**Cardiolipin externalization to the outer mitochondrial membrane acts as an elimination signal for mitophagy in neuronal cells**

**Authors:** Charleen T. Chu1\*, Jing Ji2,3†, Ruben K. Dagda1†, Jian Fei Jiang2†, Yulia Y. Tyurina2, Alexandr A. Kapralov2, Vladimir A. Tyurin2, Naveena Yanamala5, Indira H. Shrivastava6, Dariush Mohammadyani5,Kent Zhi Qiang Wang1, Jianhui Zhu1, Judith Klein-Seetharaman5,

Krishnakumar Balasubramanian2 , Andrew A. Amoscato2, Grigory Borisenko2 ,

Zhentai Huang2, Aaron M. Gusdon1, Amin Cheikhi2, Erin K. Steer1, Ruth Wang1, Catherine Baty4, Simon Watkins4, Ivet Bahar6, Hülya Bayır2, 3\* and Valerian E. Kagan2\*

**Methods**

Primary cortical neuron cultures were prepared from E16 Sprague–Dawley rats (Charles River Lab, CA), employing 0.25% trypsin with EDTA (Invitrogen, Carlsbad, CA) for 15-20 min at 37 ºC, followed by trituration in ice-cold Neurobasal medium and plating in poly-D-lysine coated plates, following protocols approved by the University of Pittsburgh InstitutionalAnimal Care and Use Committee. Experiments were performed at 7-9 DIV (>95% neurons, <5% astroglia), employing sublethal conditions (Fig. S1a; Reference[48](#_ENREF_48)) for rotenone (125 nM-250 nM x 2h), STS (100 nM x 2h) or DMSO (Sigma, MI, USA) as vehicle control.

SH-SY5Y, a human neuroblastoma cell line (ATCC, Rockville, Maryland) that expresses tyrosine hydroxylase and dopamine transporter, was maintained in antibiotic-free Dulbecco’s modified Eagle’s medium (BioWhittaker, Walkerville, Maryland) supplemented with 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, California), 15 mM HEPES, and 2 mM glutamine, with 5% CO2 at 37°C. All cells were verified mycoplasma-free using Lonza's MycoAlert mycoplasma detection kit. Cells are routinely tested every one to two months. To stimulate mitophagy, SH-SY5Y cells were treated with rotenone (1 μM), staurosporine (1 μM), 6-hydroxydopamine (120 μM), FCCP (2-5 μM), rapamycin (50 μM) or vehicle for 4 hours. HeLa cells (ATCC) expressing Parkin (Germantown, MD) were cultured in DMEM supplemented with 10% FBS. For mitophagy induction, cells were treated with 20 µM CCCP for 4 hours.

Transfections and image analysis.

Cortical neurons and SH-SY5Y were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as previously described. Plasmids included mtDsRed2 (Clontech, MA, USA), Mito-GFP (Clontech, Mountain View, CA), GFP-tagged or RFP-tagged LC3 (Dr Tamotsu Yoshimori, Research Institute of Microbial Diseases, Osaka University, Japan), mCherry-tagged p62/SQSTM1 (Zhenyu Yue, Mt. Sinai, NY), or untagged mouse phospholipid scramblase 3 in pcMV6-XL4 (Origene,Rockville, MD). Primary neurons were transfected at 4 DIV with 45 nmol of small interfering RNA (siRNA) as indicated in Table 2, using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). For HeLa cells, siRNA targeting human CLS or negative control siRNAs were transfected using RNAiMax (Life Technologies) and two distinct siRNAs targeting human PLS3 sequences beginning at nucleotide #433 or #508 (Invitrogen, Carlsbad, CA), siRNA targeting siRNA targeting human ATG8 (LC3B)[17](#_ENREF_17), human Atg7[17](#_ENREF_17) and/or scrambled nontargeting siRNA controls (Life Technologies, Carlsbad, CA) were used for SH-SY5Y cells (Table 2). All STS, rotenone, 6-OHDA, FCCP, CCCP or vehicle treatments were performed 72 h following plasmid or siRNA transfection.

Fluorescence images were captured as z-stacks (0.5 μm slices, 640 × 640 pixel resolution, 20 µs/pixel) encompassing the depth of the cell using an Olympus Fluoview 1000 confocal microscope (×60 oil immersion lens, NA: 1.42), using sequential laser imaging and Kalman filter correction. Coverslips/wells were imaged randomly, scanning for 3-5 fields in each quadrant; 20–30 neurons/cover slip or 15-55 transfected cells were imaged/experiment. Images were coded, and total numbers and numbers of colocalizing LC3 puncta were analyzed by an individual blinded to the condition. Numbers of fluorescent LC3 puncta elicited per cell, western blot analysis of LC3-II, and mitophagy analysis by colocalization of mitochondria with early and late autophagolysosomal markers were performed as described[45](#_ENREF_45), [49](#_ENREF_49).

Completion of mitophagy was analyzed by immunoblot for levels of OMM, IMM and matrix proteins (See Antibodies list below). Bafilomycin (50 nM x 1h) was used in some experiments to arrest autophagosome degradation.

Biochemical studies.

Mitochondria were prepared using a combination of differential and Percoll gradient centrifugation (Sims and Anderson, 2008). The inner (IMM) and outer (OMM) mitochondrial membrane fractions were obtained as previously described33. Samples were centrifuged (12,000g) and the pellets and supernatants collected as IMM and OMM fractions, respectively. The purity of fractions was verified by two methods, immunoblot analysis of COX4, TOM40, ANT or VDAC, and by quantitative LC-MS analysis of characteristic phospholipid distribution.

Cell lysates or fractions from isolated mitochondria were washed with PBS, lysed in 0.1% Triton X-100 with protease/phosphatase inhibitor cocktail, and protein concentration determined by Coomassie Plus Protein Assay (Pierce, Rockford, Illinois). Loading was adjusted for relative protein yields of different subcellular fractions to enable comparisons of equivalent cell numbers. Standard immunoblot procedures employed antibodies described below.

Quantitative mass spectrometry.

Individual phospholipid classes from mitochondrial inner and outer membranes were resolved using normal phase silica column chromatography (Luna, 3micron, 15 cm x 2mm i.d. Phenomenex, Inc., Torrance, CA), coupled to a Shimadzu Prominence HPLC system (Shimadzu, Inc., Kyoto, Japan), with elution at 0.2 ml/min employing a linear gradient [Solvent A: chloroform:methanol:triethylamine:acetic acid, (80:19:0.5:0.5); Solvent B: chloroform:methanol:water:triethylamine:acetic acid, (60:33.5:5.5:1.0:0.065)]. Phospholipids were analyzed on a Waters Q-TOF mass spectrometer (Waters, Inc., Milford, MA) using the following parameters: capillary voltage, 2.85 kV, negative mode; source temperature, 100OC; desolvation gas, 400 L/h; sampling cone, 60V; extraction cone: 4.5V; ion guide, 3.0V. Tuning was optimized for all lipids across the scan range. For some samples, an ion trap platform was also used for analysis. For quantitative analysis of CL, samples were mixed with an internal standard (1,1′,2,2′-tetramyristoyl-cardiolipin, Avanti Polar Lipids Inc., Alabaster, AL). Nanomoles of individual molecular species of CL were calculated by determining the ratio of the peak area of the CL molecular species of interest to the internal standard. In addition to TMCL, the following phospholipid internal standards were used with fatty acid chains as follows: phosphatidylglycerol (PG, 12:0/12:0); phosphatidylinositol (PI, 16:0/16:0); phosphatidic acid (PA, 17:0/17:0); phosphatidylethaolamine (PE, 17:0/17:0); phosphatidylserine (PS, 17:0/17:0), phosphatidylcholine (PC, 11:0/11:0) and sphingomyelin (d18:1/18:0).   The CL content was normalized as pmoles CL/nmole total mitochondrial phospholipid phosphorus or as nmoles CL/mg mitochondrial protein.

Cardiolipin exposure on the outer surface of mitochondria.

To quantify the content of CL exposed to the outside of mitochondria, we treated intact mitochondria with 0.7 U/mg protein of an anionic phospholipid-selective PLA2 (Porcine pancreatic, Sigma) [50](#_ENREF_50), in the presence of fatty acid-free human serum albumin (20 mg/ml , 4oC, 50 min) to prevent membrane damage by CL hydrolysis products such as lyso-CLs and free fatty acids [51](#_ENREF_51). Normal phase LC-MS was employed to detect lyso-hydrolysis products before and after cleavage with PLA2, as described above.

Annexin V binds anionic phospholipids in a calcium dependent manner. It has the highest affinity for CL based on the [Ca2+] required for half maximal binding, as the unique phospholipid headgroup of CL is more effective at charge clustering[16](#_ENREF_16),[17](#_ENREF_17)To validate and optimize the annexin V binding assay for externalized CL, isolated liver mitochondria[52](#_ENREF_52) were coated with different phospholipids (50 pmol/mg protein) and Annexin V binding assessed by flow cytometry in the presence of increasing [Ca2+](Fig. S2d). The assay was further validated using cells depleted of phospholipid scramblase 3, the enzyme that mediates CL redistribution to the OMM.

To measure the extent of CL externalization in response to mitophagy-inducing treatments, cells seeded on 100 mm tissue culture plates were pre-transfected with Mito-GFP or stained with 250nM Mitotracker Green FM (Invitrogen) for 45 minutes at 37°C to label mitochondria. In some experiments, SH-SY5Y cells were first transfected with control hairpin and scramblase-3 siRNA using LipofectAMINE 2000[17](#_ENREF_17), and treated 3 days later. Each independent experiment involved pooling cells from five plates/condition for mitochondrial isolation. Isolated mitochondria were incubated with Alexa 647-labeled Annexin V (Invitrogen) in buffer maintained at 15 nmol Ca2+/g protein to stain surface-exposed anionic phospholipids and then subjected to flow cytometric analysis (FACSCanto, Becton-Dickinson, Rutherford, NJ) of red fluorescence (Ex/Em, 633/660 nm) employing appropriate settings for forward light scatter and side light scatter detectors. The Alexa fluorescence from gated green fluorescent mitochondria events (Ex/Em, 488/530 nm) was used to evaluate the binding of Annexin-V to mitochondria.

Molecular modeling and sequence alignment.

Marvin Sketch (v. 5.3.6, 2010) was used for displaying chemical structures and generating 3D structures corresponding to the lowest energy conformer (<http://www.chemaxon.com>). CL was docked to the crystal structure of LC3-I (PDBid : 1UGM [8](#_ENREF_8)) using AutoDock Vina[53](#_ENREF_53) (http://vina.scripps.edu). Lipid and protein structures were converted from pdb into pdbqt format using MGL Tools[54](#_ENREF_54). LC3 was treated as the receptor and kept rigid during docking, maintaining flexibility of rotatable bonds in CL. A grid box was centered at the -11.358, 37.55, 13.575 coordinates with 40Å units in x, y and z directions to cover the entire LC3 protein. AutoDock Vina reports the 9 lowest energy conformations, which were inspected using Pymol software (www.pymol.org). All computational calculations (docking and molecular dynamics) have been done using the published LC3B structure (1UGM)[8](#_ENREF_8).

A total of 39 sequences corresponding to the LC3 family corresponding to isoforms (A-C) were downloaded from SwissProt[55](#_ENREF_55) using the criteria “*((gene:MAP1LC3B OR gene:MAP1LC3A OR gene:MAP1LC3C)) AND fragment:no*”, aligned with ClustalW[56](#_ENREF_56) and displayed using WebLogo[57](#_ENREF_57).

Please refer to SI Note for methods pertaining to the full atomic and coarse-grained molecular dynamics simulations of Supplementary Fig. S6.

Preparation of liposomes.

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,1’,2,2’-tetraoleoyl-cardiolipin (TOCL), 1,1’,2,2’-tetralinoleoyl-cardiolipin (TLCL), monolyso-tri-linoleoyl-cardiolipin (lyso-CL), 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (DOPG), were obtained fromAvanti Polar Lipids, Inc. (Alabaster, AL). Phospholipids, stored in chloroform, were mixed and dried under nitrogen, then mixed in vortex in HEPES buffer (20 mM, pH 7.4) and sonicated 3x for 30 s on ice. Liposomes were used immediately after preparation.

Phospholipid-LC3 native blue gel shift binding assay.

Recombinant human His6-LC3/MAP1LC3A (R&D Systems, Inc, Minneapolis, MN) (4 µM) was incubated in 25 mM HEPES buffer (pH 7.4) with liposomes containing different ratios of TOCL, DOPA, DOPI or DOPG, on a DOPC backbone for 10 min at RT, followed by Native Blue electrophoresis in 4-16% bis-tris gel, and silver staining using GelCode SilverSNAP kit (Fisher Scientific). In some experiments recombinant rat LC3B (gift of Yasuo Uchiyama, Juntendo University Graduate School of Medicine, Tokyo, Japan) was used. Densitometric analysis of gels was performed and percentage of monomeric LC3 in gel was determined. Lipid /LC3 ratios preventing 50% of the LC3 monomer from entering the gel were determined from analysis of the plots of percentage monomeric LC3 vs. lipid/LC3 ratio for each individual phospholipid. TLCL and lyso-CL were also employed to investigate the possible role of acyl chain numbers.

Custom synthesized (LifeTein LLC, South Plainfield, NJ) N-terminal human LC3A WT and mutant (R10R11>L10L11) peptides (amino acid sequences 1-30) (1.5 µM) were incubated in 25 mM HEPES buffer (pH 7.4) with liposomes containing 1,1′2,2′-tetraoleoylcardiolipin (TOCL)/ 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) at TOCL/LC3 ratio of 6 (10 min at RT). This was followed by Native Blue electrophoresis in 4-16% bis-tris gel, and silver staining using GelCode SilverSNAP kit (Fisher Scientific).

Mutagenesis

An N-terminal deletion mutant plasmid for GFP-LC3 (Δ1-28) was generated by PCR using forward and reverse primers that harbor a BglII restriction site and an EcoRI restriction site, respectively, and the cDNA for the first β-sheet of rat LC3 (5’TATAGAATTCTATGAACCAAGATCCCAGTGATTAT-3’). The purified DNA fragment was subjected to enzymatic digestion with BglII and EcoRI restriction enzymes and ligated onto BglII and EcoRI pre-digested pEGFP-C1 vector backbone. The GFP-LC3(R10L,R11L) (forward primer: 5’-GAG AAGACCTTCAAACAG CTCCTGAGCTTCGAACAAAGAGTG-3’, reverse primer: 5’-CACTCTTTGTTCGAAGCTCAGGAGCTGTTTGAAGGTCTTCTC-3’) and GFP-LC3(Q26L,H27L) (forward primer: 5’CGGCTCATCCGGGAGCTGCTTCCCACC AAGATCCCAG-3’, reverse primer: 5’CTGGGATCTTGGTGGGAAGCA GCTCCCGG ATGAGCCG-3’) mutant plasmids were generated by QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Lajolla, CA) according to the manufacturer’s instruction. The cDNA sequences in all mutant constructs were confirmed by DNA sequencing analysis. The expression levels of the recombinant constructs were evaluated in transfected SH-SY5Y cells by immunoblotting with anti-GFP antibody (Fig. 5k inset). The GFP-LC3 mutant containing a C-terminal glycine to alanine mutation (G120A) that is impaired for binding to autophagosomes was obtained from Dr. Tamotsu Yoshimori (Graduate School of Biosciences and Frontier Bioscience, Osaka University, Suita-Osaka, Japan)

RNAi sequences and antibodies.

The following RNAi sequences were used: siCLS (rat, CGCGAACACUAGCUAAGUAtt, GGAUUUGUUGGAUGGAUUUtt, CCCACUCACUUACAUGAUAtt, pooled, S172628, S172627, S172626, Life Technologies, Carlsbad, CA); siCLS#2 (rat, GGAUGGAUUUAUUGCUCGAtt, J-093152-09-0002, Thermo Fischer Scientific, Pittsburgh, PA; siCLS (human, GCUUAUAGUUACUAUCAUUTT, S29308, Ambion, Life Technologies, Grand Island, NY); siPLS3 #433 and #508 (human, GCCTCGAATTCCTGGTG CAGA and GGGAGACCTGTAATCGGTATGAACT, Stealth RNAi™ Pre-Designed siRNAs, Invitrogen, Grand Island, NY; siLC3B (human, 5-GAAGGCGCUUACAGCUCAA-3) and siAtg7 (human, 5 -GCCAGUGGGUUUGGAUCAA- 3) as previously described17.

Antibodies used, rabbit-anti-LC3 (1:1000, #2775, Cell Signaling, Danvers, MA; mouse-anti-LC3 (1:200, LC3-5F10: 0231-100, Nanotools); mouse-anti-TOM40 (1:1000, D-2: SC-365467), rabbit anti-TOM40 (1:1000, H-300: SC-11414), goat anti-LDH-A (1:1000, V-17: SC-27232), and rabbit anti-GAPDH (1:1000, SC-25778) from Santa Cruz Biotechnology, Dallas, Texas; rabbit-anti-MnSOD (1:1000, DD-17: S5069), mouse-anti-actin (1:2000, clone AC-40: A3853) and rabbit-anti-actin (1:2000, A2066 (C11 peptide) or A5441) from Sigma;

mouse anti-MnSOD (1:1000, clone 19: 611580, BD Pharmingen); mouse anti-human mito. antigen p60 (1:1000, MU213-UC, Biogenex, San Ramon, CA); mouse anti-p62 (1:1000 (WB), 1:200 (IC), 610832, BD Biosciences, San Jose, CA); rabbit anti-GFP (1:5000, A6455, Invitrogen, Carlsbad, CA). We thank Peter J Sims, University of Rochester School of Medicine, for the rabbit anti-human scramblase-3 (1:500).

Statistics

For fluorescence experiments, all in-focus cells, expressing the appropriate transfection marker if applicable, from 12-15 randomly captured fields per slide were analyzed. The average value per cell was determined for each independent experiment. The results from each independent experiment were then averaged and expressed as the mean of n = 3-7 independent experiments, +/- standard deviation (s.d.). The data was analyzed by one-way ANOVA for multi-group parametric comparisons or two-tailed t-tests for parametric comparisons involving two samples with similar variances. For western blot quantification, densitometry was performed and the normalized values from independent experiments averaged. Significance was set at p < 0.05.

References.

48. Tyurin, V.A. *et al.* Mass-spectrometric characterization of phospholipids and their primary peroxidation products in rat cortical neurons during staurosporine-induced apoptosis. *Journal of neurochemistry* **107**, 1614-1633 (2008).

49. Zhu, J., Dagda, R.K. & Chu, C.T. Monitoring mitophagy in neuronal cell cultures. *Methods Mol Biol* **793**, 325-339 (2011).

50. Yu, B.Z. *et al.* Structural basis of the anionic interface preference and kcat\* activation of pancreatic phospholipase A2. *Biochemistry* **39**, 12312-12323 (2000).

51. Nilsson, O.S. & Dallner, G. Transverse asymmetry of phospholipids in subcellular membranes of rat liver. *Biochimica et biophysica acta* **464**, 453-458 (1977).

52. Frezza, C., Cipolat, S. & Scorrano, L. Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nature protocols* **2**, 287-295 (2007).

53. Trott, O. & Olson, A.J. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of computational chemistry* **31**, 455-461 (2010).

54. Sanner, M.F. Python: a programming language for software integration and development. *Journal of molecular graphics & modelling* **17**, 57-61 (1999).

55. Boeckmann, B. *et al.* The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003. *Nucleic acids research* **31**, 365-370 (2003).

56. Thompson, J.D., Gibson, T.J. & Higgins, D.G. Multiple sequence alignment using ClustalW and ClustalX. *Current protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.]* **Chapter 2**, Unit 2 3 (2002).

57. Crooks, G.E., Hon, G., Chandonia, J.M. & Brenner, S.E. WebLogo: a sequence logo generator. *Genome research* **14**, 1188-1190 (2004).