# Supplementary Material

## Materials and methods

*Cell Culture*

Human epithelial HeLa cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cyt *c* deficient HeLa cells (HeLa1.2) were generated as previously described using shRNA procedure [11]. Cells were maintained in DMEM supplemented with 15% FBS, 25 mM HEPES, 50 mg/L uridine, 110 mg/L pyruvate, 2 mM glutamine, 1× nonessential amino acids, 0.05 mM 2'-mercaptoethanol, 100 U/L penicillin and 100 μg/L streptomycin in a humidified atmosphere of 5% $CO_2$:95% air at 37°C. To develop *mt*DNA deficient ρ° cells, HeLa1.2 cells were exposed to 50 ng/ml ethidium bromide for 60 days (equaling approximately 15 passages).

*Phosphatidylserine (PS) externalization assay*

Externalization of PS and cell membrane integrity were analyzed by flow cytometry using an Annexin-V/propidium iodide (PI) kit. At the end of incubation, still adherent cells were trypsinized and pooled with the cells that had already detached. Harvested cells were stained with Annexin-V-FITC and PI for 5 min in the dark prior to flow cytometry analysis. Ten thousand events were collected on a FACScanto II flow cytometer. Percentages of Annexin-V-positive cells were calculated by combining Annexin V+/PI- (early apoptotic) and Annexin V+/PI+ (late apoptotic or necrotic) cells.

*Western blotting*

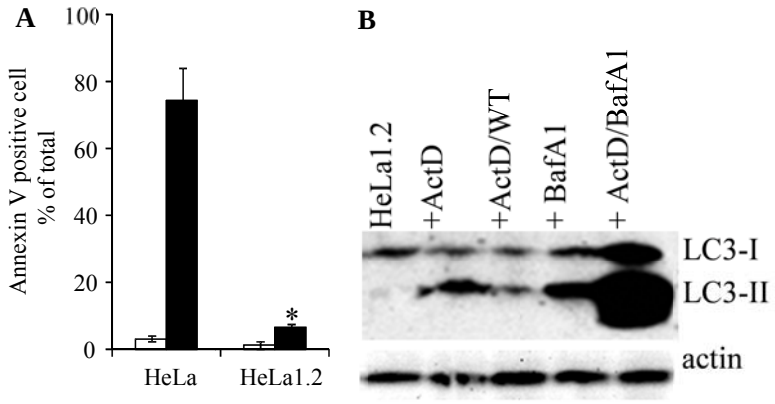
Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1 % Igepal CA-630) containing protease inhibitors cocktail (Sigma) for 1 h on ice. The samples were then centrifuged at 5, 000×g for 5 min at 4°C. The resulting supernatants (50 μg) were subjected to 15% SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was blocked with 1% casein in TBS contain 0.02% Tween-20 for 1 h, and then probed with antibodies against LC3 (1:400), COX-IV (1:2000)

and β-actin (loading control, 1:10000) followed by horseradish peroxidase-coupled detection.

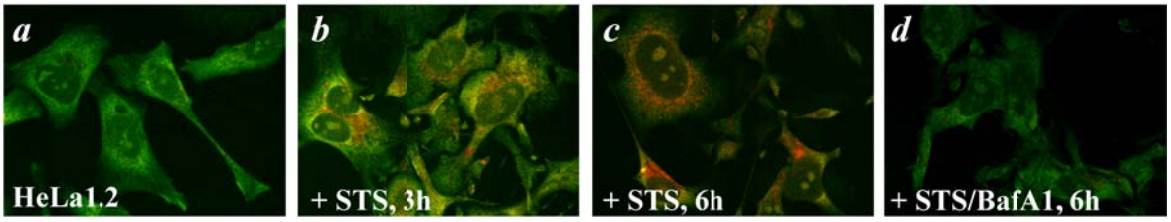
*Acridine orange staining of acidic vesicular organelles(AVO)*

The appearance and volume of the AVO was quantified using Acridine orange staining (1 μg/ml, 15 minutes, 37°C). In order to carry out a specificity control, cells were treated with 50 nM BafA1 before the addition of Acridine orange to inhibit the acidification of autophagic vacuoles. Images were obtained with an Olympus confocal microscope.

**Figures**

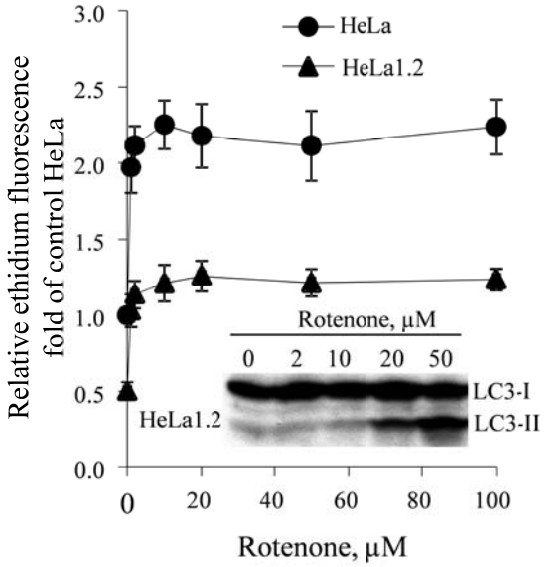



**Supplementary figure S1**. Actinomycin D induced autophagy independently of apoptosis in cytochrome c deficient HeLa1.2 cells. Cells were treated with 100 ng/mL actinomycin D for 24 h. (A) ActD-induced PS externalization in HeLa and cytochrome c deficient HeLa1.2 assessed by flow cytometry. Note that around 75% of HeLa cells were PS-positive cells after ActD treatment. In contrast, ActD-induced PS externalization was remarkably attenuated in HeLa1.2 cells (5.8%). (B) ActD-induced LC3-I/II conversion in HeLa1.2 cells examined by western blotting. The increased autophagic flux was confirmed by wortmannin (an inhibitor of PI3-kinases, WT, 0.2 μM) and Bafilomycin A1 (an lysosomal inhibitor, BafA1, 50 ng/mL). Note that a robust LC3-I/II conversion was detected in ActD-treated HeLa1.2 cells in the absence of PS externalization.




**Supplementary figure S2**. Representative confocal images of Acridine orange staining. HeLa1.2 cells were treated with 100 nM STS for 3 and 6 hour, Acridine orange was added to cells at a final concentration of 1 μg/ml for a period of 15 min. BafA1 (50 nM) was added to cells 2 h prior to addition of Acridine orange. At the end of incubation, cells were fixed with 4% paraformaldehyde and imaged using an Olympus Fluoview 1000 confocal microscope.

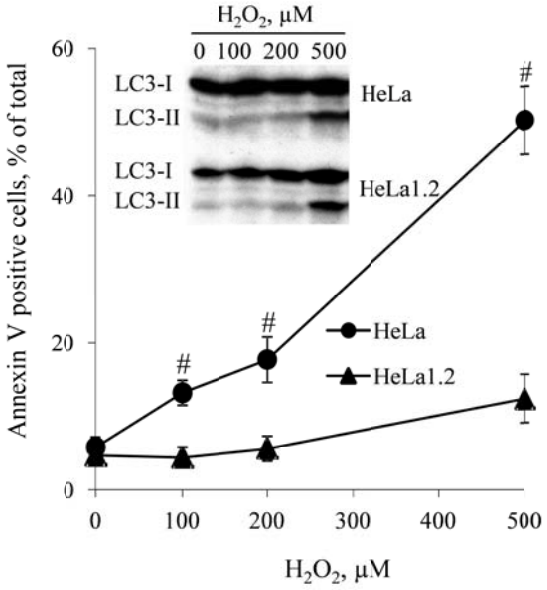
Note that treatment of HeLa1.2 cells with STS resulted in a marked increase of AVO content (*b* and *c*), which was inhibited by 50 nM BafA1 (*d*).




**Supplementary figure S3**. Dose dependent response of rotenone (0-100 μM) induced superoxide generation in HeLa cells and HeLa1.2 cells. At the end of incubation, cells were incubated with 5 μM DHE for 20 min. Ethidium fluorescence was analyzed by using a FACScanto II flow cytometer. Mean fluorescence intensity from 10,000 cells was acquired by using a 585/42 nm band-pass filter. The relative superoxide production was calculated by comparing the mean fluorescence intensity with that of control HeLa cells. Inserts are

representative western blot data of LC3-I/II conversion in rotenone treated HeLa1.2 cells. Data are means ± SD (n=3).

Note that treatment of HeLa and HeLa1.2 cells with rotenone resulted in a significantly increased oxidation of DHE compared with non-treated cells. Intriguingly, there was no significant difference in terms of rotenone induced superoxide generation within the dose range from 1 to 100 μM. Changes of LC3-I/II ratio, however, were only observed in cells treated with higher concentrations (>20 μM) of rotenone, consistent with a previous report [S1]. Despite the robust generation of superoxide in cells treated with lower concentrations of rotenone, no change of LC3-I/II pattern was detected in the absence or presence of BafA1 (Fig.2C). Therefore, there was no correlation between rotenone-induced superoxide generation and LC3-I/II conversion. It appears that rotenone-induced superoxide generation is insufficient to induce autophagy in HeLa and HeLa1.2 cells. We suggested that mechanisms different from mitochondrial ROS generation are required for rotenone-induced changes of LC3 flux.




**Supplementary figure S4.** Exogenously added $H_2O_2$ induced cell death and LC3-I/II conversion in HeLa and HeLa1.2 cells. HeLa and HeLa1.2 cells were exposed to different concentrations of extracellular $H_2O_2$ (100, 200 and 500 μM) for 18 h. Inserts are representative western blotting data of LC3-I/II conversion in $H_2O_2$ treated HeLa and HeLa1.2 cells. Data presented were means ± SD (n=3). # $p < 0.05$ *vs* HeLa ce1.2 cells under the same condition.

Note that the cell death induced by exogenously added $H_2O_2$ was greatly attenuated in cyt *c* deficient HeLa1.2 cells compared to HeLa cells, in line with the known role of cyt *c* in mitochondria-dependent mechanisms of cell death [S2]. Importantly, the LC3-I/II patterns were similar in $H_2O_2$ treated HeLa and HeLa1.2 cells. Despite the fact that the extracellularly added $H_2O_2$ could be readily detected in cells by either mito- or cyto-HyPer sensors (Fig.4A,C) and caused significantly decreased survival of HeLa cells, no LC3-I/II conversion was observed in cells exposed to either 100 or 200 μM $H_2O_2$. The accumulation of LC3-II, however, was detectable in both HeLa and HeLa1.2 cells treated with 500 μM $H_2O_2$.

**References**:


S1. Y. Chen, E. McMillan-Ward, J. Kong, S.J. Israels, S.B. Gibson, Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. J. Cell Sci. 120(2008) 4155-4166.

S2. H. Stridh, M. Kimland, D.P. Jones, S. Orrenius, M.B. Hampton, Cytochrome c release and caspase activation in hydrogen peroxide- and tributyltin-induced apoptosis, FEBS Lett. 429(1998) 351-355.