Supplementary Information

**Cardiolipin Externalization Signals Mitophagy**

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Legends for Figure S1 – Figure S7

Table S1 – Table S3

Fig. S1. Additional characterization of Rot and STS-induced mitophagy. Primary rat cortical neuron cultures were treated with rotenone at 7-9 days *in vitro* (DIV) using the indicated doses and times. Cell death was analyzed by flow cytometry of Annexin V/propidium iodide stained neurons to define sub-lethal conditions (a). N=6 independent experiments; mean +/- s.d., \*p < 0.05 vs. vehicle. Equivalent results were observed for caspase-3/7 activation (not shown), assayed using the luminescent Promega Caspase-Glo assay (Madison, WI). Rotenone elicited increased GFP-LC3 puncta (b) and colocalization with mitochondria (c), the latter of which was inhibited by siRNA knockdown of CLS (siCLS), but not scrambled siCtrl. For b,c: N=3; mean +/- s.d. \* p<0.05 vs. vehicle-treated neurons; † p<0.05 vs. respective Rot-treated siCtrl neurons. Primary cortical neurons treated with vehicle or the indicated concentrations of rotenone were analyzed for p62 protein levels by immunoblot. Densitometry indicates significant decreases in p62 levels, consistent with dose-dependent increases in autophagic flux (d). N=3; mean +/- s.d. \*p<0.01 vs. vehicle; \*\*p<0.01 vs. Rot 125 nM. Densitometry of mitochondrial proteins analyzed by immunoblot (See Fig. 1g) confirmed decreased protein levels, inhibited by co-treatment with bafilomycin (e). N=3; mean +/- s.d., \*p<0.05 vs. Ctrl; †p<0.05 vs. Rot alone. Primary neurons transfected on DIV 4 with siCLS or siCtrl were treated with STS on DIV 7 and analyzed for autophagy by LC3 shift (f) and GFP-LC3 puncta (g), and for mitophagy by colocalization analysis (h) or by loss of mitochondrial proteins (f). For g,h: N=3; mean +/- s.d. \*p<0.05 vs. respective vehicle-treated neurons; †p<0.05 vs. STS-treated siCtrl neurons. STS also elicited mitophagy in SH-SY5Y cells (i), as assessed by delivery of mitochondria to LTR-labeled lysosomes, which was inhibited by siRNA knockdown of the autophagy proteins Atg7 or LC3, as well as PLS3. \* p<0.05 vs Veh; † p<0.05 vs STS/siCtrl.

Fig. S2. Mitochondrial isolation and characterization of phospholipid content and accessibility. Primary cortical neuron cultures (DIV 7 or 8) were exposed to rotenone followed by isolation of mitochondrial IMM and OMM for LC-MS analysis. Immunoblot analysis employed the following (a): COXIV - IMM marker; TOM40 - OMM marker; pan Cadherin - cellular membrane marker; Histone H3 - nuclear marker. Western blot analysis for contact site IMM and OMM proteins ANT and VDAC, respectively, reveal no significant cross-contamination of fractions (b, Rot, 250 nM x 2h, representative of 3 experiments). Neuronal cells were treated with rotenone (250 nM) or STS (100 nM), and the IMM and OMM fractions prepared from isolated mitochondria were pooled from 3 experiments for lipid extraction and LC-MS analysis as described in Methods. Representative spectra for phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidic acid (PA) are shown for mitochondrial membranes isolated from control, rotenone- and STS-treated neurons are shown (c). In contrast to CL (Fig. 2a), only subtle changes in peak distribution, intensity, or cluster pattern of IMM and OMM PC, PE, PI and PA were observed. Isolated liver mitochondria labeled with the indicated phospholipids were mixed with Annexin V in the presence of the indicated Ca2+ concentrations. Annexin V binding was assessed by flow cytometry, indicating specificity for CL at lower Ca2+ concentrations (d). The highest concentration shown is equivalent to 300 M. N=4 experiments, mean +/- s.d. STS-treated HeLa cells exhibited increased surface accessibility of CL on isolated mitochondria probed with Annexin V (e). Inset: purity of membrane fractions. \* p<0.05 vs. control. N=3 experiments, mean +/- s.d.

**Fig. S3. Additional characterization of phospholipid scramblase 3 (PLS3) knockdowns.** Mitochondrial and cytosolic fractions from SH-SY5Y cells were immunoblotted for human scramblase-3 (hPLS3), and for the human mitochondrial antigen of 60 kDa (mito-P60) or the cytosolic lactate dehydrogenase enzyme (LDH) as compartment markers **(a)**. SH-SY5Y cells were treated with two siRNAs targeting base pairs 433-453 (#433) or base pairs 508-532 (#508), and the extent of knockdown assessed by immunoblot densitometry **(b).** n=6, ‡ p<0.05 vs. siCtrl. RNAi knockdown of PLS3 (siPLS3#508) blocks the Rot-induced colocalization of mitochondria with LTR-labeled lysosomes **(c)**. \* p<0.05 vs. Veh/siCtrl; † p<0.05 vs. Rot/siCtrl. Western blot analysis of the effects of siPLS3 on Rot-induced decreases in COX IV and MnSOD (**d**). Effects of PLS3 knockdown on basal and 6-OHDA-elicited GFP-LC3 puncta **(e).** \* p<0.05 vs. respective vehicle treated cells. There were no significant effects of PLS3 knockdown on baseline or rotenone-treated mitochondrial membrane potentials **(f).** n=8 wells/condition, representative of two independent experiments, no significant changes by ANOVA. The two siRNAs showed equivalent effects on mitophagy elicited by Rot **(g)** or by 6-OHDA (Fig. 3d). \* p<0.05 vs. Veh/siCtrl; † p<0.05 vs. Rot/siCtrl.

**Fig. S4. Additional characterization of cardiolipin synthase (CLS) knockdowns.** Treatment of primary cortical neurons with siCLS resulted in knockdown of rat CLS without affecting expression of other mitochondrial proteins **(a).** N=3, mean +/- s.d. \* p < 0.05 vs. siCtrl. CLS knockdown had no effects on membrane potential measured by TMRM staining, and did not affect the small, but significant decrease elicited by rotenone **(b).** N=3, \* p < 0.05 vs. Veh. CLS knockdown suppressed the loss of mitochondrial proteins elicited by rotenone as quantified by densitometry **(c).** N=3; \* p <0.05 vs. vehicle; † p < 0.05 vs. respective Rot/siCtrl. See also Fig. 3g-3i for representative blots and additional quantification. A second independent CLS siRNA (siCLS#2) was also effective in reducing CLS expression **(d)** and inhibiting the loss of mitochondrial proteins **(e),** with no effect on baseline or rotenone-treated membrane potentials **(f).** N=3, \* p < 0.05 vs. Veh.

**Fig. S5. The potential relationship of CL to the FCCP/CCCP-PINK1-Parkin pathway.**

A previously characterized stable PINK1-3xFlag expressing SH-SY5Y line (#24, Reference 10), was treated with FCCP (2μM x 4 hrs) beginning at 72 h after transfection with siCtrl or siPLS3. Immunoblot analysis confirms PLS3 knockdown, which had no effect on the well-characterized FCCP-induced accumulation of full-length PINK1 **(a)**. SH-SY5Y cells were co-transfected with HA-Parkin and siCtrl or siPLS3, treated with FCCP 72 h later, fixed in paraformaldehyde and immunolabeled for HA (green) and mitochondrial p60 antigen (red). Scale bar: 10μm. FCCP elicited HA-Parkin puncta, all of which colocalized with mitochondrial p60 **(b,** arrows). There were no effects of siPLS3 on basal or FCCP-induced Parkin puncta **(c)**, nor were there effects on FCCP-induced mitochondrial translocation of Parkin analyzed by immunoblot (not shown). \*p <0.05 vs. respective Veh-treated cells. While PLS3 knockdown had no effect on FCCP-induced autophagosomes **(d)**, it significantly reduced the degree of mitophagy elicited by FCCP **(e).** Overexpression of Drp1 did not rescue the inhibition of mitophagy by PLS knockdown **(e),** despite promoting significant further mitochondrial fragmentation (not shown). \* p<0.05 vs. respective Veh-treated cells; † p<0.05 vs. FCCP/siCtrl. Similarly, in Parkin-expressing HeLa cells, knockdown of CLS (siCLS #S29308) did not block the mitochondrial translocation of Parkin **(f).** Nevertheless, CCCP treatment did elicit higher levels of CL at the mitochondrial surface, as assessed using both the AnnexinV assay **(g)** and the PLA2 hydrolysis assay **(h),** which was inhibited by siCLS. \* p<0.05 vs. Veh/siCtrl. † p <0.05 vs. CCCP/siCLS. CCCP treatment of Parkin-expressing HeLa cells resulted in the loss of mitochondrial proteins MnSOD and TOM40, while RNAi knockdown of CLS partially reduced this loss **(i).** Representative of 3 independent experiments. Effective knockdown of human CLS was demonstrated in Parkin-transfected HeLa cells at 72h after siRNA treatment **(j),** which was associated with a ~30% decrease in the levels of total CL in these cells **(k).**

**Fig. S6. Dynamic modeling of LC3/CL interactions.**

LC3 and three closest CL molecules in simulation MD1 are shown at four successive snapshots **(a).** Water, ions and POPC molecules are not shown in the snapshots for clarity.  Note that LC3 enters the interaction range (less than 6 Å) of CL within 10 ns in all three simulations. Time evolution of LC3 position **(b)**, monitored as minimum distances between head group atoms on CL and residues R68-R70 (black), R10-R11 (red), K42 (green) or K5 (blue) as observed in each of the three simulations (MD1-3). Magnified snapshots illustrate LC3/CL binding poses with the indicated residues at the end of 50 ns in each of the three simulations **(c).** For clarity, only those CL molecules which interact with LC3 are illustrated. The LC3 residues interacting with CL are highlighted in stick representation and labeled. The number of CL, PA and LysoCL interacting with LC3 is graphed with time during CGMD simulations **(d).** Insert snapshots illustrate the simulated interaction of LC3 with a PA-containing bilayer. Color guide: DOPC: yellow; head groups of PA: dark blue; acyl chain of PA: light blue; LC3: purple. CGMD simulation of the interaction of CL with LC3 for 1 μs **(e)**.Snapshots illustrate the simulated interaction of LC3 with a CL-containing membrane using elastic network to preserve the protein structure (Refs. 9-10, SI Note). Note that water, ions and DOPC molecules are not shown for clarity. Color guide: head groups of CL: dark blue sticks, acyl chain of CL: light blue sticks, LC3: cyan (transparent), N-terminus of LC3 (K5, R10-R11): blue spheres, K49, R68-R69-R70: green spheres, Glu117: red sphere). Arrow indicates the C-terminal region involved in lipidation.

**Fig. S7. Uncropped western blots.** Shown in this figure are all immunoblots from which the multiblot figure panels or insets for Figures 1f, 1g, 3e, 3g, 4b, 4c, 4d, 4f, and 5k were derived. Also shown are key uncropped immunoblots comprising panels in Supplementary Figures S1, S2, S3, and S5.

Table S1. The Rotenone-Treated Cortical Neuron OMM Exhibits a Similar Number and Distribution of CL Species as the IMM.

The m/z values of the CL species for rotenone-treated neuron OMMs and for control neuron IMMs are compared for the 7 major clusters of CL detected. The table displays a similar number and distribution of CL in the OMM in rotenone treated neurons as compared to control IMMs. MS/MS analysis was performed on the major CL species from each cluster, revealing that the major CL species of each cluster from the rotenone-treated OMM was structurally identical to the corresponding species from the control neuron IMM.

**CL Species(m/z) in Rotenone-treated cortical neuron OMM**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Cluster 1** | **Cluster 2** | **Cluster 3** | **Cluster 4** | **Cluster 5** | **Cluster 6** | **Cluster 7** |
| 1372 | 1396 | 1422 | 1446 | 1474 | 1498 | 1522 |
|  | 1398 | 1424 | 1448 | 1476 | 1500 | 1524 |
|  | 1400 | 1426 | 1450 | 1478 | 1502 | 1526 |
|  | 1402 | 1428 | 1452 | 1480 | 1504 | 1528 |
|  |  | 1430 | 1454 |  |  |  |
|  |  |  | 1456 |  |  |  |
|  |  |  | 1458 |  |  |  |

Fatty acyl chain analysis for main species in OMM after rotenone.

m/z 1372, (16:1)3 (18:1)1; m/z 1400, (16:1)2 (18:1)2; m/z 1428, (16:1)1 (18:1)3; m/z 1456, (18:1)4; m/z 1478, (18:1)3 (20:4)1; m/z 1502, (18:0)1 (18:1)1 (20:4)2; m/z 1526, (18:1)2 (20:3)1 (22:6)1.

**CL Species(m/z) in control cortical neuron IMM**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Cluster 1** | **Cluster 2** | **Cluster 3** | **Cluster 4** | **Cluster 5** | **Cluster 6** | **Cluster 7** |
| 1370 | 1394 | 1420 | 1446 | 1472 | 1498 | 1522 |
| 1372 | 1396 | 1422 | 1448 | 1474 | 1500 | 1524 |
| 1374 | 1398 | 1424 | 1450 | 1476 | 1502 | 1526 |
|  | 1400 | 1426 | 1452 | 1478 | 1504 | 1528 |
|  | 1402 | 1428 | 1454 | 1480 |  |  |
|  |  | 1430 | 1456 | 1482 |  |  |
|  |  | 1432 | 1458 |  |  |  |
|  |  |  | 1460 |  |  |  |

The fatty acyl analysis for main species in IMM control

m/z 1372, (16:1)3 (18:1)1; m/z 1400, (16:1)2 (18:1)2; m/z 1428, (16:1)1 (18:1)3; m/z 1456, (18:1)4; m/z 1478, (18:1)3 (20:4)1; m/z 1502, (18:0)1 (18:1)1 (20:4)2; m/z 1526, (18:1)2 (20:3)1 (22:6)1.

**Table S2. Residues of LC3 interacting with CL as evidenced by Coarse-Grained Molecular Dynamics simulation.** The data reflect one of the prototypical simulations found in three out of four performed CGMD experiments. The first contact involves N-terminal residues F7-K8-Q9-R10-R11 [Note overlap with the favored docked conformation of F7-R10-R11 (Figure 4f,h)]. These residues stay in contact throughout the rest of the simulation. While additional electrostatic interactions form subsequently, a hydrophobic contribution develops through involvement of V33-I34-V46-F52-L53-I67 at 100ns, which is further expanded with the addition of V17-V20- L22-I31-I35-Y38-L47-V54-I64-I66-L71-F79-F80-L82-L83-I95-F108-Y110 at 150ns. A distance of < 5Å was used to identify interacting molecules **Bold**: residues that contact the bilayer after 50ns. Underlined: residues in contact after 100ns.

|  |  |  |
| --- | --- | --- |
| **Time (ns)** | **Residues** | **Number of Residues** |
| 40 | --  | 0 |
| 50 | **F7-K8-Q9-R10-R11**  | 5 |
| 100 | K5-**F7**-**Q9-R10-R11**-E14-H27-T29-K30-P32-V33-I34-R37-P45-V46-K49-T50-F52-L53-I67-R68  | 21 |
| 150 | **F7-Q9-R10-R11**-V17-V20-R21-L22-H27-T29-K30-I31-V33-I34-I35-Y38-K42-L47-K49-T50-F52-L53-V54-N59-I64-I66-R68-L71-F79-F80-L82-L83-I95-F108-Y110 | 35 |

**Table S3. Mammalian LC3B aligned with Atg8 homologs from different species.**

