Supplemental material

Figure S1. The volume of mitochondria and amounts of mitochondrial proteins were mostly unchanged under condition of starvation. HeLa cells were cultured in complete medium or Krebs-Henseleit buffer (starvation) for 18 h. Mitochondrial proteins were detected by immunofluorescence with an anti-TOMM20 antibody (**A**) and immunoblotting with antibodies against the mitochondrial proteins TOMM20, VDAC1, TIMM23, and TFAM (**B**). Bar = 10 μm. The histogram shows quantification of the band intensity for each mitochondrial protein after normalization by ACTB/β-actin, relative to the value in cells cultured in complete medium. Data are expressed as means ± SD from 3 separate experiments.

Figure S2. Observation of macroautophagy using cyto-Keima cells. (**A**) Tet-off cyto-Keima-expressing HeLa cells (cyto-Keima cells) were cultured in complete medium or Krebs-Henseleit buffer (starvation) supplemented with or without 10 mM 3-MA for 18 h. Cyto-Keima localized in the cytoplasm was excited by 440-nm light (Keima [cytosol]; red) and cyto-Keima delivered within lysosomes by macroautophagy was excited by 590-nm light (Keima [autolysosomes]; green). Bar = 10 μm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments. (**B**) Cyto-Keima cells were cultured in Krebs-Henseleit buffer (starvation) for the indicated times. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 μm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S3. The acidic puncta represent macroautophagy in cyto-Keima cells. (**A**) siRNA was used to knock down PIK3C3 in cyto-Keima cells. The cells were then cultured in complete medium or Krebs-Henseleit buffer for 18 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 μm. (**B**) siRNA efficiency in HeLa cells was

confirmed by measuring the mRNA level of *PIK3C3* by real-time PCR. Data are expressed as means ± SD of 4 PCR reactions.

Figure S4. Mito-Keima is localizes within mitochondria. Mito-Keima cells were treated with MitoTracker Red CMXRos for 10 min at 37 °C, fixed, and observed with a fluorescence microscope. The merged image was generated by combining MitoTracker (red) and mito-Keima (green). Bar = 10 µm.

Figure S5. The acidic puncta represent mitophagy in mito-Keima cells. (**A**) Mito-Keima cells were cultured in Krebs-Henseleit buffer (starvation) for 18 h, and stained for lysosomes with LysoTracker Blue. The merged image represents the combination of autolysosomes (green) and LysoTracker (Red). Bar = 10 µm. (**B**) Mito-Keima cells were transfected with a LAMP1-EGFP expression vector, cultured in Krebs-Henseleit buffer (starvation) for 18 h, and observed with a fluorescence microscope. Bar = 10 µm.

Figure S6. Paraquat does not efficiently induce mitophagy. Mito-Keima cells were cultured in complete medium, or with 10 µM paraquat in complete medium for the indicated times. Mito-Keima in mitochondria (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S7. Antimycin A does not efficiently induce mitophagy. Mito-Keima cells were cultured in complete medium, or with 1 µM antimycin A in complete medium for the indicated times. Mito-Keima in mitochondria (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S8. Oligomycin does not efficiently induce mitophagy. Mito-Keima cells were cultured in complete medium, or with 1 µM oligomycin in complete medium for the indicated times. Mito-

Keima in mitochondria (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S9. The iron-chelating drug deferiprone induces mitophagy. Mito-Keima cells were cultured in complete medium supplemented with or without 1 mM deferiprone (DFP) for 24 h. Mito-Keima in mitochondria (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm.

Figure S10. Colocalization of LysoTracker Red and TOMM20 during starvation or hypoxia. HeLa cells were cultured in complete medium, in Krebs-Henseleit buffer (starvation) for 18 h, or under hypoxic conditions for 24 h, and stained for lysosomes with LysoTracker (red). Cells were fixed and immunostained with an anti-TOMM20 antibody (green). To sharpen the image and confirm colocalization, the merged images were deconvoluted (Dec) and the boxed regions are highlighted in the right panels. Bar = 10 µm.

Figure S11. LC3 marginally colocalizes with TOMM20 during starvation. HeLa cells were cultured in complete medium or Krebs-Henseleit buffer (starvation) for 6 or 18 h. Cells were fixed and immunostained with an anti-TOMM20 antibody (green) and anti-LC3 antibody (red). To sharpen the image, the merged images were deconvoluted (Dec), and the boxed regions are highlighted in the right panels. Bar = 10 µm.

Figure S12. LC3 marginally colocalizes with acidic puncta of mito-Keima during starvation or hypoxia. Mito-Keima cells were transfected with GFP-LC3, and then cultured in complete medium or Krebs-Henseleit buffer (starvation) for 18 h, or under hypoxic conditions for 24 h. Bars = 10 µm.

Figure S13. Macroautophagy is induced by starvation and rapamycin but not by hypoxia. Cyto-Keima cells were cultured in complete medium, in Krebs-Henseleit buffer (starvation) for 4 h, in complete medium supplemented with rapamycin for 24 h, or under hypoxic conditions for 24 h.

Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S14. Macroautophagy is efficiently inhibited in ATG7&ATG12 knockdown cells. (**A**) The indicated proteins were knocked down by siRNA in cyto-Keima cells. The cells were then cultured in complete medium or Krebs-Henseleit buffer (starvation medium) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments. (**B**) siRNA efficiency in HeLa cells was confirmed by measuring the mRNA levels of the indicated genes by real-time PCR. Data are expressed as means ± SD of 4 PCR reactions.

Figure S15. LC3 accumulation is suppressed when both ATG7 and ATG12 are knocked down. (**A**) ATG7, ATG12, and ATG7&ATG12 were knocked down in HeLa cells, and the cells were cultured in complete medium or Krebs-Henseleit buffer (starvation) for 4 h. The cells were fixed, immunostained with an anti-LC3 antibody (green), and observed with a confocal laser microscope. Bar = 20 µm. The number of LC3 puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments. (**B**) LC3A/B/C were knocked down in HeLa cells by siRNA. The cell lysates were analyzed by immunoblotting using an anti-LC3 antibody and anti-ACTB/β-actin antibody (loading control).

Figure S16. Macroautophagy is a combination of conventional and alternative autophagy. RAB9A/B, ATG7&ATG12&RAB9A/B, and LC3A/B/C&RAB9A/B were knocked down in cyto-Keima cells, and the cells were cultured in complete medium or Krebs-Henseleit buffer (starvation) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are

expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S17. UBA1 and BNIP3L are related to mitophagy. (**A, B**) UBA1, MUL1, and BNIP3L were knocked down by siRNA in mito-Keima cells. The cells were then cultured in complete medium or Krebs-Henseleit buffer (starvation) for 18 h (A), or under normoxic or hypoxic conditions for 24 h (B). Bars = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments. (**C**) siRNA efficiency in HeLa cells was confirmed by measuring the mRNA levels of the indicated genes by real-time PCR. Data are expressed as means ± SD of 4 PCR reactions.

Figure S18. None of the MAPKs are related to mammalian macroautophagy. (**A**) The indicated proteins were knocked down by siRNA in cyto-Keima cells. The cells were then cultured in complete medium or Krebs-Henseleit buffer (starvation medium) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments. (**B**) siRNA efficiency in HeLa cells was confirmed by measuring the mRNA levels of the indicated genes by real-time PCR. Data are expressed as means ± SD of 4 PCR reactions.

Figure S19. MAPK1 and MAPK14 are not required for macroautophagy. Cyto-Keima cells were cultured in complete medium or Krebs-Henseleit buffer (starvation) supplemented with or without the MAP2K1/MAP2K2 inhibitor U0126 (**A**) or MAPK14 inhibitor SB203580 (**C**) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bars = 10 µm. The number of acidic puncta per cell was counted (**B, D**). Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S20. Macroautophagy was barely inhibited by knockdown of the MAPK1 upstream factors. The indicated proteins were knocked down by siRNA in cyto-Keima cells. The cells were then

cultured in complete medium or Krebs-Henseleit buffer (starvation medium) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S21. Macroautophagy was barely inhibited by knockdown of the MAPK14 upstream factors. The indicated proteins were knocked down by siRNA in cyto-Keima cells. The cells were then cultured in complete medium or Krebs-Henseleit buffer (starvation medium) for 4 h. Cyto-Keima in the cytoplasm (red) or in autolysosomes (green) was observed with a fluorescence microscope. Bar = 10 µm. The number of acidic puncta per cell was counted. Data are expressed as means ± SD of at least 30 cells from 3 separate experiments.

Figure S22. GFP-MAPK1 or GFP-MAPK14 does not colocalize with mitochondrial TOMM20 during starvation. HeLa cells transformed with EGFP, EGFP-MAPK1, or EGFP-MAPK14 expression vectors were cultured in Krebs-Henseleit buffer (starvation) for the indicated times. The cells were fixed and immunostained with an anti-TOMM20 antibody (red). Bars = 10 µm.

Figure S23. Overexpression of FUNDC1 does not induce mitophagy. (**A, B**) Mito-Keima cells were transfected with pIRES2-AcGFP1+NLS (negative control) or FUNDC1-IRES-AcGFP1+NLS, and cultured in complete medium for 24 h. Mito-Keima in the mitochondria (red), Mito-Keima in autolysosomes (green), or nuclear localized GFP (blue) was observed with a fluorescence microscope (**A**). The cell lysates were analyzed by immunoblotting using a FUNDC1 antibody and anti-ACTB/β-actin antibody (loading control) (**B**).