**Fernandez-Mosquera et al.,**

**“Mitochondrial respiratory chain deficiency inhibits lysosomal hydrolysis”**

**Supplementary Information**

**- supplementary figures S1-S9 and legends**

**- legend for supplementary videos SV1-SV3 (video files uploaded separately)**

**Figure S1.** Validation of the RC-kds as a model of mitochondrial respiratory chain deficiency. (**A**)Western blot analysis of whole-cell extracts for UQCRC1 in RC-kd and scrambled control HeLa cells, using GAPDH and HPRT as loading controls. The density of the band for UQCRC1 is decreased in RC-kds. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**B**) transcript levels of UQCRC1 measured by quantitative RT-PCR, in HeLa cells with UQCRC1 knockdown (RC-kds), calculated by the ΔΔCt method using GAPDH as a control gene. There is a significant decreased in the expression of RC-kds compared to the scrambled. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*\*\*p<0.001. (**C**)Representative measure of oxygen consumption rate (OCR), using SeaHorse extracellular flux analyzer profiling, where there is a significant decreased of the OCR in the RC-kds compared to the scrambled. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*\*\*p<0.001. (**D**) Measurement of MitoSOX red was used to estimate the levels of mitochondrial superoxide in the RC-kds compared to the scrambled control, by flow cytometry. The analysis shows a significant increase in ROS production in the RC-kd compared with the negative control. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05, \*\*p<0.005. (**E**) Mitochondrial membrane potential was used to measure relative oxygen species in the RC-kds compared to the scrambled, by flow cytometry. The analysis shows a significant decrease in mitochondrial membrane potential in RC-kd compared with the negative control. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*\*\*p<0.001.

**Figure S2.** Lysosomal mass but not lysosomal number is affected in respiratory chain deficient cells. (**A**)Column plot showing that there are no changes in the number of the lysosomes in the RC-kd in comparison with the scrambled. At least 30 cells obtained for each condition, obtained from 3 independent experiments. (**B**)Western blot analysis of whole-cell extracts for LAMP1 in RC-kd and scrambled control HeLa cells, using GAPDH as loading control. The band for LAMP1 is increased in RC-kds. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**C**)Western blot analysis of whole-cell extracts for ATP6V1A and ATP6V01A in RC-kd and scrambled control HeLa cells, using GAPDH as loading control. The band for ATP6V1A and ATP6V01A were increased in RC-kds. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**D**)Increased in lysosomal mass in RC-kds relative to scrambled cells. The cells were stained with LysoTracker Green, and the fluorescence intensity was measured by flow cytometry. The quantification of the average ± st.dev of at least 3 independent replicates (repeated tree times, representative experiment plotted) per condition is presented in the bar graph. ANOVA p-value \*\*\*p<0.001. (**E**)Lysosomal volume is increased in the *ndufs4*-/- MEFs. Representative spinning-disk microscopy images (scrambled, upper panel) stained with anti-LAMP1 antibody (secondary conjugated with Alexa Fluor 488 fluorophore). Scale bar: 5 μm. The plot shows average±s.e.m. of at least 30 cells obtained for each condition, obtained from 3 independent experiments, and also indicates the average volume and the standard deviation. T-test p-value \*p<0.05 (**F**)Increased in lysosomal mass in *ndufs4*-/- MEFs relative to WT in cell stained with LysoTracker Green, measured by flow cytometry. The quantification of the average ±st.dev of at least 3 independent replicates (repeated tree times, representative experiment plotted) per condition is presented in the bar graph. ANOVA p-value \*\*\*p<0.001.

**Figure S4**. Lysosomal biogenesis in respiratory chain-deficient cells. (**A**)Heat map showing that while under starvation there is an upregulation in the expression of lysosomal biogenesis in scrambled HeLa cells that is no present in RC-kd. (**B**)Heat map showing that while under starvation there is an upregulation in the expression of lysosomal biogenesis in wild-type MEFs that is not present in *ndusf4-/-*.

**Figure S5.** Decrease in lysosomal activity in RC-kd measured by the acridine orange phototoxicity assay. (**A**) Representative spinning disk confocal images of scrambled and RC-kds HeLa cells loaded with acridine orange. The leftmost panel is an overlay image of green and red channels showing cytoplasmic (green) and lysosomal (red) acridine orange. The right side panel shows binarized images of the red channel at the beginning of the exposure with blue light (t=0s), after 60 s, or after 120 s, in scrambled, RC-kds or RC-kds treated with the MCOLN1 activator MLSA1 20 µM for 4 h. DMSO was used as vehicle control. (**B**) Representative time-lapse image of one lysosome in each condition tested from t=0 s to t=120 s, under repeated exposure to blue light. (**C**) intensity of the lysosomal acridine orange fluorescence, normalized to the first frame, during recurrent exposure to blue light (left panel) and quantification of the area under the curves (right panel). The plot shows the average±s.e.m. of at least 10 cells per condition. ANOVA adjusted p-value \*p<0.05

**Figure S6**. Regulation of AMPK activity in RC-kds. (**A**)Western blot analysis of whole-cell extracts of scrambled and RC-kd HeLa cells treated with the complex III inhibitor antimycin for 4h or with DMSO as vehicle control. The total and phosphorylated levels of AMPK and TSC2 were monitored, and the ratios p-AMPK:AMPK and p-TSC2:TSC2 are presented in the column plots on the right side. ANOVA p-value \*p<0.05. (**B**) Western blot analysis of whole-cell extracts for folliculin and folliculin-interaction partner FNIP1 in HeLa cells treated with complex III inhibitor antimycin or DMSO as vehicle control for the time periods indicated, using GAPDH as loading control. Column plots below show average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**C**) transcript levels of *FLCN*, *FNIP1* and *FNIP2* measured by quantitative RT-PCR, in scrambled HeLa cells treated with antimycin or vehicle control for the time periods indicated, calculated by the ΔΔCt method using GAPDH as a control gene. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**D**) transcript levels of *FLCN*, *FNIP1* and *FNIP2* measured by quantitative RT-PCR, in *ndufs4-/-* and corresponding WT MEFs, calculated by the ΔΔCt method using *GAPDH* as a control gene. There is a significant increase in the expression of FLCN, FNIP1 and FNIP2 in *ndufs4-/-* MEFs compared to WT. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**E**) transcript levels of *FLCN*, *FNIP1* and *FNIP2* measured by quantitative RT-PCR, in 6-week old brains of *ndufs4-/-* and corresponding WT, calculated by the ΔΔCt method using GAPDH as a control gene. There is a significant increase in the expression of folliculin and FNIP1 in *ndufs4-/-* brain compared to WT. Column plot shows average plus standard deviation of at least 3 independent experiments, with duplicates in each experiment. T-test p-value \*p<0.05. (**F**) Effect of folliculin knockdown in RC-kds and scrambled HeLa cells. FLCN was silenced by siRNA, and the transcript levels of *TFEB* and TFEB target genes were measured by quantitative RT-PCR, in RC-kds and scrambled HeLa cells. The transcript levels were calculated by the ΔΔCt method using GAPDH as a control gene. As shown before, there is a significant decrease in the expression of TFEB-related genes RC-kds compared to the scrambled cells. However, when FLCN is silenced, an increase in the expression of these genes is evident (denoted by a change in the color code towards red tones) in both scrambled and RC-kds. The heatmap shows average of at least 3 independent experiments. (G) Effect of folliculin knockdown on the lysosomal proteolytic activity measured by DQ-BSA degradation, in RC-kds and scrambled HeLa cells. The column plot shows the average and s.e.m. of 12 biological replicates per experiment, in 3 independent experiments. ANOVA p-value relative to control cells \*p<0.05.

**Figure S7.** Inhibition of PIKFYVE causes lysosomal enlargement. (**A**) Lysosomal size increases after 4h YM2010636 1 μM treatment (PIKFYVE inhibitor). Representative spinning disc microscopy images of HeLa cells (scrambled and RC-kds and with the PIKFYVE inhibitor YM2010636 or DMSO as vehicle control), stained with anti-LAMP1 antibody (secondary conjugated with Alexa Fluor 488 fluorophore). Scale bar: 8 μm. (**B**) Column plot on shows the average ± s.e.m. lysosomal volume across at least 20 cells obtained for each condition, obtained from 2 independent experiments. ANOVA p-value \*p<0.05, \*\*p<0.05, \*\*\*p<0.005.

**Figure S8**. AMPK activity in *ndufs4-/-* brain. (**A**)Western blot analysis of whole tissue homogenates for ACC and ACAC-P in the brain of *ndufs4-/-* mice, using HPRT as loading control. The bands for ACAC-P were decreased in *ndufs4-/-* tissues. (**B**) Column plot showing a significant decrease in p-ACAC/ACC:ACAC/ACC ratio in brain of 6-weeks-old *ndufs4-/-* mice compared to the wild type. T-test p-value \*p<0.05.

**Figure S9.** Inhibition of MTORC1 in RC-kd does not resolve lysosomal enlargement. (**A**)Lysosomal size increases after 4 h Torin1 20 nM treatment (MTORC1 inhibitor). Representative spinning disc microscopy images of HeLa cells (scrambled and RC-kds and with the PIKFYVE inhibitor Torin1 or DMSO as vehicle control), stained with anti-LAMP1 antibody (secondary conjugated with Alexa Fluor 488 fluorophore). Scale bar: 8 μm. The column plot on the right shows the average ± s.e.m. lysosomal volume across at least 20 cells obtained for each condition, obtained from 2 independent experiments. ANOVA p-value \*p<0.05, \*\*p<0.05, \*\*\*p<0.005.

**Video SV1.** Increase in cytoplasmic Ca2+ concentration, measured by the intensity of Ca2+ sensor Fluo-4-AM, upon treatment of HeLa scrambled and RC-kd with GPN, which permeabilizes the lysosomal membrane and thus releases lysosomal Ca2+ to the cytoplasm.

**Video SV2.** Decrease in lysosomal activity in RC-kd measured by the acridine orange phototoxicity assay.This movie shows the time progression, under progressive exposure to blue light, of the acridine orange staining of the lysosomes, with discrete lysosomal disintegration events, for scrambled control, RC-kds and RC-kds treated with the MCOLN1 activator MLSA1 20 µM for 4 h. DMSO was used as vehicle control. The speed of red staining decrease (acridine orange appears red in the lysosomes) is lower in the RC-kds, and normalized to control levels when the RC-kds are treated with MLSA1.

**Video SV3.** Increase in cytoplasmic Ca2+ concentration, measured by the intensity of Ca2+ sensor Fluo-4-AM, upon treatment of WT and AMPK DKO MEFs (prkaa1-/-prkaa2-/-) with GPN, which permeabilizes the lysosomal membrane and thus releases lysosomal Ca2+ to the cytoplasm.