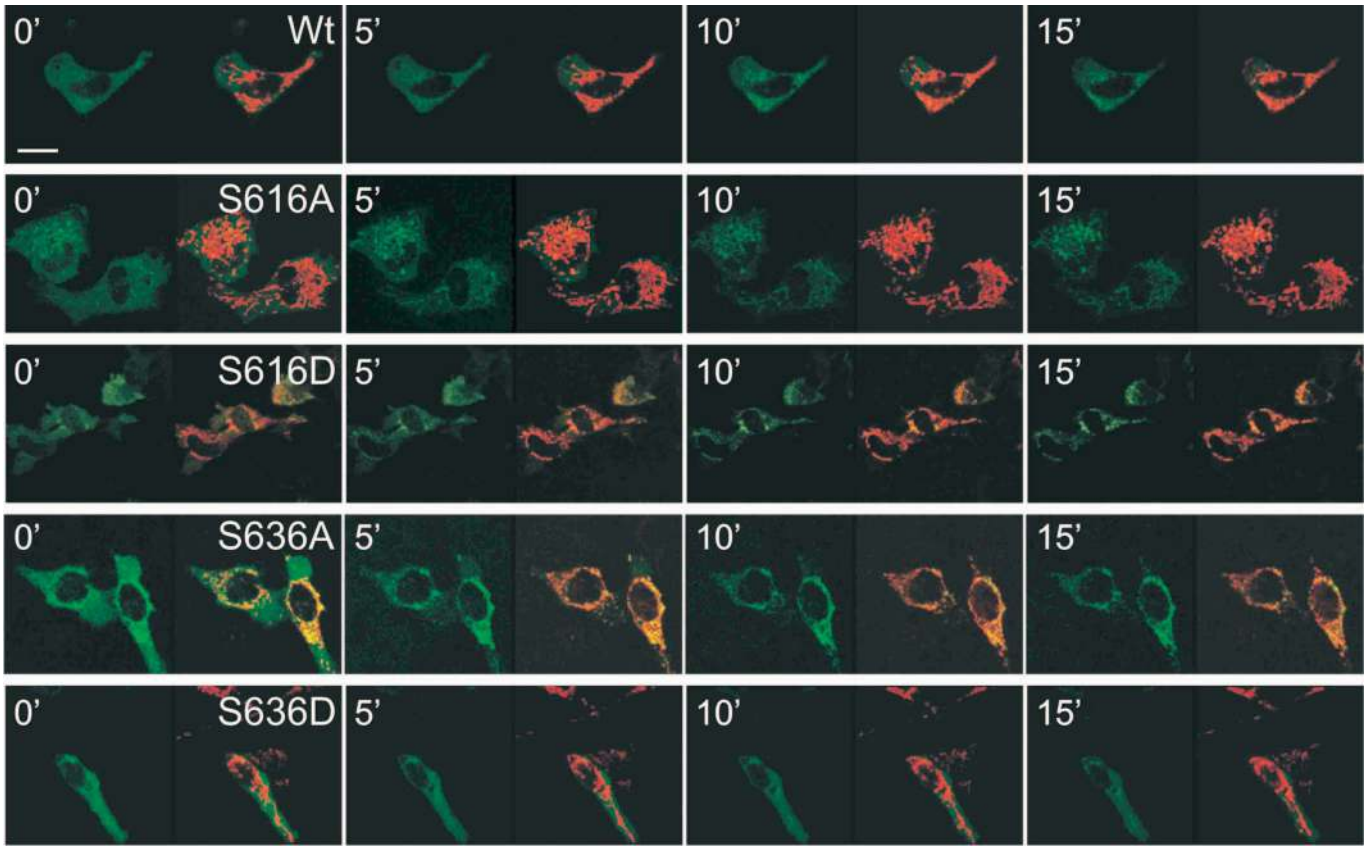

Fig. S2. Real time confocal imaging of wt and mutant Drp1-YFP translocation to mitochondria in response to FCCP. Representative frames acquired at the indicated times from real time confocal imaging of mtRFP (red) and Drp1-YFP (green) in HeLa cells cotransfected with mtRFP and wt or the indicated mutants of Drp1-YFP. Yellow in the merge images indicates colocalization. At t = 3 min, cells were treated with 2 μM FCCP.

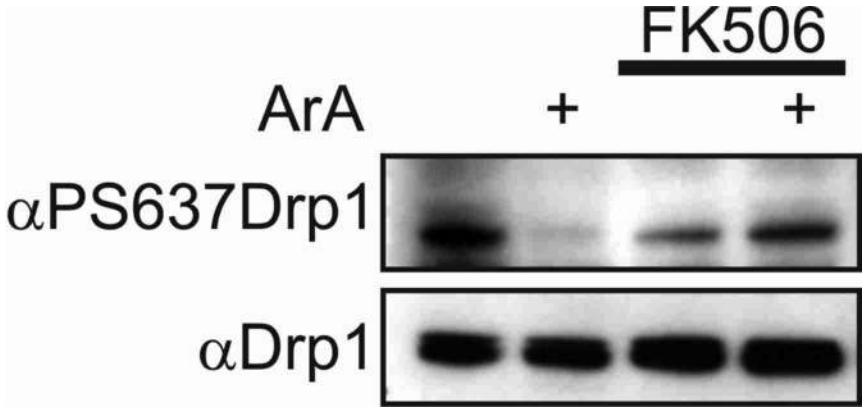

Fig. S3. ArA causes calcineurin-dependent dephosphorylation of Drp1 on Ser-637. HeLa cells were treated where indicated with 20 μM ArA for 15 min, lysed in CPBS buffer supplemented with protease and phosphatase inhibitors and equal amounts of proteins (50 μg) were separated by SDS/PAGE and immunoblotted with the indicated antibodies. Where indicated, cells were pretreated for 15 min with 0.6 μM FK506. α-PS637Drp1: α-phosphorylated serine 637.

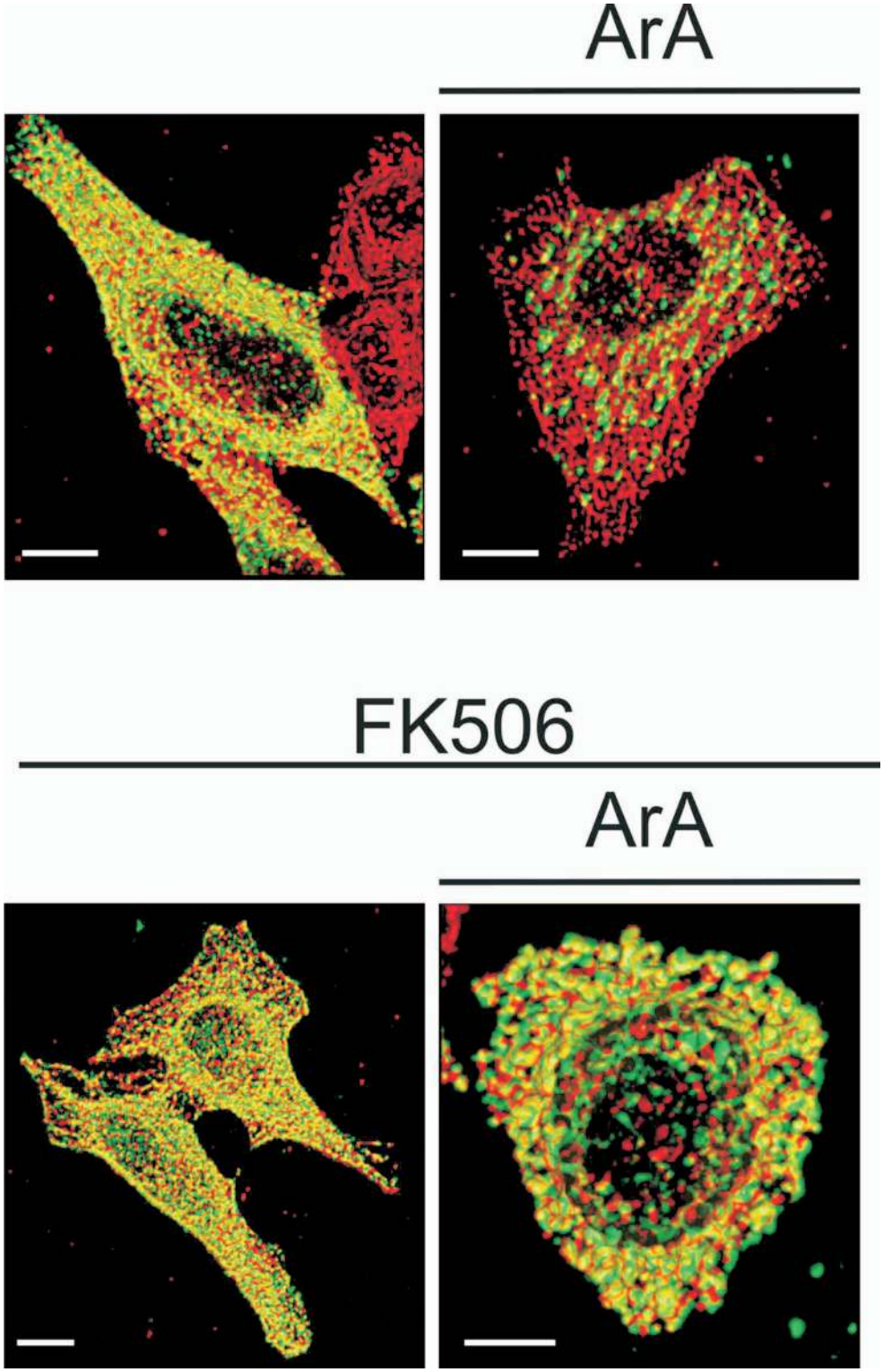

Fig. S4. Volume rendered 3D reconstructions of Drp1-YFP and anti-tubulin fluorescence. HeLa cells were transfected with Drp1-YFP and after 24 h fixed and immunostained with anti-tubulin. Where indicated, cells were preincubated with 0.6 μM FK506 and treated with 20 μM ArA for 15 min. For confocal *z*-axis stacks, 24 h after transfection cells were excited with exposure times of 50–100 msec using a 60 $\times$ 1.4 NA Plan Apo objective, and stacks of 20 images separated by 0.3 μm along the *z*-axis were acquired. 3D reconstruction and volume rendering of the stacks were performed with the appropriate plug-in of ImageJ package (National Institutes of Health, Bethesda). Red represents tubulin and green Drp1-YFP; yellow indicates colocalization.