Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner

Magdalena Frank, Stéphane Duvezin-Caubet, Sebastian Koob, Angelo Occhipinti, Ravi Jagasia, Anton Petcherski, Mika O. Ruonala, Muriel Priault, Benédicte Salin, Andreas S. Reichert

1. Introduction

Mitochondria are double-membrane enclosed organelles of eukaryotic cells fulfilling central roles in energy metabolism; in the synthesis of amino acids, lipids, and iron sulfur clusters; in ion homeostasis and in thermogenesis; and are central players in regulating apoptosis. Mitochondrial dysfunction is strongly linked to numerous neurodegenerative and muscular disorders, obesity, diabetes, cancer, and to aging [1]. Minimizing mitochondrial dysfunction is thus of major importance for counteracting the development of numerous human disorders and the aging process [2–4]. Such a quality control of mitochondria does indeed occur naturally at different levels [2]. On the molecular level, chaperones and mitochondrial proteases exert an important role in preventing misfolding and aggregation of proteins within mitochondria. On the organelar level dysfunctional mitochondria are recognized and degraded within cells by autophagy. Mitochondria can be degraded both by non-selective autophagy and by mitophagy, a selective type of autophagy. Engulfment of mitochondria by autophagosomes is observed under starvation conditions and under numerous conditions, in particular when mitochondrial function is impaired [5–12]. Several studies of the past years have shown that mitophagy plays a major role in the specific recognition and removal of damaged mitochondria (for review see [13,14]). In the meantime, a number of factors required for mitophagy have been identified and their role in this process has

Abstract

Mitochondrial dysfunction is linked to apoptosis, aging, cancer, and a number of neurodegenerative and muscular disorders. The interplay between mitophagy and mitochondrial dynamics has been linked to the removal of dysfunctional mitochondria ensuring mitochondrial quality control. An open question is what role mitochondrial fission plays in the removal of mitochondria after mild and transient oxidative stress; conditions reported to result in moderately elevated reactive oxygen species (ROS) levels comparable to physical activity. Here we show that applying such conditions led to fragmentation of mitochondria and induction of mitophagy in mouse and human cells. These conditions increased ROS levels only slightly and neither triggered cell death nor led to a detectable induction of non-selective autophagy. Starvation led to hyperfusion of mitochondria, to high ROS levels, and to the induction of both non-selective autophagy and to a lesser extent to mitophagy. We conclude that moderate levels of ROS specifically trigger mitophagy but are insufficient to trigger non-selective autophagy. Expression of a dominant-negative variant of the fission factor DRP1 blocked mitophagy induction by mild oxidative stress as well as by starvation. Taken together, we demonstrate that in mammalian cells under mild oxidative stress a DRP1-dependent type of mitophagy is triggered while a concomitant induction of non-selective autophagy was not observed. We propose that these mild oxidative conditions resembling well physiological situations are thus very helpful for studying the molecular pathways governing the selective removal of dysfunctional mitochondria.

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been analyzed to varying extent [15–19]. NIX, a BH3 domain containing protein [20,21] was reported to act as a mitochondrial receptor required for mitochondrial clearance in reticulocytes [22]. Several reports strongly indicate that Parkinson’s disease (PD) is linked to impaired removal of dysfunctional mitochondria by mitophagy and that this process depends on at least PINK1 and PARKIN, two factors known to be causally linked to Parkinson’s disease [23–29]. Overexpression of PARKIN, an E3-like ubiquitin-ligase, and treatment of cells with the protonophore CCCP that dissipates the mitochondrial membrane potential led to a complete disappearance of mitochondria within 48 h [29]. PARKIN-dependent mitophagy of depolarized mitochondria was shown to depend on PINK1, a mitochondrial kinase [23,24] as well as on p62 and NIX [30].

Non-selective autophagy, as well as mitophagy, has been shown to be triggered by reactive oxygen species (ROS) in response to nerve growth factor deprivation, rapamycin, or starvation [for review see [14,31,32]]. Mitochondria represent a major physiological source of ROS known (1) to cause oxidative damage to basically all cellular components, including DNA, proteins, and lipids, but also (2) to serve as important signaling molecules for the induction of autophagy [14,31]. Albeit it is clear that high ROS levels stimulate autophagy and mitophagy, it was also suggested that mitophagy functions as a negative regulatory feedback mechanism by reducing mitochondria-derived ROS production [31,33]. Furthermore, it should be noted that in all cases, at least to our knowledge, when mitophagy was induced by ROS, the increase in ROS levels was usually several fold and simultaneously involved the induction of non-selective autophagy [14,31]. Also, mitochondrial depolarization induced by CCCP, by NIX overexpression [30], or by downregulation of PINK1 [26], not only induced mitophagy but also non-selective autophagy. It is not clear what levels of ROS are actually required to induce mitophagy and whether this is necessarily always linked to an induction of non-selective autophagy.

Another important question is how dysfunctional mitochondria are initially distinguished from the functional ones. The dynamic nature of mitochondrial morphology [34,35] was linked to this process as e.g. mitochondrial dysfunction leads to a fast proteolytic processing and inactivation of the fusion factor OPA1, and to the fragmentation of dysfunctional mitochondria [36–38]. It was hypothesized that such a spatial separation of dysfunctional mitochondria from the intact mitochondrial network could act as a mechanism to prevent further damage (e.g. caused by reactive oxygen species) and is a prerequisite for removal of damaged mitochondria from the cell [38–41]. Indeed, in mammalian cells it was reported that under normal growth conditions [41,42], after downregulation of PINK1 [26], or during starvation [43,44], mitophagy is inhibited when mitochondrial fission was blocked. Mitochondrial fragmentation by overexpression of hFis1 was shown to promote mitophagy [45]. Whether fission of mitochondria is a prerequisite for the removal of dysfunctional mitochondria under other conditions is currently unclear. For example, studies in Saccharomyces cerevisiae have revealed contradicting results regarding the roles of known mitochondrial fission factors in mitophagy. One study reported that starvation-induced mitophagy was reduced in a strain lacking the mitochondrial fission factor Dnm1 but not when the other factors, Mdv1, Fis1, or Caf4 were absent [17]. Another study reported that none of these four factors impacted mitophagy [15] consistent with our own observations [46].

Thus, it remains to be investigated whether mitochondrial fission is generally required for mitophagy or only under certain circumstances. Conditions that lead to a general induction of bulk autophagy, such as starvation or serum deprivation, are known to induce also mitophagy. However, it is not clear whether in these cases the molecular steps for mitophagy are identical to situations when primarily compromised mitochondria have to be recognized and degraded selectively. Furthermore, it is at all possible to identify conditions that allow induction of mitophagy while non-selective autophagy is not triggered simultaneously?

Here we show that in mammalian cells mild and transient oxidative stress leads to the induction of mitophagy whereas non-selective autophagy was only found at basal levels suggesting that mitophagy can be induced independently of non-selective autophagy. The low levels of ROS generated by our treatments suggest a ROS signaling cascade that primarily triggers mitophagy. We further provide evidence that mitochondrial fission is necessary for the induction of mitophagy under mild oxidative stress and that on the contrary starvation-induced hyperfusion of mitochondria counteracts mitophagy.

2. Material and methods

2.1. Cell culture

Cell lines were grown under standard conditions in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/l glucose supplemented with 10% fetal bovine serum. Cell culture reagents were obtained from PAA Laboratories. HeLa cells stably expressing CD63-GFP and mitoDsRed or GFP-LC3 and mitoDsRed were kindly provided by Aviva Tolkovsky [11]. Atg5−/− mouse embryonic fibroblasts and the corresponding wild type cells were kindly provided by Noboru Mizushima [47].

2.2. Plasmids, transient transfection and lentiviral transduction

Transient transfections were performed using FuGENE HD (Roche Applied Science) according to the manufacturer’s instructions. Cells were seeded on round glass coverslips in 24-well-plates one day before transfection. The plasmids pEGFP-C1-rLC3 was a kind gift from T. Yoshimori. The plasmid CMV_IRES_mitoDsRed was obtained by replacing the GFP gene in the CSC.cPPT.hCMV.GFP.WPRE vector [48,49] with IRES-mitoDsRed.T4, encoding a N-terminal fusion protein consisting of amino acids 2–29 of the human cytochrome c oxidase subunit VIII and DsRed.T4. The DNA fragment IRES-mitoDsRed.T4 was obtained by PCR from the CAG-IRES-mitoDsRed.T4 vector described earlier [50]. The DRP1dn (K38A) coding sequence was subcloned from the pcDNA3–DRP1dn (K38A) plasmid described earlier [51] and cloned into the HIV-1 lentivirus-based vector CMV_IRES_mitoDsRed using the SfiI and Pmel restriction sites to obtain CMV_DRP1dn_IRES_mitoDsRed. Lentiviruses were produced in HEK293FT cells according to Zufferey et al. [52]. The cells were assayed two days after transient transfection or 2–6 days after lentiviral transduction.

2.3. Cell treatments

Induction of mild oxidative stress: unless indicated differently, the cells were treated either with 1 μM rotenone (Sigma) in growth medium for 1 h or with 3.3 mM hydrogen peroxide (AppliChem) in growth medium for 10 min, followed by 30 min of recovery in fresh growth medium. Autophagy was induced by amino acids and serum starvation: the cells were washed with PBS and incubated for 6 h in Hank’s Buffered Salt Solution (HBSS) purchased from Invitrogen and buffered with 2.2 g/l NaHCO3 (Merck). The cells were processed for analysis as described below or within the text and the figure legends. Cell viability/cell toxicity was determined as follows: the cells were analyzed as described below or within the text and the figure legends.
2.4. Determination of ROS levels

Superoxide anion levels were measured using the fluorescent dye DHE (dihydroethidium) in a final concentration of 5 μM. Cells were seeded at a constant density of 1 × 10⁴ cells per well in a 96-well plate 24 h prior to treatment. Cells were treated as described above, then DHE was supplemented to the growth medium (or HBSS for starvation conditions), incubated for 10 min at 37 °C, the medium was exchanged to PBS and the DHE fluorescence was measured using a plate reader (Infinite M200Pro, Tecan Deutschland GmbH, Germany) set to 518 nm excitation and 605 nm emission wavelength.

2.5. Confocal fluorescence microscopy

Cells were grown for 3 days on round glass coverslips in 24-well plates. In the case of HeLa cells stably expressing GFP-LC3 and mitoDsRed, E64d, pepstatin A and leupeptin (all purchased from Sigma) were added (each at 10 μg/ml) together with the treatments or vehicle, to inhibit degradation of GFP-LC3 in autophagosomes. In the case of HeLa cells stably expressing CD63-GFP and mitoDsRed the addition of these inhibitors was omitted as the CD63-GFP signal as well as the mitoDsRed signal was not markedly reduced by lysosomal proteases. The drugs were prepared from DMSO stock solutions, except leupeptin that was dissolved in PBS. After the treatments, the cells were fixed at 37 °C for 15 min in 3.7% paraformaldehyde in prewarmed growth medium and rinsed three times with PBS. In the case of MEF cells, they were further permeabilized and immunostained with mouse anti-cytochrome c monoclonal antibody (clone GH2.B4, dilution 1:800; BD Biosciences) and Cy3-conjugated donkey anti-mouse IgG (H+L) (dilution 1:250; Jackson ImmunoResearch). All coverslips were mounted with aqua polymount antifade reagent (Polysciences) according to the manufacturer's instructions. The cells were imaged in a multi-track scanning mode using an inverted LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Inc.) equipped with a 63×/1.4 oil DIC plan-apochromat objective. Cy3 and DsRed were excited at 543 nm and emission using a BP 560–615 nm filter was recorded. GFP was excited at 488 nm and emission using a BP 505–530 nm filter was recorded. Single confocal planes were acquired with an optical slice thickness of 1 μm and the images were further processed using ImageJ.

2.6. Quantification of autophagy and mitophagy by confocal microscopy

To monitor mitophagy in HeLa cells, two cell lines were used: (i), HeLa cells coexpressing mitochondrially targeted DsRed (mitoDsRed) and GFP-LC3, the autophagosomal marker LC3 fused with GFP, and (ii), HeLa cells coexpressing mitoDsRed and CD63-GFP, a late endosome/lysosomal marker protein fused with GFP [11]. To monitor mitophagy in MEFs, the cells were transfected with a plasmid expressing GFP-LC3 fusion protein and mitochondria were detected by immunostaining of cytochrome c. This allowed us to monitor macroautophagy of mitochondria at two distinct stages, namely at an early stage when mitochondria are engulfed by autophagosomes (mitoDsRed and GFP-LC3 positive structures) and at a later stage when those structures have already been fused with lysosomes (mitoDsRed and CD63-GFP positive structures). Mitophagy was quantified by counting the number of GFP-positive vesicles per cell that contained mitochondria in at least 30 cells for each condition.

2.7. Analysis of mitochondrial morphology

HeLa cells were cultured, transiently transfected with the CMV-IRES-mitoDsRed or CMV-DRPdn-IRES-mitoDsRed constructs, and treated with H₂O₂ (3.3 mM for 10 min, wash out and 30 min recovery) or rotenone (1 μM for 1 h) and fixed. Mitochondrial morphology was quantified as tubular, at least one mitochondrial tubule of 10 μm in length or more is present in a cell. All other cells were counted as harboring fragmented mitochondria. Representative images of these categories are shown in Fig. 2C, left panel. In each condition the proportion of cells with tubular versus fragmented mitochondria from three independent experiments (>120 cells were analyzed for each condition and experiment) was calculated. For mouse cells, Atg5⁻/⁻ and Atg5⁺/⁺ mouse embryonic fibroblasts were seeded in 24-well plates and transiently transfected with pEGFP-Mito (Clontech Laboratories, USA) using Effectene (Qiagen, Germany) according to the manufacturer’s instructions, prepared for fluorescence microscopy after 24 h, and mitochondrial morphology was categorized as follows: tubular, more than 60% of the mitochondria show a tubular length of at least 2–3 μm or more in a cell; fragmented, no mitochondrial tubules larger than 2–3 μm in length are present in a cell; intermediate, mitochondrial morphology is neither tubular nor fragmented. In each condition the proportion of cells with tubular, intermediate, and fragmented mitochondria from three independent experiments (36 to 112 cells were analyzed for each condition and experiment) was calculated. Determination of relative mitochondrial mass and of mitochondrial hyperfusion was performed on focal plane images of HeLa cells expressing GFP-LC3 and mitoDsRed. The Trainable Segmentation plug-in for Fiji (http://fiji.sc/) was used to extract foreground mitochondrial signal and the area of segmented mitochondria was calculated with ImageJ. Mitochondria covering an area of at least 20 μm² were considered hyperfused. Structures with area less than 0.2 μm² were considered as segmentation artifacts and excluded from the analysis.

2.8. Electron microscopy

HeLa cells stably expressing CD63-GFP and mitoDsRed were seeded on 6-well plates, infected with CMV_GFP_IRES_mitoDsRed or CMV_DRPdn_IRES_mitoDsRed lentiviruses, and cultured for 3 days. The cells were then treated as described before and consecutively fixed with 4% glutaraldehyde in PBS for 30 min and with 2% OsO₄ in PBS for 1 h, and washed with PBS (pH 7.4). The samples were then post-fixed with 2% uranyl acetate in water. After repeated washing steps the cells were dehydrated with a graded series of ethanol and progressively embedded in Epon resin. Thin sections (80 nm) were contrasted with lead citrate and observed at 80 kV with a Hitachi 7650 transmission electron microscope. The number of autophagic structures (autophagosomes, characterized by double membrane-surrounded material with an electron density comparable to the cytosol; and autophagolysosomes, characterized by double membrane-surrounded material with a higher electron density) per cell was counted in at least 10 cells per condition.

2.9. Determination of long-lived protein degradation

Degradation of long-lived proteins was measured according to Ogier-Denis et al. [53]. HeLa cells stably expressing CD63-GFP and mitoDsRed were seeded on 6-well plates, infected with CMV_GFP_IRES_mitoDsRed or CMV_DRPdn_IRES_mitoDsRed lentiviruses, and cultured for two days. The cells were then labeled for 24 h in complete medium containing 0.1 μCi l-[¹⁴C]-valine/ml/well. After three rinses with PBS, radioactivity was pre-chased for 1 h in complete medium in the presence of an excess of l-valine (10 mM) to remove the contribution of short-lived protein degradation. After three more rinses with PBS, cells were then treated as indicated below. They were incubated for 6 h in HBSS (starvation) or in complete medium, both supplemented with an excess of l-valine. For the treatments, the cells were either incubated for 6 h in DMEM + Val containing 1 μM rotenone, or alternatively, they were treated for 10 min with 3.3 mM H₂O₂ in DMEM + Val and incubated further for 5 h and 50 min in fresh DMEM + Val without H₂O₂. After that supernatants were collected and precipitated with trichloroacetic acid. The precipitated proteins were washed, dissolved, and counted in a liquid scintillation spectrometer.
3.1. Mild and transient oxidative stress induces mitophagy in human cells

We were interested in determining to what extent autophagy contributes to degradation of mitochondria in HeLa cells under mild oxidative stress conditions. The conditions used for H$_2$O$_2$ were reported to induce only mild and transient oxidative stress and to result in only moderately elevated ROS levels that were comparable to normal physical activity [54]. The concentration of rotenone used was reported to fully inhibit complex I activity leading to formation of ROS in mitochondria but without grossly reducing mitochondrial membrane potential [55]. We confirmed in two different mammalian cell lines that the applied conditions induced ROS formation only moderately as compared to nutrient starvation (Fig. 1A), a condition known to increase cellular ROS levels grossly [31,32]. Moreover, none of these conditions had an effect on cell vitality (Fig. 1B). Next, the amount of mitochondria undergoing autophagy was quantified by two assays using confocal fluorescence microscopy. We determined the number of mitochondria (labeled by a matrix-targeted mitoDsRed) colocalizing with GFP-LC3 or, alternatively, with CD63-GFP (Fig. 2A). These two markers are commonly used to monitor autophagy: Atg8/LC3 decorates autophagic vesicles at all steps throughout the maturation process, while CD63 is associated with late endosomes/lysosomes and autophagolysosomes [11,56,57]. To ascertain a true colocalization of mitochondria and these markers we determined the intensity profiles of red and green fluorescence signals within several 3D-image stacks (Fig. 2A). Only mitochondria clearly surrounded by LC3- or CD63-positive GFP signals were considered to colocalize with those structures. It should be noted that this appearance is morphologically clearly distinct from previously reported GFP-LC3 aggregates [56,58] and from the situation when autophagosomes are formed in close apposition to the mitochondrial outer membrane as observed recently during starvation [59]. We noted that the colocalization events were relatively rare (2–3 events per cell with LC3; 15 events per cell with CD63; see Fig. 2B control) as compared to the estimated total

**Fig. 1.** Analysis of ROS formation and cell viability. A, Indicated cell lines were starved for 6 h (HBSS), or treated with 3.3 mM H$_2$O$_2$ (10 min, 30 min recovery) or 1 [$\mu$M] rotenone (1 h), or left untreated (control). A, ROS formation was analyzed by assessing the formation of DHE derived fluorescence. The results are shown as mean±s.d. (n=4). B, The amounts of viable cells after the indicated treatments were determined by counting detached, Trypan blue stained and non-stained cells. Non-stained cells were counted as viable whereas stained and detached cells were counted as non-viable. The mean values of two experiments are shown. Error bars indicate respective minimal and maximal values.

**Fig. 2.** Mild and transient oxidative stress induces mitophagy in human cells. A–D, HeLa cells stably expressing mitoDsRed and either CD63-GFP or GFP-LC3 were analyzed by confocal microscopy to quantify mitophagy and mitochondrial morphology. A, Representative CD63-GFP-positive structure containing mitoDsRed is shown in consecutive stacks (top left) and an overview of the corresponding cell is shown (bottom left). Engulfment of mitochondria within GFP-positive structures was validated by determining the fluorescence intensity profiles (green and red) along a line crossing these structures (right panel) through 3D stacks. B–D, HeLa cells stably expressing mitoDsRed and CD63-GFP (left panel), or mitoDsRed and GFP-LC3 (right panel), were transiently transfected or transduced, respectively, with the lentiviral construct CMV_IRES_mitoDsRed to increase the signal for mitoDsRed and to be used as a proper control (−DPR1dn) for the experiments shown in Fig. 4. Cells were treated with 3.3 mM H$_2$O$_2$ (10 min, 30 min recovery) or 1 [$\mu$M] rotenone (1 h), or left untreated (control), and fixed. B, Single focal planes of fluorescence images are shown. 1st row, red channel; 2nd row, green channel; 3rd row, merged; 4th row, blow-up. Examples of mitochondria-containing autophagic vesicles that were ascertained in 3D stacks as described in A are shown by arrowheads. C, Mitochondrial morphology for HeLa cells expressing mitoDsRed after indicated treatments. Representative images of HeLa cells with different mitochondrial morphologies (left panel) and corresponding categories (tubular, intermediate, or fragmented) are shown (see Material and methods for details). Right panel; quantification of mitochondrial morphology according to the three categories defined in left panel and the Material and methods. Quantification is based on three independent experiments (>120 cells were analyzed for each condition and experiment) given as mean±s.d. (n=3). D, Mitophagy was assessed by counting the number of mitochondria encapsulated in GFP-positive structures. The results of at least three independent experiments (>30 cells were analyzed per condition in each experiment) are shown as mean per cell±s.d. (n=3). E, Relative mitochondrial mass was assessed after indicated treatments in GFP-LC3 and mitoDsRed expressing Hela cells (see Fig. 1B, right panels) from confocal sections in three independent experiments (>120 cells were analyzed for each condition and experiment) given as mean±s.d. (n=3). The area covered by mitochondria was normalized to the total cell area analyzed for each experiment. Statistically significant differences and corresponding p-values using a two-sided student’s T-test are indicated.

acid (12% TCA). Proteins from the adherent cells were precipitated by adding 10% TCA into the wells. Radioactivity was quantified in a scintillation liquid analyzer Tri-carb 2100TR (Packard). 1-[$\text{I}^{14}$C]-valine release was calculated as a percentage of the radioactivity in the supernatant to the total cell radioactivity.

2.10. SDS-PAGE and Western blot analysis

Equal protein amounts of total cell extracts were separated by SDS-PAGE and analyzed by immunoblotting using the following antibodies: anti-actin (cell signaling, 4967), anti-p62 (Santa Cruz Biotechnology, sc-25575), and anti-LC3 (Abcam, ab51520). Quantification of Western blots was done using a ChemiDoc XR+ system with the Quantity One software (BioRad, Germany).
amount of LC3-positive and CD63-positive structures. In order to test whether low levels of oxidative stress lead to an induction of mitophagy we analyzed HeLa cells treated either for 1 h with rotenone, or for 10 min with H$_2$O$_2$ followed by incubation without H$_2$O$_2$ for 30 min. Oxidative stress generated in bulk by H$_2$O$_2$ or intramitochondrially by rotenone led to a significant increase in mitochondria with fragmented appearance (Fig. 2BC) consistent with an earlier report [54]. The extent of mitochondria targeted to the autophagy machinery was stimulated under these conditions as the number of mitochondria colocalizing with GFP-LC3 was increased about 2.3-fold (Fig. 2BD, right panels). Colocalization of mitochondria with CD63-GFP was increased about 1.6-fold (Fig. 2BD, right panels). As

**Fig. 3.** Mitophagy in mouse embryonic fibroblasts is induced by mild and transient oxidative stress and depends on ATG5. Wild type MEF (Atg5$^{+/+}$) and Atg5$^{-/-}$ knock-out MEF cells were cultured, transiently transfected with pEGFP-LC3 (green), treated with 3.3 mM H$_2$O$_2$ (10 min, 30 min recovery) or 1 µM rotenone (1 h), or left untreated (control), fixed and immunostained against cytochrome c (red). A, Single focal planes of fluorescence images are shown. 1st row, red channel; 2nd row, green channel; 3rd row, merged. B, Mitochondrial morphology of wild type MEF (Atg5$^{+/+}$) and Atg5$^{-/-}$ knock-out MEF cells was determined from three independent experiments (36 to 112 cells were analyzed for each condition and experiment) given as mean ± s.d. (n = 3). C, Mitophagy in murine cells was assessed by counting the number of mitochondria encapsulated by GFP-LC3-positive structures per cell. The results are shown as mean per cell ± s.d. (n ≥ 10 cells per condition). Examples of autophagosome (A), autophagolysosome (AL), and multivesicular bodies (MVB) are indicated.

**Fig. 4.** Mild oxidative stress specifically induces mitophagy but not bulk autophagy. A, HeLa cells stably expressing mitoDsRed and GFP-LC3 were starved for 6 h in HBSS, treated with 3.3 mM H$_2$O$_2$ (10 min, 30 min recovery) or 1 µM rotenone (1 h), or left untreated (control) either in the absence (−Baf/Inh) or in the presence (+Baf/Inh) of bafilomycin, leupeptin, and E64d to block autophagic degradation of substrates. Total cell extracts were prepared for all samples after a total time of six hours, and analyzed by SDS-PAGE and immunoblotting with indicated antibodies. One representative experiment is shown. B, Quantification of the relative accumulation of LC3-II and p62 under the indicated conditions shown in panel A. Band intensities were normalized to actin levels and the relative increase of LC3-II and p62 upon adding autophagosomal/lysosomal inhibitors was calculated for each condition separately. The quantifications of three immunoblots are shown as mean ± s.d (n = 3). CD, HeLa cells stably expressing mitoDsRed and CD63-GFP were transduced with the lentiviral construct CMV_GFP_BRES_mitoDsRed serving as a control (−DPR1dn) for the experiments shown in Fig. 5. Cells were starved for 6 h in HBSS, or treated with 3.3 mM H$_2$O$_2$ (10 min, 30 min recovery) or 1 µM rotenone (1 h), or left untreated (control), fixed and analyzed by electron microscopy. C, EM pictures are shown. Top, overview; bottom, blow-up. D, Autophagy was assessed from the EM pictures by counting the number of autophagic structures (autophagosomes and autophagolysosomes) per cell section. The results are shown as mean per cell ± s.d. (n ≥ 10 cells per condition). Examples of autophagosome (A), autophagolysosome (AL), and multivesicular bodies (MVB) are indicated with arrowheads. E, Quantification of protein turnover by measuring the degradation and release of [14C]valine derived from long-lived proteins. Pulse-labeled HeLa cells stably overexpressing mitoDsRed and CD63-GFP were either left untreated (control), starved (HBSS), or treated with rotenone (1 µM), each for 6 h, or treated with H$_2$O$_2$ (3.3 mM) for 10 min and allowed to recover for 5 h 50 min after washout of the reagent. The results of three independent experiments are shown as mean percentage of free radioactive valine released into the medium ± s.d (n = 3). Statistically significant differences and corresponding p-values using a two-sided Student’s T-test are indicated.
stimulation of mitophagy was already observed after rather short time periods we conclude that increased sequestration of mitochondria to the autophagosomal/lysosomal compartment is induced rapidly after mild and transient oxidative stress. We next determined whether and to which extent mitochondrial mass is reduced when applying mild oxidative stress. For this, we quantified the average mitochondrial area covered per cell in confocal sections of fluorescence images obtained from the experiment described above (Fig. 2B, right panels). Mild
oxidative stress led to a modest, yet significant, reduction in mitochondrial mass (Fig. 2E). Taken together, the three assays show that mitophagy is induced by mild oxidative stress in HeLa cells.

3.2. Mild and transient oxidative stress induces mitophagy in murine cells

We next asked whether our observations are also valid in a different organism/cell type and depend on an essential component for autophagosome formation, ATG5. For this, we applied the same conditions as described above to wild type Atg5+/+ mouse embryonic fibroblasts (MEF) and Atg5−/− knockout MEF cells [60]. We confirmed that the applied conditions did only moderately induce ROS formation compared to nutrient starvation (Fig. 1A) and that none of these conditions influenced cell vitality (Fig. 1B). We noted that significantly fewer cells showed tubular mitochondria in the absence of ATG5 (Fig. 3AB) suggesting that impaired macroautophagy promotes the accumulation of dysfunctional mitochondria. This is consistent with another study showing that this cell line is characterized by a reduced respiratory capacity and an increased heterogeneity of mitochondrial membrane potential [41]. Wild type MEF cells showed a similar increase in colocalization of GFP-LC3 with mitochondria after addition of H2O2 or rotenone (Fig. 3AC) as described above for HeLa cells (Fig. 2BD), a finding that supports our results in a murine cell line. When Atg5−/− knockout MEF cells were subjected to the same treatments, no increase in the amount of mitochondria colocalizing with GFP-LC3 was observed. Furthermore, under normal as well as oxidative stress conditions the basal levels of colocalization of GFP-LC3 with mitochondria in Atg5−/− knockout cells were strongly reduced when compared to wild type cells (Fig. 3AC). Similar results were obtained when mitophagy was analyzed via colocalization of mitoDsRed with CD63-GFP (data not shown). This confirms that colocalization of mitochondria with GFP-LC3 or with CD63-GFP depends on ATG5 which is in line with the general view that mitophagy uses the macroautophagic pathway. In summary, we conclude that under mild and transient oxidative stress mitophagy is induced in murine and human cell lines to a similar extent and relies on the macroautophagic machinery.

3.3. Mild oxidative stress specifically induces mitophagy but not non-selective autophagy

We further tested whether mild oxidative stress conditions induce mitophagy specifically or whether also non-selective autophagy is triggered. To this end, HeLa cells expressing mitoDsRed and GFP-LC3 were treated with H2O2 or rotenone as above. As a control we induced bulk autophagy by starvation, a condition known to increase autophagic flux [31,56,61]. The cells were treated as above either in the presence (+Baf/Inh) or absence (−Baf/Inh) of lysosomal inhibitors and the accumulation of LC3-II and p62 was assessed after a total time of six hours. We observed that inhibiting lysosomal protein degradation led to the accumulation of LC3-II and p62 in all cases (Fig. 4AB). The relative accumulation of LC3-II and p62 was most pronounced when cells were starved for six hours as compared to control cells. Moreover, compared to control conditions mild oxidative stress does neither appear to increase nor to block autophagic flux (Fig. 4AB). Taken together, the autophagic flux appears higher after nutrient-starvation in comparison to the control conditions or to mild oxidative stress.

Next, we investigated whether the different treatments impact the number of autophagic structures per cell. In order to avoid the many pitfalls reported previously regarding quantification of GFP-LC3 puncta [56,58,62], we applied electron microscopy where different types of autophagic structures can be visualized and quantified well. Electron micrographs showed that treatment of HeLa cells with H2O2 or rotenone does not lead to an apparent increase in the amount of autophagosomes and autophagolysosomes whereas a 6-fold increase in the number of these structures was observed under starvation conditions (Fig. 4CD). None of the treatments altered the ratio between autophagosomes and autophagolysosomes grossly (data not shown) suggesting that the individual maturation steps were not affected. We generally noted the presence of numerous multi-vesicular bodies (MVBs); however, their abundance was not altered in any of the conditions applied (data not shown) and thus was not considered further. Altogether, upon starvation the autophagic flux as well as the number of autophagosomes and autophagolysosomes was highly increased. By contrast, upon mild oxidative stress both of these parameters did not change compared to control conditions consistent with our results described in Fig. 4AB. We certainly cannot exclude a moderate increase in the total number of autophagosomes under these conditions as these structures are quite abundant and a small increase would be difficult to detect. In order to further corroborate these results we quantified the degradation of long-lived proteins by a pulse-chase experiment after applying mild and transient oxidative stress or after starvation. The amount of released radiolabeled valine was determined after a total time of 6 h for all conditions. In line with our earlier results, mild oxidative stress conditions did not induce a detectable increase in bulk protein degradation whereas starvation did (Fig. 4E). We are aware that the methods applied here are limited in their ability to detect minor changes in non-selective autophagy, whereas mitophagy can be detected in a very sensitive manner as only few mitochondria colocalize with autophagosomes and a relative increase would be more readily detectable. Nevertheless, mitophagy was increased about 2-fold upon mild oxidative stress and such an increase in non-selective autophagy would certainly have been detectable by the methods used here. In summary, several lines of evidence demonstrate that the mild and transient oxidative stress conditions specifically trigger mitophagy while non-selective autophagy was apparently not increased concomitantly.

3.4. Inhibition of mitochondrial fission prevents induction of selective mitophagy

The specific induction of mitophagy by mild oxidative stress was accompanied by mitochondrial fragmentation (Fig. 2BC). In order to test whether mitochondrial fission is required for selective mitophagy under these conditions we expressed a dominant-negative variant of the fission factor DRP1− (DRP1dn− = DRP1K38A−) in HeLa cells. Expression of DRP1dn led to the formation of a highly interconnected network (Fig. 5AB) consistent with earlier reports [63,64] and blocked mitochondrial fragmentation upon treatment with H2O2 or rotenone (Fig. 5AB). In addition, induction of mitophagy by mild oxidative stress was blocked (Fig. 5AC). Under normal growth conditions, we noted that DRP1dn did not reduce the extent of colocalization of mitochondria with GFP-LC3 (Fig. 5C, right panel) and only slightly reduced the colocalization of mitochondria with CD63-GFP (Fig. 5C, left panel). We conclude that impairing fission of mitochondria prevents the induction of selective mitophagy under mild and transient oxidative stress. Moreover, it does not appear to grossly impair basal turnover of mitochondria under non-inducing conditions. To assess whether DRP1 function is specifically required for mitophagy or alternatively also for non-selective autophagy we analyzed cells expressing DRP1dn under mild oxidative stress and starvation conditions by electron microscopy (Fig. 5DE). Furthermore, we determined the extent of bulk degradation of long-lived proteins by a pulse-chase experiment (Fig. 5F). Both approaches revealed that expression of DRP1dn did not impair bulk autophagy after starvation demonstrating the specific impact of impaired mitochondrial fission on mitophagy. Furthermore, the number of autophagosomes and autophagolysosomes per cell was not increased upon mild and transient oxidative stress independent of the presence or absence of DRP1dn (Figs. 4D and 5E). Taken together, we conclude that mitochondrial fission is required for the specific induction of mitophagy under mild and transient oxidative stress but not for non-selective autophagy.
Using the same assays as applied above we asked to which extent fission is required for mitophagy during starvation. Starvation was chosen as it is a standard condition known to induce both autophagy and mitophagy. Indeed, starvation led to a significant ~1.7-fold increase in mitophagy that was prevented by expression of DRP1dn (Fig. 6ABC). It is noteworthy that DRP1dn apparently did not affect basal turnover of mitochondria (Fig. 6AB), similarly to our previous observations (Fig. 5AC). Starvation-induced mitophagy (~1.7-fold) was significantly less pronounced in comparison to the ~2.3-fold induction of mitophagy after H2O2 or rotenone treatment (Fig. 6C), although the number of autophagosomes/autophagolysosomes was increased by ~6-fold upon starvation as compared to mild oxidative stress conditions (see Fig. 4CD). Therefore, considering the high level of non-selective autophagy the mitophagy induction of ~1.7-fold upon starvation appears rather low when compared to mild oxidative stress conditions. Moreover, consistent with other studies [43,44,65], mitochondria formed a highly tubular, interconnected, and hyper-fused network under starvation conditions (Fig. 6AD). These results

**Fig. 5.** Induction of selective mitophagy depends on mitochondrial fission. A, HeLa cells stably expressing mitoDsRed and CD63-GFP (left panel), or mitoDsRed and GFP-LC3 (right panel), were transiently transfected or transduced, respectively, with the lentiviral construct CMV_DRPdn_IRES_mitoDsRed (+DRP1dn), treated with 3.3 mM H2O2 (10 min, 30 min recovery) or 1 µM rotenone (1 h), or untreated (control), and fixed. Controls without expression of DRP1dn (−DRP1dn) are shown in Fig. 2B. Single focal planes of fluorescence images are shown in A. 1st row, red channel; 2nd row, green channel; 3rd row, merged; 4th row, blow-up. Examples of mitochondria-containing autophagic vesicles that were ascertained in 3D stacks as described in Fig. 2A are shown by arrowheads. B, Mitochondrial morphology of these cells expressing DRP1dn was determined. C, Mitophagy was assessed by counting the number of mitochondria encapsulated in GFP-positive structures as described in Fig. 2. The results of at least three independent experiments (>30 cells were analyzed per condition in each experiment) are shown as mean per cell ± s.d. (n ≥ 3). Controls without expression of DRP1dn (−DRP1dn) from Fig. 2BC are shown again for comparison. DE, HeLa cells stably expressing mitoDsRed and CD63-GFP were transduced with the lentiviral construct CMV_DRPdn_IRES_mitoDsRed and treated and analyzed by electron microscopy as described in Fig. 4CD. D, EM pictures are shown. Top, overview; bottom, blow-up. E, Autophagy was assessed from the EM pictures by counting the number of autophagic structures (autophagosomes and autophagolysosomes) per cell section. The results are shown as mean per cell section ± s.d. (n ≥ 10 cells per condition). F, Quantification of protein turnover by measuring the degradation and release of l-[14C]-valine derived from long-lived proteins. HeLa cells stably overexpressing mitoDsRed and CD63-GFP were transduced with the lentiviral construct CMV_DRPdn_IRES_mitoDsRed (+DRP1dn) or with CMV_IRES_mitoDsRed (−DRP1dn). The pulse-labeled cells were starved (HBSS) for 6 h or left untreated (control). The results of three independent experiments are shown as mean percentage of free radioactive valine released into the medium ± s.d (n = 3).
support the view that starvation-induced hyperfusion of mitochondria counteracts excessive degradation of mitochondria.

4. Discussion

The molecular mechanisms ensuring the removal of dysfunctional mitochondria are proposed to be crucial to a number of human disorders including Parkinson’s disease [23–29] and aging [3]. Still, the mechanistic details linking mitochondrial dynamics and selective mitophagy remain largely unclear. In particular, which conditions induce mitophagy specifically; i.e. is mitophagy under all circumstances inherently linked to non-selective autophagy or not? Are the molecular details for mitophagy during normal growth or under starvation conditions identical to situations when compromised mitochondria have to be recognized and degraded selectively? Can mitophagy occur independently of non-selective autophagy at all?

Our study demonstrates that mitophagy is specifically induced after mild and transient oxidative stress. We propose that under these conditions mitophagy occurs in a truly selective manner and can proceed without detectable increase in non-selective autophagy. Especially when dysfunctional mitochondria are only present at low frequency within a cell it would not make much sense to stimulate excessively the macroautophagic machinery. These findings enable us and others to better distinguish mitophagy-specific effects from non-selective autophagy in mammalian cells.

ROS are well known to be important signaling molecules required to induce the formation of autophagosomes [31,32]. For example, amino acid starvation as well as growth factor deprivation rapidly induces high levels of ROS required for induction of non-selective autophagy. Also depolarization of mitochondria by CCCP or by overexpression of NIX leads to ROS formation and subsequent induction of autophagy [30]. Here we show that the ROS levels induced by starvation are considerably higher than those induced by rotenone or hydrogen peroxide as applied here. Our finding that the oxidative stress conditions applied here are relatively mild is fully consistent with an earlier report using H₂O₂ in a very similar way [54]. In that study, ROS levels were shown to be increased to a level comparable to that triggered by physical activity. Our data suggest that with low levels of ROS mitophagy is induced selectively and regulatory pathways of autophagy operates independently or that can be triggered in a very sensitive and specific manner. To our knowledge, the data presented here show for the first time that cells have the intrinsic capability to degrade specifically mitochondria without grossly inducing macroautophagy at the same time. We certainly cannot fully exclude that non-selective autophagy is induced to a minor, not detectable, extent. Still, we can safely conclude that non-selective autophagy is not induced at a magnitude similar to the induction of mitophagy that was induced about 2-fold. Our assays, in particular the very sensitive pulse-chase assay, would have detected such an increase. Thus, mitophagy is indeed specifically induced by mild oxidative stress.

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**Fig. 5 (continued).**

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exclusively. We propose that the low H$_2$O$_2$ and rotenone concentrations applied over short incubation times do not massively damage mitochondria, but rather initiate a ROS signaling cascade, leading to the induction of selective mitophagy. This in turn would promote the selective removal of damaged mitochondria, explaining the observed recovery after removal of the oxidative stress [54]. This observation is also consistent with the current view that mitophagy acts as a mechanism to reduce mitochondria-derived ROS and by that turn off or limit excessive induction of autophagy [31]. Thus, the increase in mitophagy combined with a rather mild oxidative stress could well

Fig. 6. Starvation induces DRP1-dependent mitophagy and hyperfusion of mitochondria. HeLa cells stably expressing mitoDsRed and GFP-LC3 were transiently transfected with CMV_IRES_mitoDsRed (−DRP1dn) or CMV_DRPdn_IRES_mitoDsRed (+DRP1dn). The cells were starved for 6 h (HBSS) or left untreated (control) and fixed. A, Single focal planes of fluorescence images are shown. 1st row, red channel; 2nd row, green channel; 3rd row, merged; 4th row, blow-up. Examples of mitochondria-containing autophagic vesicles that were ascertained in 3D stacks as described in Fig. 2A are shown by arrowheads. B, Mitophagy was assessed by counting the number of mitochondria encapsulated in GFP-LC3-positive structures. The results of at least three independent experiments (>30 cells were analyzed per condition in each experiment) are shown as mean per cell ± s.d. (n ≥ 3). C, The relative induction of mitophagy was calculated from panel B (−Drp1dn) and from Fig. 2D, right panel, for the indicated conditions. D, The extent of mitochondrial hyperfusion was assessed by determining the number of mitochondria covering an area larger than 20 µm$^2$ per focal plane normalized to the total mitochondrial area analyzed in three independent experiments. The results are shown as mean ± s.d. (n = 3). Statistically significant differences and corresponding p-values using a two-sided student’s T-test are indicated.
explain why in our study non-selective autophagy is not induced concomitantly as one may have expected based on earlier studies when ROS levels were massively increased. Our data certainly do not contradict the view that prolonged and more excessive ROS formation in mitochondria also triggers autophagy. Moreover, at higher ROS concentrations this and other quality control systems might just be overloaded, resulting in permanent damage and reduced cell viability as described previously [54].

It has been reported that mitochondrial fission is required for mitophagy under basal growth conditions [41], after downregulation of PINK1 [26], and after starvation [43,44]. Our study shows that mitochondrial fission is also required for the induction of mitophagy under mild and transient oxidative stress and confirms that fission is required for mitophagy induced by starvation. Noteworthy, mitochondrial turnover at basal levels is apparently not affected by mitochondrial fission. The latter is consistent with a study from Dagda and colleagues [26] but differs from the results by Twig and colleagues reporting that under normal growth conditions mitophagy was reduced to about 30% of control upon inhibition of mitochondrial fission with 3-MA. This discrepancy could be attributed to the different cell lines used or to deviating growth conditions. In particular, the duration when mitochondrial fission was markedly different. In the study by Twig and colleagues it was ~10 to 21 days whereas here it was only ~2–6 days. Considering that increased oxidative damage to mitochondrial proteins was observed when mitochondrial fission was permanently inhibited [41] it is well possible that intracellular oxidative stress arose during prolonged inhibition of mitochondrial fission which could then contribute to the observed discrepancies with the present study. As we and others do not see a detectable impact of mitochondrial fission on basal turnover of mitochondria we propose that a stimulation of mitophagy by specific triggers represents a selective form of mitophagy while the basal turnover of mitochondria rather appears to be non-selective. The latter implies that either engulfment of mitochondria into autophagosomes at basal levels is fission-independent or that even after expressing dominant-negative DRP1 fission proceeds at a low level yet compatible with basal turnover but not with increased turnover of mitochondria after induction of selective mitophagy. The role of mitochondrial fission for mitophagy not only appears to depend on the condition used but also depends on the organism under investigation. We have recently shown that fission is dispensable for rapamycin-induced mitophagy in S. cerevisiae [46]. There are several possibilities explaining this apparent discrepancy. One possibility is that the mitochondrial network in mammalian cells is simply too large and interconnected to be efficiently degraded without the additional fragmentation of mitochondria. Also cells that undergo few or no cell divisions (post-mitotic) might in particular depend on intracellular division mechanisms for mitochondria — a problem that yeast obviously is lacking. Another possibility is that yeast has other, not yet detected, mechanisms to perform mitochondrial fission. Alternatively, mammalian cells may have the intrinsic ability to degrade mitochondria by autophagy in the absence of functional mitochondrial fission machinery but the conditions required for this mechanism have not been determined yet. We would certainly not exclude the latter possibility especially given the fact that basal turnover of mitochondria appears to proceed normally when fission is blocked. Furthermore, several studies using Drosophila indicate that mitophagy is influenced by altering mitochondrial dynamics. For example, the fusion factor Mfn was shown to be a substrate of PARKIN [68], and promoting mitochondrial fission was shown to partially rescue mitochondrial dysfunction [69,70]. Future studies will have to answer whether fission is required for mitophagy in general, only in specific organisms, or under specific conditions such as mild oxidative stress and starvation.

We further observed that starvation leads to hyperfusion of mitochondria whereas fragmentation was observed when mitophagy was specifically induced by H2O2 or rotenone. Also induction of mitophagy was less pronounced upon starvation when compared to the latter conditions. Hyperfusion of mitochondria was earlier reported to occur after starvation and under various stress conditions distinct from those applied here [65]. It was suggested as an initial stress response promoting mitochondrial ATP production and cell survival. Two recent studies have demonstrated that hyperfusion of mitochondria upon starvation counteracts mitochondrial degradation [43,44]. Considering these results and our own observations hyperfusion of mitochondria apparently protects them from degradation. What might be the physiological reason for this observation? Possibly, hyperfusion of mitochondria counteracts the excessive removal of mitochondria by autophagy which ensures that not too much of the mitochondrial mass is lost. This may be of particular importance during starvation, a condition in which ROS levels are high and non-selective forms of autophagy are strongly induced. Conversely, such a protective mechanism would not be required, or even is expected to be detrimental, when damaged mitochondria accumulate and need to be selectively targeted for degradation. In line with this idea, under mild and transient oxidative stress conditions we did not observe an increased hyperfusion of mitochondria. It was shown earlier that mitochondrial dysfunction indeed causes the proteolytic inactivation of the fusion factor OPA1 [37,38,71]. Thus, we propose that forcing mitochondria to undergo hyperfusion acts as a mean of joining all functional organelles in a large and interconnected network. By that the damaged mitochondria are spatially excluded, which promotes their efficient removal.

In conclusion, our study demonstrates that mitophagy can be triggered selectively by mild oxidative stress in a DRP1-dependent fashion. These findings strengthen the view that mitochondrial dynamics and mitophagy are part of a complex pathway ensuring the functionality of mitochondria that we are just beginning to understand.

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