MITOCHONDRIAL COMMUNICATION IN PHYSIOLOGY, DISEASE AND AGING

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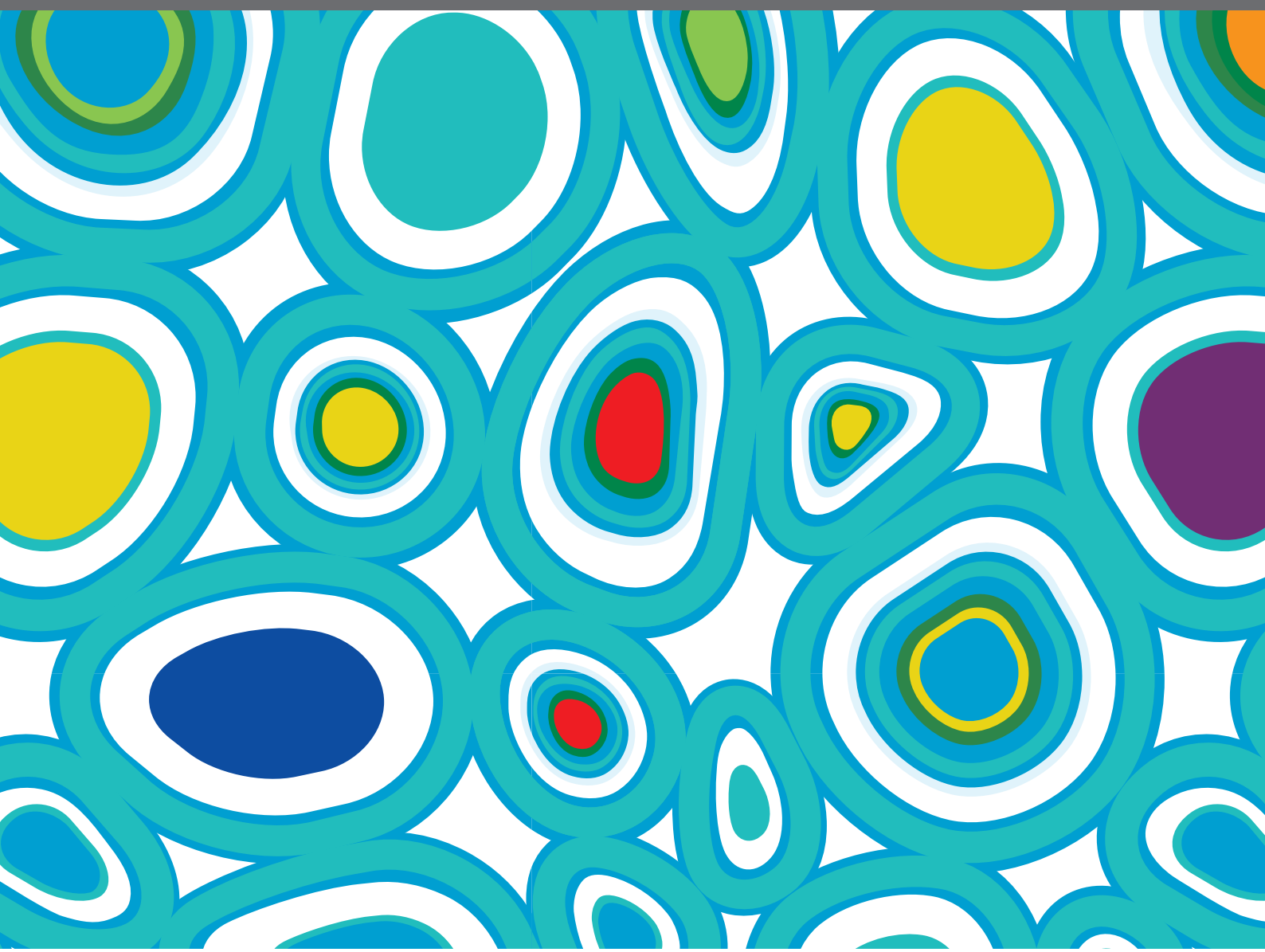

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MITOCHONDRIAL COMMUNICATION IN PHYSIOLOGY, DISEASE AND AGING

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Table of Contents

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EDITORIAL
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Editorial: Mitochondrial Communication in Physiology, Disease and Aging

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Keywords: mitochondria, aging, age-related disease, lysosome, endoplasmic reticulum

Editorial on the Research Topic

Mitochondrial Communication in Physiology, Disease and Aging

Mitochondria hold the key to many biological functions, ranging from the long-known role in ATP production to metabolic control, cellular signaling and regulation of cell death (Raimundo, 2014). Many of these functions rely on complex signaling pathways elicited by perturbations of diverse aspects of mitochondrial function (Raimundo, 2014; Shadel and Horvath, 2015). Notably, it has recently started to be unveiled, that mitochondrial functions are supported by their communication with other cellular organelles (e.g., endoplasmic reticulum, lysosomes) and processes (e.g., autophagy, senescence).

In general, organelle communication can be manifested by the formation of direct contact sites via membrane apposition, as well as via signals released by one organelle that trigger a signaling pathway regulating the function or homeostasis of another organelle (Diogo et al., 2018). The research topic "Mitochondrial communication in physiology, disease and aging" (2017–2018) brought together an ensemble of perspectives on how mitochondria communicate with the intra and extracellular surroundings, in different model organisms. In this context, Pon and colleagues address the role of interorganellar mitochondrial tethers in aging using budding yeast as a model (Pernice et al.). Mitochondrial tethers are critical for non-uniform segregation of mitochondria during asymmetrical cell division in yeast, allowing the daughter cell to inherit fitter, and the mother to retain high-functioning mitochondria. It is possible that other cell types characterized by asymmetrical cell division, like human mammary stem-like cells, may display similar tether-dependent mechanisms of mitochondrial segregation.

The best characterized mitochondrial interaction is the one involving the contact sites between mitochondria and the endoplasmic reticulum (ER) (Cohen et al., 2018). These structures are conserved from yeast to humans, and they are important for the transfer of Ca^{2+} and lipids between the two organelles (Eisenberg-Bord et al., 2016). While many proteins that form the mitochondria-ER tethers have been identified, less is known about how the assembly or maintenance of these tethers is regulated upon stimulation. Giacomello and colleagues review how proteins are recruited to the mitochondria-ER contact sites and discuss their physiological role in these interfaces, as well as their role in stress signaling (Ilacqua et al.).

As mentioned above, there are interactions between mitochondria and ER that do not rely on contact sites. One such example is the activation of the mitochondrial unfolded protein response (UPRmt) in response to mitochondrial stress, as reviewed by Callegari and Dennerlein. Furthermore, this pathway is part of broader mitochondria-ER interaction spectrum. Mitochondria

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and ER both respond to changes in proteostasis status in each other cellular compartment resulting in a cell-wide response to compartmentalized proteostasis failure or improvement (Perić et al., 2016). Interestingly, this process relies on the crosstalk between Hsp82 and TORC1 complex, providing another example of communication between pathways in regulation of a cell homeostasis (Perić et al., 2017).

Another organelle interaction that has lately gathered attention is the mitochondria-lysosome crosstalk. Similar to the case of ER, contact sites between mitochondria and lysosomes have been described both in lower (Elbaz-Alon et al., 2015) and higher eukaryotes (Wong et al., 2018; Cioni et al., 2019). Germain and colleagues present a comprehensive discussion on the tethers between mitochondria and lysosomes as well as on the metabolic and signaling crosstalk between the two organelles (Todkar et al.). This question is further addressed by Mittelbrun and colleagues, who also consider the endosomal interactions with mitochondria and their implications for extracellular signaling (Soto-Heredero et al.). It is noteworthy to point out that acute and chronic defects in mitochondria have opposite regulatory effects on lysosomal biogenesis and function. While under acute mitochondrial stress activation of TFEB/MITF-dependent lysosomal (and, likely, mitochondrial) biogenesis is observed, chronic mitochondrial stress results in repression of both lysosomal function and biogenesis, resulting in the cytoplasmic accumulation of dysfunctional lysosomes with decreased hydrolytic capacity (Fernández-Mosquera et al., 2017; Fernandez-Mosquera et al., 2019). The role of mitochondria-lysosome crosstalk is also considered in the context of diseases. Plotegher and Duchen explore the impact of this organelle "duo" in Parkinson's disease (Plotegher and Duchen), while Fernandez-Checa and colleagues comprehensively integrate the mitochondria-lysosome crosstalk in the pathology of a lysosomal storage disease (Torres et al.). In both of these cases, primary defects in lysosomes result in perturbation of mitochondrial homeostasis and function. While this is often entirely attributed to the impairment of the autophagic pathway, new findings revealed the existence of a signaling pathway triggered in lysosomal sphingolipidosis (storage diseases of the lysosomal catabolism pathway), which results in the repression of mitochondrial biogenesis (Yambire et al., 2019). Moreover, one natural consequence of lysosomal impairment is the perturbation of the autophagy pathway, with consequent decrease in mitophagy. The vast majority of the studies in autophagy and mitophagy have been carried out in cultured cells, while gathering *in vivo* data from model organisms, particularly mammals, has been hampered by a lack of appropriate tools. In this research topic, Poulton and colleagues presented a novel tool to assess mitophagy in mice, and applied it to a mouse model of a mitochondrial disease, namely autosomal dominant optic atrophy (Diot et al.).

Finally, several signaling pathways that are active upstream and downstream of mitochondria were also addressed in this research topic, in multiple model organisms. For example, this includes the role of mitochondrial retrograde response in induction of yeast filamentous growth in a conditioned environment (Gonzalez et al.). On the other hand, using mouse embryonic fibroblast cell lines, presenilin-2, a protein involved in Alzheimer's disease (AD), is shown to have a role in the regulation of mitochondrial function in the context of the electron transport chain maintenance (Contino et al.). These results may contribute to the research of AD pathology, in particular of the AD-related metabolic decline. Lastly, an important role of the mitochondrial inner membrane as an independent signaling platform is carefully dissected by Dudek.

Altogether, this research topic has integrated mitochondrial function into a complex but comprehensive network with other cellular organelles and processes. While this view is evolving, due to the increasing attention that this field is awarded, it provides a time-stamp on how mitochondrial communication in physiology, pathology and aging is seen in 2018.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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ORIGINAL RESEARCH
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Validating the RedMIT/GFP-LC3 Mouse Model by Studying Mitophagy in Autosomal Dominant Optic Atrophy Due to the OPA1Q285STOP Mutation

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Background: Autosomal dominant optic atrophy (ADOA) is usually caused by mutations in the essential gene, OPA1. This encodes a ubiquitous protein involved in mitochondrial dynamics, hence tissue specificity is not understood. Dysregulated mitophagy (mitochondria recycling) is implicated in ADOA, being increased in OPA1 patient fibroblasts. Furthermore, autophagy may be increased in retinal ganglion cells (RGCs) of the OPA1^{Q285STOP} mouse model.

Aims: We developed a mouse model for studying mitochondrial dynamics in order to investigate mitophagy in ADOA.

Methods: We crossed the OPA1^{Q285STOP} mouse with our RedMIT/GFP-LC3 mouse, harboring red fluorescent mitochondria and green fluorescent autophagosomes. Colocalization between mitochondria and autophagosomes, the hallmark of mitophagy, was quantified in fluorescently labeled organelles in primary cell cultures, using two high throughput imaging methods Imagestream (Amnis) and IN Cell Analyzer 1000 (GE Healthcare Life Sciences). We studied colocalization between mitochondria and autophagosomes in fixed sections using confocal microscopy.

Results: We validated our imaging methods for RedMIT/GFP-LC3 mouse cells, showing that colocalization of red fluorescent mitochondria and green fluorescent autophagosomes is a useful indicator of mitophagy. We showed that colocalization increases when lysosomal processing is impaired. Further, colocalization of mitochondrial fragments and autophagosomes is increased in cultures from the OPA1^{Q285STOP}/RedMIT/GFP-LC3 mice compared to RedMIT/GFP-LC3 control mouse cells that were wild type for OPA1. This was apparent in both mouse embryonic

fibroblasts (MEFs) using IN Cell 1000 and in splenocytes using ImageStream imaging flow cytometer (Amnis). We confirmed that this represents increased mitophagic flux using lysosomal inhibitors. We also used microscopy to investigate the level of mitophagy in the retina from the OPA1^{Q285STOP}/RedMIT/GFP-LC3 mice and the RedMIT/GFP-LC3 control mice. However, the expression levels of fluorescent proteins and the image signal-to-background ratios precluded the detection of colocalization so we were unable to show any difference in colocalization between these mice.

Conclusions: We show that colocalization of fluorescent mitochondria and autophagosomes in cell cultures, but not fixed tissues from the RedMIT/GFP-LC3, can be used to detect mitophagy. We used this model to confirm that mitophagy is increased in a mouse model of ADOA. It will be useful for cell based studies of diseases caused by impaired mitochondrial dynamics.

Keywords: mitophagy, mouse model, OPA1, ADOA, mitochondrial fragmentation, high content imaging

INTRODUCTION

Mitochondria are important for cells, not just for generating energy, calcium regulation and key biosynthetic processes including synthesis of iron Sulfur centers, but also for apoptosis, signaling, and response to cellular stress (Suomalainen and Battersby, 2018). Mitochondria form a dynamic reticulum in cells, with portions of this network constantly fusing and dividing (Legros et al., 2002). Mitochondrial location and transport are particularly important in neurons, individual mitochondria moving along microtubules in the cytoplasm to synapses and other parts of the cell requiring energy (Li et al., 2004). These dynamics are under the control of molecular players such as Drp1 and Fis1 for fission and mitofusins (Legros et al., 2002) and OPA1 for fusion (Chen and Chan, 2006). Hence mitochondrial diseases are mechanistically diverse and do not necessarily manifest clear evidence of impaired ATP synthesis. For instance, the evidence for respiratory chain dysfunction in mitochondrial optic neuropathies may be very subtle (Yu-Wai-Man et al., 2002). Nevertheless, these are important diseases that impair vision, resulting in lifelong disability.

Exactly how mutations in both mitochondrial DNA (mtDNA), encoding subunits of the respiratory chain and in nuclear genes involved in mitochondrial biogenesis can cause optic neuropathies with rather similar phenotypes is poorly understood. Retinal ganglion cells (RGCs), forming the optic nerve and transmitting visual information to the brain, are the cell type that is affected in both Leber Hereditary Optic Neuropathy (LHON) and Autosomal dominant optic atrophy (ADOA) respectively. Autophagy (a type of cellular quality control) is important for the maintenance of RGCs: even a slight reduction in retinal autophagy levels can alter the capability of RGCs to respond to axonal stress (Boya, 2017) which can be rescued by activating autophagy with rapamycin (Rodríguez-Muela et al., 2012).

ADOA is the commonest inherited optic neuropathy (prevalence 1:25,000) resulting in a bilateral, symmetrical and painless loss of vision, color vision defects, central visual field loss and atrophy of the optic disc. It is a slowly progressive neuropathy, currently irreversible and untreatable. Over 200 mutations in the essential gene OPA1 have been identified, comprising about 60% of patients with ADOA (Yu-Wai-Man et al., 2014). The OPA1 gene encodes a ubiquitous protein involved in mitochondrial dynamics. It plays a crucial role in the regulation of the mitochondrial network and cristae morphology, in oxidative phosphorylation, maintenance of mitochondrial membrane potential (MMP), in apoptosis and in neuronal maturation (Bertholet et al., 2013). Furthermore, upregulation of OPA1 can protect against some types of mitochondrial disease (Civiletto et al., 2015). We showed that mitophagy (a subtype of autophagy for maintaining mitochondrial quality) can be upregulated by OPA1 knock down (Liao et al., 2017). When knock down is profound, mitochondrial DNA (mtDNA) depletion and respiratory chain dysfunction are seen in primary cultures of fibroblasts (Liao et al., 2017) and cortical primary neurons (Bertholet et al., 2013). When OPA1 is over-expressed it can ameliorate defects in the respiratory chain (Civiletto et al., 2015; Varanita et al., 2015). We showed that mitophagy is dysregulated in fibroblasts from patients with either mitochondrial (Dombi et al., 2016) or autosomal inherited (Liao et al., 2017) optic neuropathies. We therefore sought to develop a mouse model to investigate the role of OPA1 in the mitochondrial dynamics of RGCs. We used the B6;C3-Opa1(Q285STOP) mouse which has a heterozygous mutation located in exon 8 immediately before the central dynamin-GTPase domain (Davies et al., 2007). This mutation halves the expression of OPA1 protein in all tissues, including the retina, on Western blot analysis (Davies et al., 2007). Heterozygous mutants show a slow onset of degeneration in the optic nerve, preceded by retinal ganglion cell dendropathy (Williams et al., 2010). Furthermore, the mice demonstrate a reduction in visual function on testing with the optokinetic drum and the circadian running wheel (Davies et al., 2007).

Dysfunctional mitochondria can be recycled by a specific type of autophagy, called mitophagy. The damaged mitochondrial fragment is targeted to a developing autophagosome, called a

phagophore, which engulfs the mitochondrion forming a so-called "mitophagosome" (Eid et al., 2016a). This then fuses with a lysosome generating a "mitophagolysosome" (Eid et al., 2016a) that acidifies and degrades its contents, including the mitochondrion. Key stages in this process have been exploited to highlight markers as a readout for mitophagy. For instance, both Finkel (Sun et al., 2015) and Ganley (McWilliams et al., 2016) used genetically encoded fluorescent proteins, targeted to mitochondria, that respond to the drop in pH following fusion with the lysosome (with mKeima and mCherry respectively). These are endpoint and steady state assays, respectively. Because they both depend on lysosomal acidification they are particularly useful for highlighting defects in lysosomal processing, late in the mitophagy process.

We however started our studies before either of these pH sensitive models was available. In studying the effects of OPA1 knock down on mitophagy we postulated that mitochondrial fragmentation might drive mitophagy and hence were specifically interested in engulfment of mitochondria by autophagosomes in the earliest stages of this process. We therefore exploited a tool developed by Mizushima, who visualized autophagosomes by tagging their molecular hallmark, LC3-ll with green fluorescent protein (GFP) (Mizushima et al., 2004). We made mice in which monomeric red fluorescent protein (mRFP) was targeted to mitochondria and crossed these with Mizushima's LC3-GFP mice (Mizushima et al., 2004) to visualize colocalization of mitochondria and autophagosomes. Hence we focussed on an earlier stage of mitophagy than the other two assays (Sun et al., 2015; McWilliams et al., 2016). We developed this as a readout for mitophagy driven by OPA1 knock down, in which we anticipated that excessive mitochondrial fragmentation, apparent in RGCs of this mouse model (Williams et al., 2012), drives mitophagy (Liao et al., 2017).

MATERIALS AND METHODS

Media and Chemicals

DMEM glucose free (11966-025), DAPI (D1306), and goat anti-rabbit AlexaFluor 488 (A-11008) fluorescent secondary antibodies were purchased from Life Technologies. Primary mouse anti-PDH (sc-377092) antibody was purchased from SantaCruz Biotechnology. DMEM high (4.5g/L) glucose (D6546), Galactose (G5388), and the pharmacological agents E64d (E8640), Pepstatin A (77170), and Chloroquine (C6628) were purchased from Sigma Aldrich. Penicillin and Streptomycin (P4458) were purchased from Sigma Aldrich.

Genetic Modification of Mouse Embryos

RedMIT mice, developed by collaborator FI, express mRFP downstream of the COX VIII targeting peptide, driven by the EF1α promoter (ubiquitous expression). Hence mitochondria appear red when viewed by fluorescence microscopy (**Figure 1**). Mouse ES cells (129 background) were transduced with a VSV pseudotyped pHR'SIN-cPPT-SE lentivirus (Demaison et al., 2002) in which the human EF1a promoter drives expression of a fusion gene containing the mitochondrial localization signal of COX VIII and RFP. A clone with low RFP expression in which viability/function was not impaired by RFP was selected by FACS to produce the mouse by microinjecting ES cells into embryos for founders.

Mouse Embryonic Fibroblasts Production

A pregnant RedMIT-GFP-LC3 or RedMIT-GFP-LC3-OPA1^{Q285STOP} mouse was sacrificed on embryonic day 13.5 or 14.5 and the uterine horns dissected. The excess fat was trimmed away before opening the uterus carefully to release the conceptuses. The embryonic membranes and placenta were removed from each embryo. The head and internal organs were removed and the remainder minced as finely as possible using a sterile scalpel. It was incubated in trypsin for 5 min at 37°C.

After a vigorous shake to dissociate the tissue, large debris were allowed to settle before the supernatant was transferred to a tube containing DMEM medium + 10% FCS + penicillin/streptomycin (250 μM). The trypsin extraction was repeated three times and the cells split into four 75 cm^2 flasks. The medium was changed the following day to remove further debris. When the flasks were confluent, each flask was passaged into a 175 cm^2 flask. Once confluent, these cells were harvested and frozen in 1 ml aliquots at about 10^7 cells/ml for storage.

High-Throughput Microscopy and Mitophagy Assessment

For IN Cell 1000 analysis, mouse embryonic fibroblasts (MEFs) were seeded at 10^4 cells per well in a Nunc F 96-well plate (Thermo Scientific) and treated in the indicated conditions for 24 h. Cells were fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min before DAPI staining (dilution 1/5,000 for 5 min). The cells were imaged using the IN Cell 1000 analyzer (500 cells acquired per well) and raw images processed and parameters obtained using a previously published (Diot et al., 2015) customized protocol in the IN Cell Developer toolbox (GE Healthcare Life Sciences).

We used Imagestream, which we previously validated for detecting mitophagy (Liao et al., 2017), for analysis of splenocytes. Spleens were removed immediately after death and placed in individual sterile dishes with 1 mL of cell culture media. The spleen cells were separated out by mechanical disruption and divided equally between two 25 cm^3 flasks in 5 mL of DMEM. Chloroquine was added to a concentration of 20 μM to one of the flasks and both flasks incubated overnight at 37°C. Floating and trypsinized cells were filtered through a 30 μm filter and fixed in 4% PFA for 15 min at room temperature. After permeabilization in 0.4% Triton for 3 min, cells were washed in PBS and resuspended in 50–100 μL of FACS buffer for analysis with Imagestream (1,000–5,000 cells in each condition from three mice in each condition).

To identify colocalization of autophagosomes and mitochondria as an indicator of mitophagy we used Amnis IDEAS software, counting the numbers of LC3 positive puncta that colocalized with mitochondria, using a "threshold" mask for detecting mitochondrial location. Duplicate analyses using threshold masks of either 30 or 70% of the range of intensity values as defined by the starting mask was used to exclude pixels.

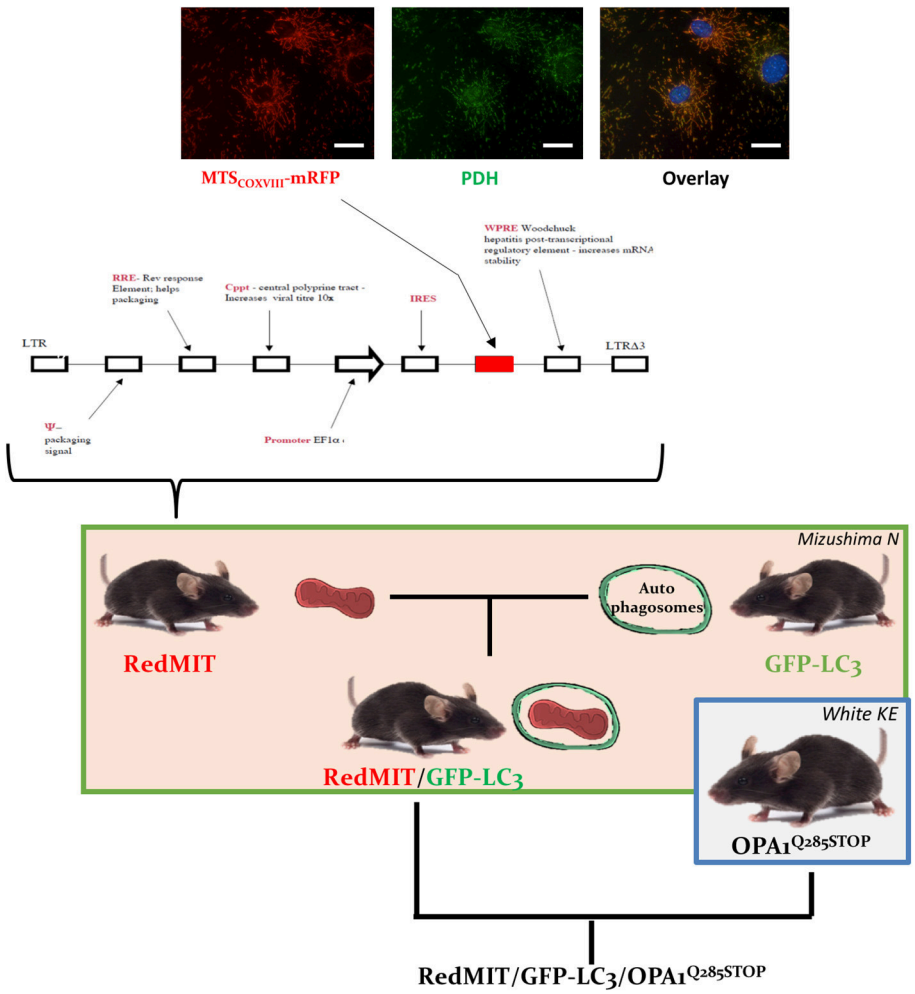

FIGURE 1 | The RedMIT-GFP-LC3-OPA1^{Q285STOP} mouse. The RedMIT mouse has been genetically engineered by random insertion of the illustrated construct. The mRFP fluorescent protein is fused to the mitochondrial targeting sequence of COX VIII and its expression perfectly colocalizes with PDH (panel, scale bar = 20 μm). Expression is under the control of the EF1α promoter. Homozygous females were then crossed to GFP-LC3 males (Mizushima et al., 2004) to obtain our RedMIT-GFP-LC3 mouse model. Once both fluorescent markers were homozygous, RedMIT-GFP-LC3 mice were crossed to the OPA1^{Q285STOP} mice (White et al., 2009).

Oxygen Consumption Measurement

MEFs were plated at a density of 50,000 per cells per well in black 96 well plates with clear bottoms (Falcon Corning). Cells were left for 7 h to attach and then media switched to media with glucose (25 mM) or galactose (10 mM) and incubated in a standard 37°C −5% CO_2 incubator. A parallel plate was set up for Hoechst quantification to allow normalization for cell number. After 16 h media were replaced with fresh media containing the MitoXpress xtra oxygen probe (Luxcel Biosciences) and overlayed with mineral oil. The oxygen consumption assay was carried on in a BMG OMEGA plate reader equilibrated at 37°C and monitored for at least 4 h. Initial oxygen consumption rates (fluorescence life time) were calculated in the linear phase of the assay and standardized to cell number measured using Hoechst on the parallel plate.

Mouse Husbandry and Ethics Statement

All animals were housed and managed in accordance with the United Kingdom's Home Office protocols, covered by the Animals (Scientific Procedures) Act 1986. The protocol was approved by the Oxford University Committee on Animal Care and Ethical Review, University of Oxford Medical Sciences division (Project licenses 3002208 and 3003213).

Mice were housed in conventional wire-top polycarbonate cages, with a 12:12 light:dark cycle at temperatures between 19 and 23°C and relative humidity 55 ± 10%. Food and water were offered ad libitum. The facility is free of *MHV, EDIM, MVM, MPV, PVM, Sendai, TMEV, ectomelia, LCMV, Mad 1, and 2, MCMV, reovirus 3, Citrobacter rodentium, Clostridium piliforme, Corynebacterium kutscheri, Mycoplasma, Pasteurellaceae, Salmonella, beta-hemolytic streptococci,*

Streptococcus pneumoniae, Streptobacillus moniliformis, endoparasites, and *ectoparasites. Helicobacter* and *MNV* are present in this facility.

Confocal Microscopy

MEFs were plated onto 0 thickness coverslips and treated as described in the main text. Four percent paraformaldehyde was used for fixation (10 min, room temperature). Cells were permeabilized and washed in 0.1% Triton-Tris buffered saline three times before mounting on slides using Vectashield (Vector Labs). Images were acquired on an upright Leica SP5 confocal microscope equipped with the appropriate filters and sequential 488, and 568 nm laser illumination.

For mouse eyes, four samples (2x RedMIT-GFP-LC3 and 2x RedMIT-GFP-LC3-OPA1^{Q285STOP}) were harvested from perfused-fixed mice and cryostat eye sections cut at 10 μm, lightly counter-stained with DAPI (1:30,000 dilution; 5 min) and sealed using aqueous glycerol-based mountant with a No. 1.5 coverslip. They were examined using a Zeiss LSM 700 inverted confocal microscope with a plan-Apo 63x NA 1.4 oil-immersion objective. The optical section thickness was set at 1.0 micrometer, and as far as was practicable the optic nerve head was examined. The maximum Pearson product moment correlation coefficient values were recorded across all four slides, using the colocalization software in the Zeiss Zen Black, Zen 2.3 SP1 version 14.0.0.0.0 with the scatter-plot threshold set to three times the standard deviation of the mean value of the background pixels, as investigated by Barlow et al. (2010).

Live Cell Imaging

MEFs, cultured and treated as described above, were plated into a 35 mm MatTek dish, and supplemented with 10 mM Hepes to buffer pH during live cell imaging. A custom Olympus IX81 inverted microscope equipped with temperature control (Solent scientific), LED illumination (Cairn Research), a Semrock quad-band filter set (bandpass filter (DS/FF01-387/485/559/649-25), dichroic quad-edge beam splitter (DS/FF410/504/582/669-Di01-25x36), and quad bandpass emission filter (DS/FF01-440/521/607/700-25) and simultaneous dual image acquisition with two C-1900 EMCCD's (C1900, Hamamatsu, Japan) mounted after a beam splitter (Dual View C2, Photometrics) controlled thorough CellR (Olympus, Japan) with a x60 oil immersion lens (Olympus, NA 1.42). Simultaneous red and green images were acquired as a z-stack every 30 s to enable subsequent Weiner filter deconvolution (CellR). A single z-plane is selected for the overlay time series shown with post-processing (time stamping and compression) for publication using Videomach (gromeda.com).

RESULTS

Engineering the RedMIT-GFP-LC3 Mouse

In order to visualize mitochondrial fate, we first generated a mouse expressing mRFP fused to the COX VIII mitochondrial targeting sequence (**Figure 1**), engineered by random insertion into embryonic stem cells. Mice that were homozygous for the insert, located in the *pkn1* gene (**Figure S1**) appear to be normal with no noticeable effects observed on lifespan or litter size.

We confirmed the mitochondrial localization of mRFP in MEFs derived from these mice (**Figure 1**) and observed an O_2 consumption similar to that of wild type MEFs, suggesting that genetic modification had not significantly affected mitochondrial function (**Figure 2A**).

To visualize mitochondria specific autophagy, i.e., mitophagy, we crossed this mouse model with the previously described mouse expressing LC3, the hallmark of autophagosomes, tagged with GFP protein (Mizushima et al., 2004). This double fluorescent labeling had no noticeable effect on mice, either reproduction (**Table S1**), lifespan or litter size.

Then we generated MEFs and imaged them using the high throughput imaging system employed previously (Diot et al., 2015) to explore mitochondrial dynamics, autophagy and mitophagy in this mouse model (**Figures 2B,C**). Like oxygen consumption (**Figure 2A**), cell growth (not shown) was not affected by the expression of the two fluorescent markers. We routinely CCCP to uncouple mitochondria as a positive control for oxygen consumption. Given the non-significant trend to lower uncoupled respiration in the RedMit/LC3-GFP MEFs, we have not excluded a subtle defect.

MEFs were treated with CCCP (carbonyl cyanide m-chlorophenyl hydrazone) to induce mitophagy or with chloroquine to block the final step of the autophagy pathway and analyzed using our IN Cell system (**Figure 2B**). We showed that an induction of mitophagy, or a block in the late stages of mitophagy both result in an increase in the mitophagy signal detected (colocalization of mitochondrial fragments with autophagosomes, **Figure 2**, $p < 0.05$), consistent with previous results (Diot et al., 2015).

As with other cell types, MEFs responded to growth in glucose-free galactose media and starvation media by an induction of mitophagy and mitochondrial fragmentation (Malik et al., 2017). Because the basal level of mitophagy in glucose-free, galactose-based media, is frequently higher than in media containing glucose (but NS in **Figure 2B**) we run our assay in both regular and galactose-based media. Using chloroquine to inhibit lysosomal acidification (25 μM for 16 h) thus blocking the late steps of the auto/mitophagy pathway, we induced a similar accumulation of mitochondria colocalising with autophagosomes in galactose and glucose- containing media (**Figure 2C**). Data were normalized by log transformation prior to ANOVA. Chloroquine significantly increased colocalization of mitochondrial fragments with autophagosomes expressed as a proportion of mitochondrial area ($p < 0.01$, effect of galactose NS).

Finally, we used confocal microscopy to confirm and illustrate our observations with better quality pictures than the ones produced with the high throughput imaging system. **Figure 3** illustrates autophagosomes engulfing mitochondria in cells grown in glucose-free galactose media. These cells can be monitored in real time using time lapse imaging, as we did not observe any significant bleaching of the fluorescent signals when the cells were imaged every 30 s for 8 h, enabling tracking of mitophagy in living cells (**Video S1**).

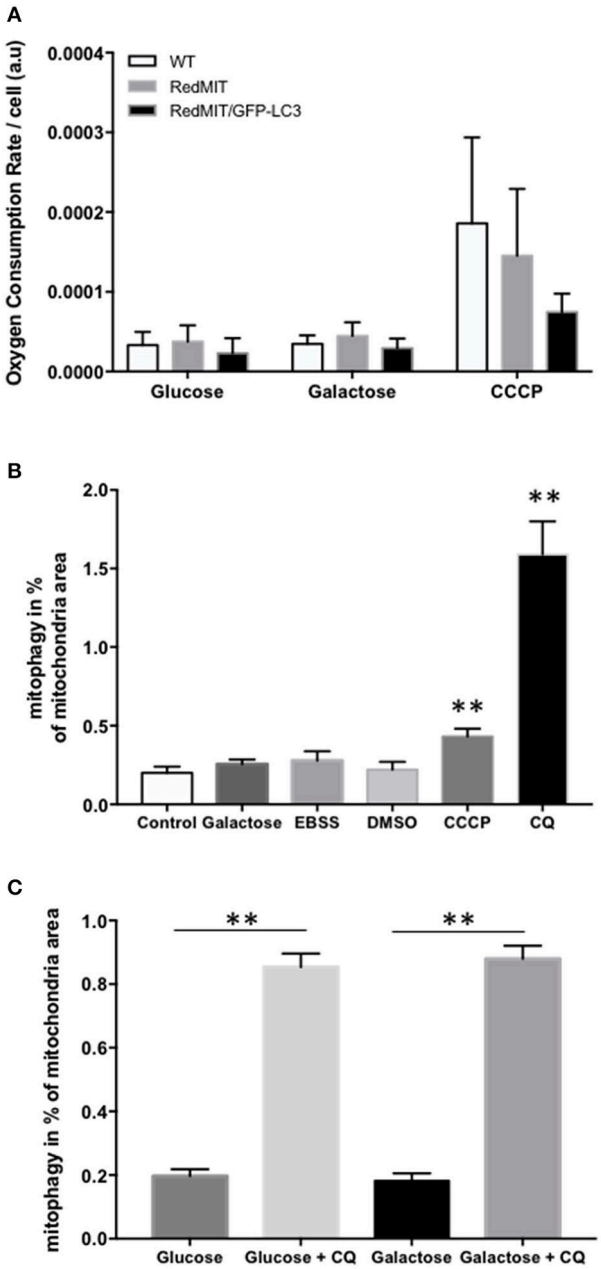

FIGURE 2 | Validation of the RedMIT-GFP-LC3 Mouse model for detecting mitophagy (see Diot et al., 2015 for detailed validation of high throughput imaging to quantify mitophagy). **(A)** MEFs from WT, RedMIT and RedMIT-GFP-LC3 mice were used to clarify whether expressing mRFP in mitochondria impaired their function. No significant differences in oxygen consumption were observed either in glycolytic (Glucose), oxidative (Galactose), or uncoupled (CCCP) conditions [mean + SD, $n = 2$, t-test $p =$ 0.625–0.684–0.289 for wt vs. RedMIT-GFP-LC3/0.832–0.559–0.714 for wt vs. RedMIT/0.532–0.409–0.369 for RedMIT vs. RedMIT-GFP-LC3 (glucose—galactose—CCCP, respectively)]. **(B)** Using our IN Cell 1000 high-throughput microscopy assay (Diot et al., 2015) we validated detection of mitophagy in RedMIT-GFP-LC3 MEFs grown for 2 h in glucose media (control), glucose-free galactose-based media (galactose), starvation media (EBSS), media containing vehicle (DMSO), glucose media supplemented with 10 μM CCCP (CCCP), and glucose media supplemented with 25 μM chloroquine (CQ). After fixation, an increase colocalization between mitochondria and LC3 was observed when using an inhibitor (chloroquine, CQ) of mitophagy, and when using CCCP compared to DMSO control (mean + SEM, at least 500 cells counted per condition, $^{**}p < 0.01$, t-test). **(C)** Using IN Cell 1000, chloroquine significantly increased colocalization of mitochondrial fragments with autophagosomes expressed as a proportion of mitochondrial area in both regular (glucose) and glucose-free galactose based media ($^{**}p <$ 0.01, t-test, effect of medium NS, error bars are SE at least 500 cells counted).

(Continued)

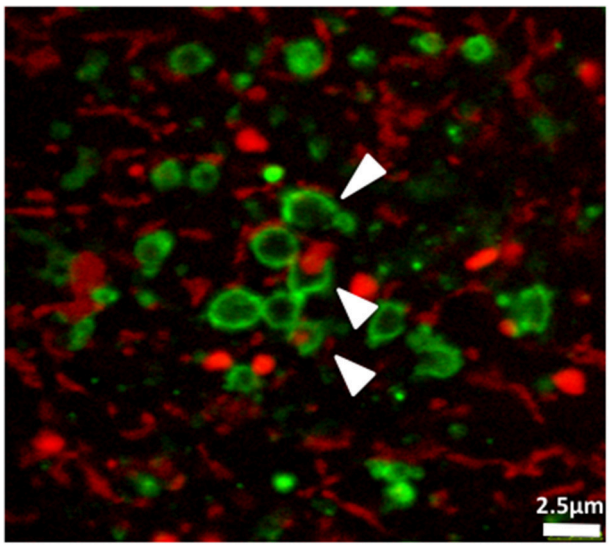

FIGURE 3 | The early stages of mitophagy can readily be demonstrated in RedMIT-GFP-LC3 MEFs. RedMIT-GFP-LC3 MEFs were cultured in glucose-free galactose media (galactose). After fixation, the cells were imaged using a Leica SP5 confocal microscope with a 63X lens and further digital zoom when needed. Autophagosomes forming around a mitochondrion are observed under these "energetic stress" growth conditions. The white arrowheads show different phases of autophagosome formation (green) around mitochondrial cargo (red). Similar observations in whole cells images have been made in Liao et al. (2017).

Together these results confirm that MEFs from the RedMIT-GFP-LC3 mouse are a useful model for the *in vitro* assessment of mitochondrial dynamics.

The RedMIT-GFP-LC3-OPA1^{Q285STOP} Mouse

We previously showed that mitophagy is increased in fibroblasts from patients with bi-allelic OPA1 mutations (compound heterozygotes Liao et al., 2017). In order to understand the mechanisms underlying this mitophagy dysregulation we crossed our RedMIT-GFP-LC3 mouse with a model of the DOA disease, the OPA1^{Q285STOP} mouse. It is already known that autophagosomes are increased in RGCs in the OPA1^{Q285STOP} mouse (White et al., 2009) and electron microscopy suggests increased mitophagy (Sarzi et al., 2012). But to our knowledge, it is not yet clear whether mitophagic flux is increased. We previously observed increased mitophagy in fibroblasts from ADOA patients in whom we had demonstrated

OPA1 deficiency, and therefore wished to explore whether mitophagy was increased in the OPA1^{Q285STOP} mouse model, potentially explaining the increased autophagosomes in RGCs. We generated RedMIT-GFP-LC3-OPA1^{Q285STOP} MEFs, to investigate the effects of OPA1 knock down on autophagy and mitophagy (**Figure 4**). We confirmed that the abundance of OPA1 in the resulting MEFs (**Figure S2**) was lower than in OPA1 wild type MEFs, as expected (Davies et al., 2007). Increased counts of colocalized mitochondria with autophagosomes in the OPA1 ± MEFs (**Figure 4A**) suggested increased mitophagy. Because this increased colocalization could be due to either activated mitophagy or slowed turnover we assessed mitophagic flux by adding lysosomal inhibitors. We thus blocked the late steps of the autophagy pathway using E64d and pepstatin A over a time-course of 24 h (**Figure 4B**).

The accumulation of autophagosomes (expressed as the summed area of LC3-ll positive punctae per cell) is significantly greater in the OPA1 mutant compared to the OPA1 wt ($n = 3$) as shown in **Figures 4A,B**. This increase was significant ($p < 0.01$, Data were normalized by log transformation prior to ANOVA). As the area of autophagy detected is similar in both the OPA1 wild type and mutant at baseline, but is greater in the OPA1 mutant following inhibitors, this indicates that in steady state conditions, the flux of autophagy is increased in the OPA1 mutant cells compared to the wild type. Similarly, the lysosomal inhibitors significantly increased colocalization of mitochondrial fragments with autophagosomes expressed as a proportion of mitochondrial area (**Figure 4C**) in the OPA1 mutant cells ($p < 0.01$, effect of galactose NS). This result is consistent with our previous results in fibroblasts from patients with bi-allelic OPA1 mutations of OPA1 (Liao et al., 2017).

Together these results show that the OPA1^{Q285STOP} mutation dysregulates both autophagy and mitophagy. Hence this mouse model is useful for studying the underlying mechanisms of the dysregulated mitophagy induced by OPA1 dysfunction in isolated cells.

Investigation of Fixed Mouse Splenocytes

In order to assess these effects in whole organisms we investigated mouse splenocytes, since large numbers of cells can readily be harvested. This time we used Imagestream, another high content imaging system, in which FACS is coupled to a fluorescence microscope, to investigate colocalization of mitochondria and autophagosomes. We had previously validated this method for detecting mitophagy in human fibroblasts (Liao et al., 2017). Briefly, the sorted and individualized splenocytes in the flow cytometer are imaged with an integrated fluorescence microscope. The pictures are then analyzed using masks and the colocalization between mitochondria and autophagosomes assessed. Again, we demonstrated a significantly higher level of colocalization in OPA1 mutants compared to OPA1 wild type ($p < 0.01$, **Figure 5**). This result confirms the induction of mitophagy by the OPA1^{Q285STOP} mutation in the mouse. We therefore investigated the organ mainly affected by dysregulated mitophagy, i.e., the retina.

Mouse Retina Investigation

As DOA affects the RGCs we investigated autophagy and mitophagy in the retina. For this, four samples from the RedMIT-GFP-LC3-OPA1^{Q285STOP} mice and the RedMIT-GFP-LC3 control mice were harvested after fixation by perfusion. Cryostat eye sections were cut at 10 μm and examined using a Zeiss LSM 700 inverted confocal microscope with a plan-Apo 63x NA 1.4 oil-immersion objective. The GFP, which was expected to be targeted to autophagosomes, was homogenously and non-specifically expressed throughout the retinal tissue, as was the mRFP fluorescent mitochondrial signal (**Figure 6**). Furthermore, the expression levels of fluorescent proteins were weak. The results from each slide were very similar; it was not possible to conclude that colocalization between the GFP and mRFP occurred, as the image signal-to-background ratios precluded the detection of colocalization with a Pearson correlation coefficient values being either very weakly negative, or very weakly positive. Indeed, the maximum Pearson product moment correlation coefficient values recorded across all four slides were +0.22 and −0.16. We did not detect consistent differences in other tissues examined (**Figure S3**). In summary, the high background and the weak red transgene expression precluded any significant colocalization analysis.

DISCUSSION

Mouse Model Demonstrates Increased Mitophagy in Primary Cell Cultures

We have developed a mouse with genetically encoded fluorescent proteins mRFP and GFP directed to mitochondria and autophagosomes respectively (**Figure 1**). We have shown that colocalization of these tags can be used as a readout for mitophagy in live cells (**Figure 2**), using two different high throughput imaging systems (**Figures 4**, **5**). In live cells from the well characterized mouse model of ADOA (OPA1 ^{Q285STOP}) (Davies et al., 2007) which develops an adult-onset dendropathy and impaired vision, we have shown that baseline mitophagy is increased in splenocytes (**Figure 5**) and mitophagic flux is increased in MEFs (**Figure 4**). Colocalization of mitochondria with autophagosomes was investigated in retina using confocal microscopy (10 μm cryostat sections).

These data validate both our model and our high throughput imaging method for quantifying mitophagy. They are consistent with our suggestion that OPA1 knock down causes an excessive mitochondrial fragmentation, and that this activates mitophagy (Liao et al., 2017).

This Model Visualizes an Earlier Stage in Mitophagy Than Do Other Mouse Models

There are considerable technical difficulties with visualizing mitophagy, because it is transient and occurs at low frequency. We set up the high throughput imaging system for quantifying mitophagy in cells (Diot et al., 2015) that we have used in this study. In order to study the mechanisms underlying pathogenesis further, with a view to testing therapeutic strategies *in vivo*, we decided to develop a mouse model allowing us *in vivo*

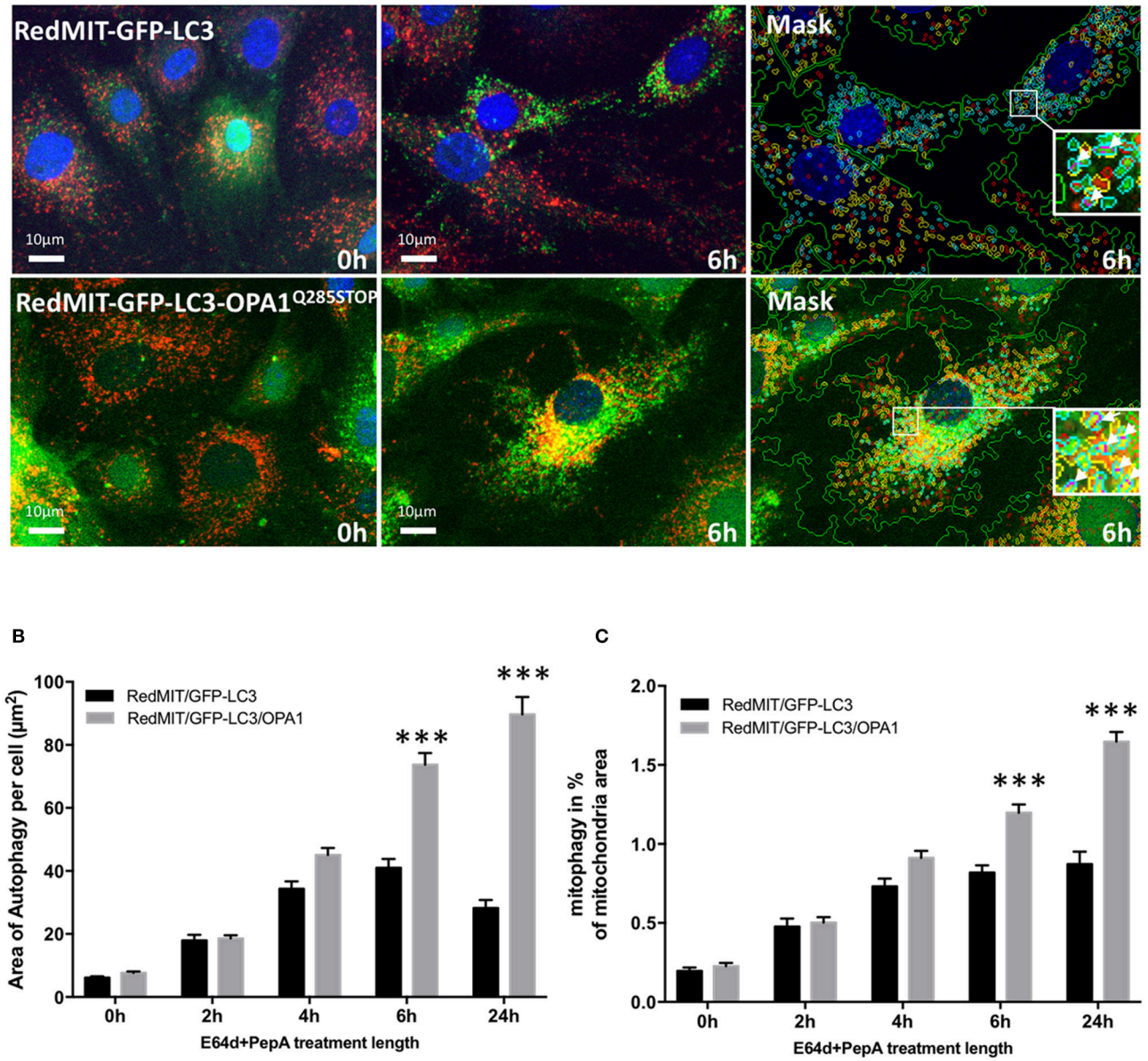

FIGURE 4 | The OPA1^{Q285STOP} mutation induces mitophagy in MEFs. **(A)** Representative images of the IN Cell 1000 high content imaging acquisition system. The cells from both RedMIT-GFP-LC3 and RedMIT-GFP-LC3-OPA1^{Q285STOP} were grown in glucose media (0 h) supplemented with E64d/Pepstatin A for 6 h before fixation. The images are analyzed using a homemade protocol developed using the IN Cell developer toolbox (Diot et al., 2015) resulting in a "mask" picture (cyan: autophagosomes; red: "short" mitochondria; yellow: "long" mitochondria; purple: "colocalization" between autophagosome and mitochondria signals). The white arrows in the insets indicate the colocalization events between mitochondrial and autophagosome signal. **(B)** Lysosomal inhibitors E64d/Pepstatin A were added to cells growing in glucose media to block the processing of autophagolysosomes. As shown on the graph a greater accumulation of autophagosomes is observed in MEFs from the RedMITGFP-LC3-OPA1^{Q285TOP} mouse compared to the RedMIT-GFP-LC3-OPA1$^{+/+}$ mouse, indicating a greater flux of autophagy when the OPA1 mutation is present. (at least 500 cells counted, regression $p < 0.05$). **(C)** In similar conditions, a greater flux of mitophagy (mitochondrial fragments colocalizing with autophagosomes) is observed with the OPA1 mutation: the rate of accumulation of mitophagosomes is 0.73 ± 0.21 (SEM) for wild type and 1.96 ± 0.22 for OPA1 (at least 500 cells counted, regression $p < 0.02$). ***$p < 0.001$ at specified time points.

imaging of mitophagy. All three models employed to date use genetic manipulation to express fluorophores (Sun et al., 2015; McWilliams et al., 2016). While two of these can be used both in live and fixed cells, the mt-Keima is not compatible with fixation. The RedMIT-GFP-LC3 mouse model visualizes mitochondrial fragments engulfed by autophagosomes, at an earlier time point in mitophagy than the other mouse models that use mt-Keima and mCherry, which demonstrate fusion with lysosomes. Our model thus complements the previously published models (Sun et al., 2015; McWilliams et al., 2016), which are steady state

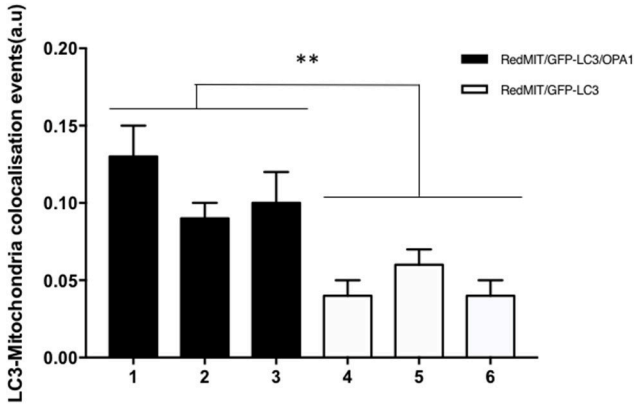

FIGURE 5 | Baseline mitophagy is increased by the OPA1^{Q285STOP} mutation in mouse splenocytes. Fixed splenocytes (1,000–5,000 per mouse) from three RedMITGFP-LC3-OPA1^{Q285TOP} mice (designated 1–3) and three RedMIT-GFP-LC3 mice (designated 4–6) mice were prepared and analyzed using the Imagestream (Amnis) system, error bars are SE of technical replicates. In line with the results observed in MEFs, colocalization between mitochondria and LC3 is increased in the OPA1 mutant mice. (*t*-test, $^{**}p < 0.01$).

and endpoint assays, respectively. Both the mt-Keima and the mCherry constructs are functionally inert, but our GFP tag is linked to expression of LC3, an important part of the mitophagy process. Furthermore, our study is limited by our use of the EF1α promoter which drives expression of mRFP. While this promoter is suitable for studies of developing embryos, being ubiquitous in most cell types (Chambers et al., 1998), it is less suitable for studies of post-mitotic tissues such as muscle. Hence the mitochondria of post-mitotic tissues were poorly visualized using mRFP alone, after post-natal day 40.

While we are able to demonstrate increased mitophagic flux in primary cultures, it is not easy to demonstrate this in whole animals using any of the existing models (Sun et al., 2015; McWilliams et al., 2016).

Microscopic Examination of Tissue Sections Added No Support to Data From Primary Cultures

Despite excellent technical input, we were unable to visualize the colocalization of mitochondria and autophagosomes, that was apparent in MEFs and splenocytes, in cryostat sections of retina or other tissues. We consider three possible explanations for this. Firstly, our model is not sufficiently sensitive. On fusion with lysosomes, GFP is bleached by a drop in pH. Colocalization of the GFP and mRFP signals is therefore short lived. This appears less problematic in cultured cells, where levels of mitophagic flux can be increased by activators, than it is in fixed tissue sections, where colocalization events are less frequent and the signal-to-background ratio sub-optimal. Secondly there are many different subtypes of mitophagy (Lemasters, 2014) and we do not yet know whether the read-out of our high throughput imaging method, that increases with OPA1 knockdown (Liao et al., 2017) is BNIP3 dependent. Thirdly, it is possible that mitophagy is not actually increased in RGCs in OPA1 knock down. Indeed, Belenguer showed that BNIP3 dependent mitophagy is actually decreased in a neuronal model of OPA1 knock-down (Moulis et al., 2017). Given that we previously showed that mitophagy declines with maturity in fibroblasts (Diot et al., 2015), we suggest that their neuronal cultures may not reflect *in vivo* mature RGCs.

The Importance of Activated Mitophagy in ADOA

Up until now, much of the evidence for increased mitophagy in ADOA has been indirect. Electron microscopy of RGCs is consistent with increased autophagy (White et al., 2009). OPA1 knock down in cultured primary cortical neurons impairs maturation, resulting in reduced mtDNA, and abundance of cytochrome oxidase (Bertholet et al., 2013), consistent with, but not attributed to, activated mitophagy. Using the same system other authors have suggested that BNIP3-dependent mitophagy may be decreased rather than increased in OPA1 knock down (Moulis et al., 2017), but these authors did not measure mitophagic flux. We postulate that the increase in mitophagy that we have demonstrated may be different from the BNIP3-dependent type studied by Belenguer (Moulis et al., 2017) as we have never detected accumulation of Parkin. The latter appears to require profound depolarization to a level that does not occur *in vivo*. Furthermore, it has become increasingly clear that there are more than one, and potentially several different types of mitophagy. Some of these are dependent on Eid et al. (2016b), and others are independent of, PINK1/Parkin (Lemasters, 2014). Indeed, recent data show that PINK1/Parkin knockdown do not diminish basal mitophagy, even in dopaminergic cells, in ether mice (McWilliams et al., 2018) or flies (Lee et al., 2018). Hence their role may be confined to specific stresses (Eid et al., 2016b) and/or differentiation (Sarraf and Youle, 2018).

The severe phenotypes caused by mutations in genes regulating mitochondrial dynamics highlight the importance of mitochondrial fusion and fission in maintaining cellular, particularly neuronal, health (Schwarz, 2013; O'Mealey et al., 2017). Many disorders of mitochondrial dynamics involve neurodegeneration, including central (Ryan et al., 2015; Dombi et al., 2016; Haack et al., 2016) and peripheral nervous system (Züchner et al., 2006; Liao et al., 2017) as well as severe malformation syndromes (Cullup et al., 2013). While mechanisms of cellular aging are clearly important (Diot et al., 2016; Lang et al., 2017), the precise cause of the neurodegeneration is rarely clear.

While increased mitophagy may exert effects on local energy supply within the cell, it is increasingly apparent that significant mitochondrial stresses can be signaled to other parts of the cell. For instance, the UPRmt is a stress response pathway acting as a "checkpoint" for mitochondrial fitness that signals the nucleus (Callegari and Dennerlein, 2018). Acute and chronic mitochondrial respiratory chain deficiency differentially regulate lysosomal biogenesis (Raimundo et al., 2016). Signaling of the acute response requires both TFEB and AMPK. Given that OXPHOS deficiency also results in AMPK-dependent mitochondrial fragmentation (Toyama et al., 2016), mitochondrial dynamics could contribute to the lysosomal response. In the case of OPA1 (Sarzi et al., 2018), mitochondrial

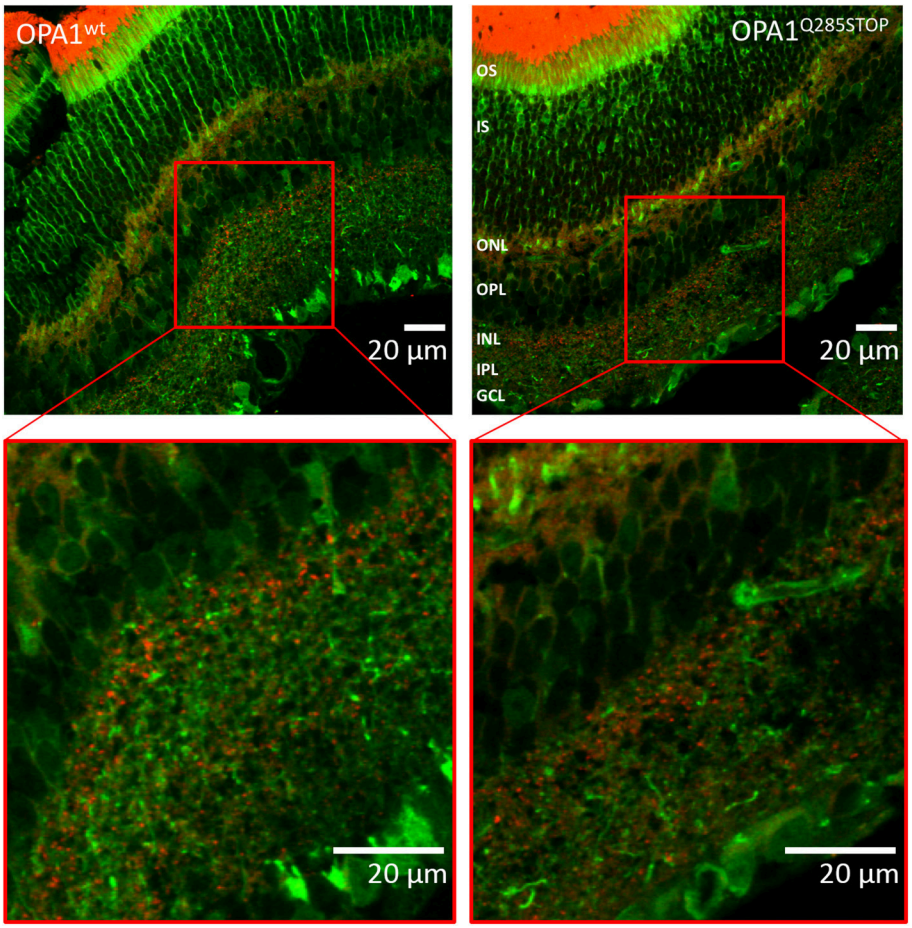

FIGURE 6 | Confocal images of transgenic OPA1wt and OPA1 Q285STOP mouse retina sections expressing LC3-GFP and RedMIT Representative images of the retina from the RedMIT-GFP-LC3-OPA1$^{+/+}$ **(Left)** and the RedMIT-GFP-LC3-OPA1^{Q285STOP} **(Right)** mice sections show autophagosomes with GFP-tagged LC3 and RedMIT tagged mitochondria. Sections were cut at 10 µm and visualized using a Zeiss LSM 700 inverted confocal microscope with a plan-Apo 63x NA 1.4 oil-immersion objective. Red boxes indicate the area magnified in each inset. RedMIT were observed from the ONL toward the IPL in both OPA1^{wt} *and* OPA1 ^{Q285STOP}. No difference in GFP-LC3 or mitochondrial mRFP is seen between OPA wild type and mutant (zoomed insets, bottom panels). In particular, no colocalization was observed between GFP-LC3 and RedMIT in both OPA1^{wt} *and* OPA1^{Q285STOP} (Pearson correlation coefficient of +0.22 and −0.16; $n = 4$). Scale bars: 20 µm OS, outer segments; IS, inner segments; ONL, outer nucleus layer; OPL, outer plexiform layer; INL, inner nucleus layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

fragmentation is apparent before loss of dendrites in RGCs while MMP is maintained (Williams et al., 2010). Hence delivery of mitochondria to the regions of the cell requiring energy may be as important as mitochondrial quality. OPA1 levels affect mitochondrial membrane cristae structure (Alavi et al., 2007) and potentially impact on apoptosis, though this in not apparent in fibroblasts (Liao et al., 2017). Others have suggested that OPA1 might be important for mtDNA maintenance (Elachouri et al., 2011) but it does not appear to be a component of the nucleoid. We documented mtDNA depletion in OPA1 knock down and suggest that this is caused by excessive mitochondrial fragmentation increasing mitophagy beyond the level that it is able to maintain mitochondrial quality. This may recapitulate the excessive mitophagy seen in fibroblasts treated with phenanthroline. This treatment depleted mtDNA copy number to 15% of baseline along with a halving in mitochondrial mass without a significant benefit to the quality of mtDNA (Diot et al., 2015). Activated mitophagy may thus become excessive in its demands on mitochondrial biogenesis, by potentially impairing the ability of the RGCs to generate sufficient mitochondria for dendritic growth and/or response to stress.

CONCLUSION

In conclusion, we developed the RedMIT-GFP-LC3 mouse model in which colocalization of fluorescent mitochondria and autophagosomes can be used as a readout to detect mitophagy. We used this model to confirm that mitophagy is increased in cell cultures of a mouse model of ADOA. Autophagy is critically important for optic nerve survival (Rodríguez-Muela et al., 2012) and increased mitophagy may generate cellular demands that are important in neurodegeneration. This model will thus be useful

for further studies of neurodegeneration caused by impaired mitochondrial dynamics.

AUTHOR CONTRIBUTIONS

AD, TA, JS, CL, JC, RN, RG, YG, CW, SS, MD, ED, SW, TE and MV performed the experiments. JP, KM, and AD designed the experiments. JP, JS, TL, FI, KM, and AD analyzed the results. JP, AD, JS, KM, and MD wrote this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2018.00103/full#supplementary-material

Figure S1 | Mapping of the mRFP transgene. The insertion of the mRFP transgene was investigated by paired-end sequencing. The insert is located in the 3rd exon of the *pkn1* gene, which has been confirmed by PCR using a common forward primer (green) and two different reverse primers; one (red) 1,500 bp from the forward primer in the wild type genome (blue) and one in the mRFP insert, approximately 800 bp from the forward primer. The 1,500 bp PCR only works in the wild type genome and the 800 bp only when the insert is present.

Figure S2 | Analysis of OPA1 expression in the MEFs used. Protein extracts were prepared from wt, RedMIT-GFP-LC3 and RedMIT-GFP-LC3-OPA1^{Q285STOP} MEFs and analyzed on a 8% acrylamide gel. The OPA1 signal was detected using a rabbit anti-OPA1 antibody (abcam ab42364) and revealed using a polyclonal goat anti-rabbit secondary antibody (Dako P0448) coupled to an ECL detection kit (WESTAR Supernova HRP Detection Substrate, Geneflow K1-0068) according to the manufacturer's instructions and imaged with G:BOX (Syngene).

Figure S3 | Images from sections of brain and spleen from control, RedMIT/GFP-LC3, and OPA1^{Q285STOP}/RedMIT/GFP-LC3. Brain **(Bottom)** and spleen **(Top)** from non-fluorescent (control), RedMIT/GFP-LC3 and OPA1^{Q285STOP}/RedMIT/GFP-LC3 mice were sectioned (10 μm) and imaged on a Zeiss LSM 700 inverted confocal microscope with a plan-Apo 63x NA 1.4 oil-immersion objective. The fluorescent signals, GFP-LC3 and mitochondrial mRFP, are visible but no clear colocalization between them could be visualized in the views shown. High throughput imaging identified infrequent colocalization that was more marked in OPA1^{Q285STOP}/RedMIT/GFP-LC3 mice than RedMIT/GFP-LC3 mice (**Figure 5**). Scale bars = 10 μm.

Table S1 | mRFP and GFP transgenes do not affect mice reproductive success.

Video S1 | Movie of the RedMIT-GFP-LC3 MEFs. Live RedMIT-GFP-LC3 MEFs have been imaged using a custom Olympus IX81 inverted microscope equipped with temperature control (Solent scientific) every 30 s for 8 h. This can be used to quantify the early stages of mitophagy in real time.

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REVIEW
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Sensing the Stress: A Role for the UPR^{mt} and UPR^{am} in the Quality Control of Mitochondria

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Mitochondria exist as compartmentalized units, surrounded by a selectively permeable double membrane. Within is contained the mitochondrial genome and protein synthesis machinery, required for the synthesis of OXPHOS components and ultimately, ATP production. Despite their physical barrier, mitochondria are tightly integrated into the cellular environment. A constant flow of information must be maintained to and from the mitochondria and the nucleus, to ensure mitochondria are amenable to cell metabolic requirements and also to feedback on their functional state. This review highlights the pathways by which mitochondrial stress is signaled to the nucleus, with a particular focus on the mitochondrial unfolded protein response (UPR^{mt}) and the unfolded protein response activated by the mistargeting of proteins (UPR^{am}). Although these pathways were originally discovered to alleviate proteotoxic stress from the accumulation of mitochondrial-targeted proteins that are misfolded or unimported, we review recent findings indicating that the UPR^{mt} can also sense defects in mitochondrial translation. We further discuss the regulation of OXPHOS assembly and speculate on a possible role for mitochondrial stress pathways in sensing OXPHOS biogenesis.

Keywords: mitochondria, UPR signaling pathways, mitochondrial translation, mitochondrial signaling, cytochrome c oxidase

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INTRODUCTION

The eukaryotic cell is composed of different organelles, each fulfilling a variety of specific functions. In the last decades, organelles, such as the nucleus, the ER and mitochondria, have been extensively investigated as separate units. Nevertheless, emerging evidence describes how organelles are connected and how they communicate with each other. Mitochondria, which are the main cellular energy producers in the form of ATP, have attracted a particular focus. However, mitochondria are also involved in many other essential cellular processes such as Ca^{2+} homeostasis, Fe-S cluster biogenesis or the synthesis of critical metabolites, such as NADH/NAD^+ and succinate/α-ketoglutarate (McBride et al., 2006). Hence, mitochondria are now perceived as key cellular signaling organelles. In fact, they physically interact with the majority of membrane bound organelles within the cell (Eisenberg-Bord et al., 2016; Valm et al., 2017). The most well studied of these contact sites is between the mitochondria and the ER (Kornmann et al., 2009; Elbaz-Alon et al., 2015; Lewis et al., 2016; Cohen et al., 2017), but mitochondria are also in constant exchange with other organelles such as lysosomes (Raimundo, 2014; Diogo et al., 2017), lipid droplets (Nguyen et al., 2017), and peroxisomes (Sugiura et al., 2017). Consequently, many signaling pathways triggered by mitochondria during physiological or pathological situations have

been identified, which impact fundamental cellular processes such as autophagy, cell division, cell differentiation, or anti-viral signaling (Liu and Butow, 2006; Koshiba, 2013; Xu et al., 2013). Hence, many initiated stresses can provoke the activation of mitochondrial stress responses and many fundamental aspects regarding the molecular function of involved factors remain unclear. Specifically, it remains unclear how mitochondrial signals are transported and how and where those signals originate. We review recent findings on the mitochondrial initiated stress response pathways of the UPR^{mt} (the mitochondrial unfolded protein response) and UPR^{am} (the unfolded protein response activated by mistargeting of proteins) within the context of mitochondrial translation and impaired OXPHOS assembly. Therefore, first we will introduce the UPR^{mt} and UPR^{am} pathways. In the following sections we will discuss these pathways with a specific focus on how they could originate by reduced mitochondrial translation and disturbed OXPHOS biogenesis, taking cytochrome *c* oxidase as an example.

DEALING WITH PROTEOTOXIC STRESS: THE UPR^{MT} AND UPR^{AM}

The human mitochondrial genome contains more than 1300 proteins (Calvo et al., 2016). While only 13 proteins are encoded within mitochondria, the majority (>99%) are nuclear-encoded, synthesized in the cytosol and are imported into the organelle. The transport of these precursor proteins is facilitated by various import machineries, excellently summarized by two recent review articles of Wasilewski et al. (2017) and Wiedemann and Pfanner (2017). As a consequence, mitochondria receive a constant influx of proteins that need to be matured and assembled into functional complexes. To functionally integrate mitochondria into the cellular network, signaling pathways are required that monitor mitochondrial fitness and enable a coordination of mitochondrial function with cellular demands. A major signaling route occurs between mitochondria and the nucleus (Wasilewski et al., 2017; Melber and Haynes, 2018). This is particularly important since the accumulation of unassembled precursor proteins inside or outside mitochondria leads to proteotoxic stress and eventually to cell death (Ryan and Hoogenraad, 2007; Topf et al., 2016; Wasilewski et al., 2017). Two major signaling pathways have been identified that monitor the precise and timely delivery of cytosolic precursors to the mitochondria; the mitochondrial unfolded protein response (UPR^{mt}) and the unfolded protein response activated by mistargeting of proteins (UPR^{am}).

The Proteotoxic Stress Induced Cascade Originating Within Mitochondria—UPR^{mt}

The accumulation of misfolded or damaged proteins within the mitochondria can incite a range of proteotoxic stresses. For example, excess OXPHOS complex constituents leads to the generation of harmful sub-complexes, resulting in loss of membrane potential, or oxidative stress in the form of ROS production (Fernández-Vizarra et al., 2009; Fox, 2012; Soto et al., 2012; Timón-Gómez et al., 2017). One considerable possibility for the accumulation of non-assembled OXPHOS subunits could be defects in mitochondrial translation (see section Proteotoxic signaling cascades can be activated by defects in mitochondrial translation). One of the first lines of defense against such mitochondrial perturbations is the activation of the UPR^{mt} pathway, which is, besides defects in mitochondrial translation or OXPHOS biogenesis, responding to various mitochondrial stresses. The UPR^{mt} represents a conserved pathway between nematodes, flies, and mammals (Ryan and Hoogenraad, 2007; Quirós et al., 2016; Topf et al., 2016; **Figure 1A**). It is assumed that in nematodes, damaged or unassembled proteins are degraded by the AAA$^+$-matrix protease CLPP-1, which would lead to the accumulation of peptides within the mitochondrial matrix (Haynes et al., 2007). However, if CLPP-1 exclusively degrades damaged or unassembled proteins is not clear, but all CLPP-1 generated peptides are derived from mitochondrial proteins. The transport of these peptides to the cytosol by the ABC transporter HAF-1 initiates signaling cascades outside mitochondria (Haynes et al., 2010). The stress activated bZIP transcription factor ATFS-1 seems to play a key role during these processes. In standard physiological conditions, ATFS-1 is localized to the mitochondrial matrix, where it is constitutively degraded by the AAA$^+$-protease LON (Nargund et al., 2012). However, upon loss of import efficiency, such as during UPR^{mt} activation, ATFS-1 accumulates in the cytosol and, as it has a nuclear localization signal, it is relocalized to the nucleus where it acts as a transcriptional regulator (Nargund et al., 2012, 2015). ATFS-1 controls the expression of over 500 genes that impact several cellular processes (Nargund et al., 2012, 2015; Lin et al., 2016; Melber and Haynes, 2018). Among them are immune regulators [e.g., the antibacterial factor-related peptide 2 (Abf-2) (Nargund et al., 2012), metabolic enzymes [e.g., glutaminase (Nargund et al., 2015)] or additional transcription factors, such as the bZIP transcription factor skinhead-1 (Skn-1) (Nargund et al., 2012, 2015).

Additionally, multiple mitochondrial encoding genes, influencing the synthesis of proteotoxic stress related proteins (e.g., the 60 kDa heat shock protein HSP60 or the 70 kDa heat shock protein HSP70), as well as proteins of the oxidative phosphorylation machinery and TCA enzymes are targeted by ATFS-1 during mitochondrial stress (Nargund et al., 2015; Melber and Haynes, 2018). How ATFS-1 controls expression is not clear, however, studies in nematodes have shown that the homeobox transcription factor DVE-1, its cofactor the Ubiquitin-like protein UBL-5, the nuclear co-factor LIN-65, and the histone methyltransferase MET-2 are all involved (Tian et al., 2016a). These proteins are usually localized to the cytosol, but are recruited to the nucleus upon activation of the UPR^{mt} (Benedetti et al., 2006; Haynes et al., 2007; Tian et al., 2016a,b). LIN-65, together with MET-2, actively remodel chromatin structures, likely to enable binding of transcription factors (Merkwirth et al., 2016; Tian et al., 2016a,b). The transcription factors DVE-1 and ATFS-1 can then bind to the reorganized chromatin where, in a cascade of parallel pathways, they reprogram cell expression (Tian et al., 2016a).

Recently, the human homolog of ATFS-1, activating transcription factor 5 (ATF5), was identified (Fiorese et al.,

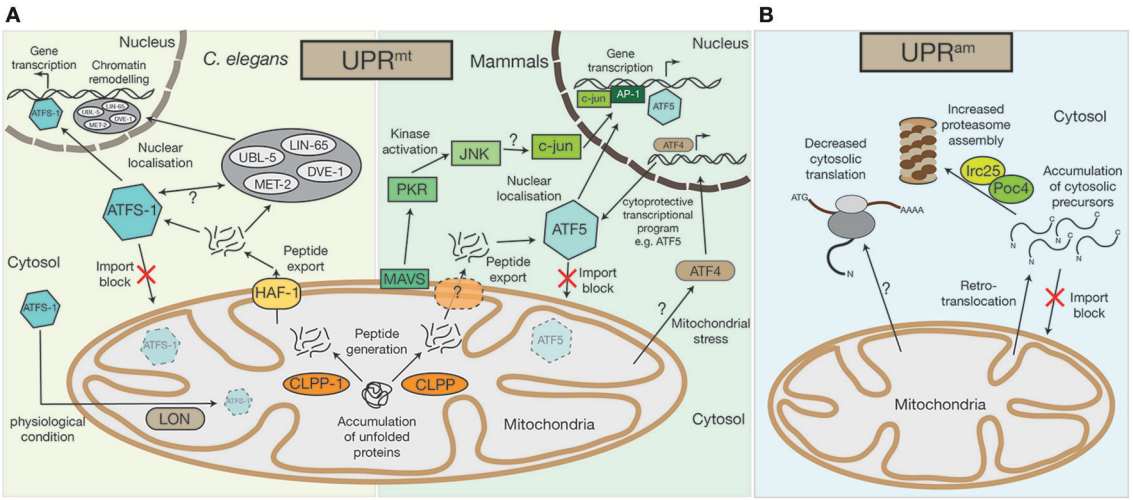

FIGURE 1 | Mitochondrial UPR^{mt} and UPR^{am} stress response pathways. **(A)** An accumulation of unfolded proteins inside the mitochondrial matrix triggers the UPR^{mt} in both mammals and in nematodes. Accumulated proteins are likely processed by the CLPP protease and exported out of the mitochondria, a process that in *C.elegans* requires the HAF-1 protein. While under physiological conditions ATFS-1 gets imported into mitochondria and constitutively degraded by the AAA^{+}-protease LON, exported peptides activate the transcription factor ATFS-1/ATF5 in the cytosol, which translocates to the nucleus to alter the cell's transcriptional program, particularly affecting the transcription of mitochondrial proteins. In *C.elegans* the UBL-5, LIN-65, and MET-2 proteins also translocate to the nucleus upon UPR^{mt} activation where they facilitate the binding of transcription factors, ATFS-1 and DVE-1, by chromatin remodeling. A second UPR^{mt} pathway has also been observed in mammals, whereby UPR^{mt} activation is sensed by the mitochondrial antiviral signaling protein MAVS by an unknown mechanism, which then triggers the activation of PKR, which in turn phosphorylates the c-Jun N-terminal kinase, JNK. JNK activates c-jun (also by an unknown mechanism), which translocates to the nucleus and, together with AP-1, alters gene transcription. The bZIP transcription factor AFT4 has also been linked to mitochondrial stress response pathways. Localized to the nucleus, ATF4 activates a complex cytoprotective transcriptional program, e.g., by transcriptional control of ATF5. **(B)** In lower eukaryotes, the accumulation of cytosolic precursors from either a block in mitochondrial import, from mislocalization, or from retrotranslocation out of the mitochondrial intermembrane space, can instigate the UPR^{am}. This accumulation enhances activity of the Irc25/Poc4 chaperone complex, which is required for assembly of the proteasome. Increased proteasome assembly causes the rapid degradation of accumulated proteins. In an independent stress response mechanism observed in mammals, there is a decrease in cytosolic translation, which is coupled to increased proteasome activity, but the molecular mechanisms underlying this response remain to be defined.

2016). Similarly to ATFS-1, ATF5 also localizes to the nucleus upon UPR^{mt} activation. ATF5 has been found to be increased in patients with mitochondrial disorders (Endo et al., 2009; Tyynismaa et al., 2010; Torres-Peraza et al., 2013; Yap et al., 2016) and has an anti-apoptotic function, since it increases the expression of the B-cell lymphoma protein (BCL-2), which antagonizes apoptosis (Persengiev, 2002; Fiorese et al., 2016). Recently, another bZIP transcription factor, the activating transcription factor 4 (ATF4), was also linked to the mitochondrial stress response (Martínez-Reyes et al., 2012; Quirós et al., 2017; Melber and Haynes, 2018). Although, the exact relationship between ATF5 and ATF4 remains unclear, ATF4 has been postulated as ATF5 transcription factor (Melber and Haynes, 2018). Interestingly, a recent publication by Quirós et al. (2017) implied that ATF4, in contrast to ATF5, does not trigger the UPR^{mt} directly, but instead elicits a cytoprotective transcriptional program, which is part of a more general cell stress response known as the integrated stress response (ISR).

However, UPR^{mt} activation between nematodes and mammals seems to be conserved to a large extent, e.g., the Clp protease, CLPX, also induces the UPR^{mt} in mammals (Al-Furoukh et al., 2015), but a second, kinase regulated, stress response mechanism seems to exist in human cells that has yet to be detected in nematodes. Within this pathway, the protein kinase R (PKR), which is likely activated by the outer mitochondrial membrane antiviral signaling protein MAVS, phosphorylates the c-Jun N-terminal kinase JNK2 (Rath et al., 2012; Jacobs and Coyne, 2013). Next, JNK2 activates c-Jun, a component of the transcription factor AP-1, which then initiates a nuclear transcriptional response (Horibe and Hoogenraad, 2007; Rath et al., 2012).

However, our understanding of the UPR^{mt} pathways in nematodes and mammals is far from complete. Until now, we do not understand how peptides that are transported by HAF-1 from the mitochondrial matrix to the cytosol activate the UPR^{mt}. Nor is it clear whether there are specific peptides that are required for UPR^{mt} activation, or what triggers the differential localization of ATFS-1/ATF5 from mitochondria to the nucleus. Furthermore, the mechanism that provokes the translocation of DVE-1, UBL-5, LIN-65, and MET-2 to the nucleus following initiation of the UPR^{mt} remains elusive. Similarly, for the human UPR^{mt} kinase activated system, the activation of the PKR kinase by MAVS remains enigmatic and requires further investigation.

Stress Induced Signaling During Protein Mislocalization—UPR^{am}

The second major signal initiation pathway during mitochondrial impairment has only been verified in lower eukaryotes, but

there are some indications that similar pathways exist in mammalian cells (Papa and Germain, 2011; Wrobel et al., 2015; Wasilewski et al., 2017). Dysfunction of the mitochondrial import machinery eventually leads to cell death. However, if the import of cytosolic synthesized precursor proteins is only mildly impaired, a cytosolic protective program is activated. This "UPR^{mt} activated by mistargeting of proteins" (UPR^{am}), involves the activation of the cytosolic proteasome (Wrobel et al., 2015) (**Figure 1B**). An increase of mitochondrial precursor proteins in the cytosol triggers the UPR^{am}, leading to increased proteasome assembly by the enhanced activity of the proteasome assembly factors Irc25 and Poc4, which degrades excess proteins (Wrobel et al., 2015). The UPR^{am} protective stress response pathway seems not to be specific for a defined subset of precursor proteins, but rather represents a general mitochondrial dysfunction monitoring mechanism. Interestingly, it has been suggested that the UPR^{am} can also be activated by peptides and proteins that back-slide from the mitochondrial intermembrane space to the cytosol (Bragoszewski et al., 2015; Wasilewski et al., 2017). This indicates that the UPR^{mt} and UPR^{am} are either activated simultaneously, or that the presence of former mitochondrial matrix localized peptides induces the UPR^{mt}, while retrotransported intermembrane space proteins activate the UPR ^{am}.

As mentioned, the UPR^{am} pathway has not been identified in higher eukaryotes, but an increase in proteasomal activity has been observed following proteotoxic stress (Papa and Germain, 2014). UPR^{mt} activation in invertebrates can decrease cytosolic translation, which has not been demonstrated in mammalian cells, yet. However, since cytosolic translation decreases upon mitochondrial dysfunction regardless of an accumulation of mitochondrial precursor proteins within the cytosol (Wang and Chen, 2015; Wrobel et al., 2015; Topf et al., 2016) it is tempting to speculate that besides the UPR ^{mt} or UPR^{am} other stress response pathways are present in mammalian cells that can influence cytosolic translation. This decrease in cytosolic translation is likely elicited by a reduction of cytosolic 80S ribosomes, due to the reduced export of the 60S subunits from the nucleus (in yeast mediated by the nucleolar GTP binding protein, Nog2) (Wasilewski et al., 2017).

In conclusion, we are only beginning to shed light into the complex mechanisms of mitochondrial stress response pathways and it will be a challenge for the next decade to explore in detail how these signaling pathways are connected to aging processes and human disorders.

PROTEOTOXIC SIGNALING CASCADES CAN BE ACTIVATED BY DEFECTS IN MITOCHONDRIAL TRANSLATION

The link between dysfunctional mitochondrial translation and the activation of the UPR^{mt} was first discovered in *C. elegans* and is nicely summarized by Suhm and Ott (2017). In 2003, an siRNA library screen, aiming to identify proteins that are affecting the lifespan of worms, was performed (Lee et al., 2003). Interestingly, some of the identified proteins (e.g., MRPS5) are involved in mitochondrial gene expression and depletion leads to reduced ATP levels and a disruption of the mitochondrial network. However, worms also exhibited increased resistance against stress inducing reagents, such as H_2O_2 (Lemieux et al., 2001; Lee et al., 2003). Later, experiments in mice and *C. elegans*, discovered that knockdown of the mitoribosomal subunit, MRPS5, leads to mitonuclear protein imbalance and UPR^{mt} activation (Houtkooper et al., 2013). Remarkably, the life span of these animals was increased (Houtkooper et al., 2013). The observed molecular phenotypes reflected an activation of the UPR^{mt}, one of the hallmarks being a reduction in the synthesis of respiratory chain subunits, attributed to the presence of the peptide-transporter HAF-1 (Haynes et al., 2010).

The same UPR^{mt} activation was observed by inhibiting mitochondrial translation with mitochondrial translation inhibitors, such as doxycycline or chloramphenicol (Houtkooper et al., 2013). Surprisingly, the inhibition of mitochondrial translation with different pharmacological agents does not always lead to the same consequences in nuclear gene expression. For example, upon treatment of human cells with actinonin, a peptide deformylase inhibitor that disrupts mitochondrial translation by stalling mitoribosomes and degrading mitoribosomal proteins, there was a decrease in mitochondrial mRNA and rRNA levels (Richter et al., 2013; **Figure 2**). This was not observed upon mitochondrial translation inhibition by other structurally diverse antibiotics, such as doxycycline or chloramphenicol (Battersby and Richter, 2013; Richter et al., 2013). The stalling of mitoribosomes by actinonin provokes an accumulation of mitochondrial translation products within the inner membrane, ultimately resulting in oxidative stress and finally, loss of membrane potential (Richter et al., 2013).

Treatment with these different classes of mitochondrial translation inhibitors suggests that actinonin activates an alternative pathway to the UPR^{mt}, due to two main observations. First, the inhibition of mitochondrial translation with chloramphenicol did not show proliferation defects and second, alterations in the gene expression profile of actinonin treated cells were not entirely comparable to those previously described during UPR^{mt} induction, one of the main differences being that mitochondrial ribosomal proteins were not upregulated (Battersby and Richter, 2013; Richter et al., 2013). Hence, it was suggested that actinonin treatment leads to the production of abnormal mitochondrial translation products, that cause inner membrane permeabiliation and mitochondrial fragmentation, thereby activating a retrograde signaling pathway independent of the UPR^{mt} (Battersby and Richter, 2013; Richter et al., 2013; Suomalainen and Battersby, 2017). The molecular nature of this retrograde signal is unknown. However, evidence exists that the processing of the OPA1 protein, which is involved in mitochondrial fusion, is required, since OPA1 processing was altered upon actinonin treatment (Richter et al., 2013).

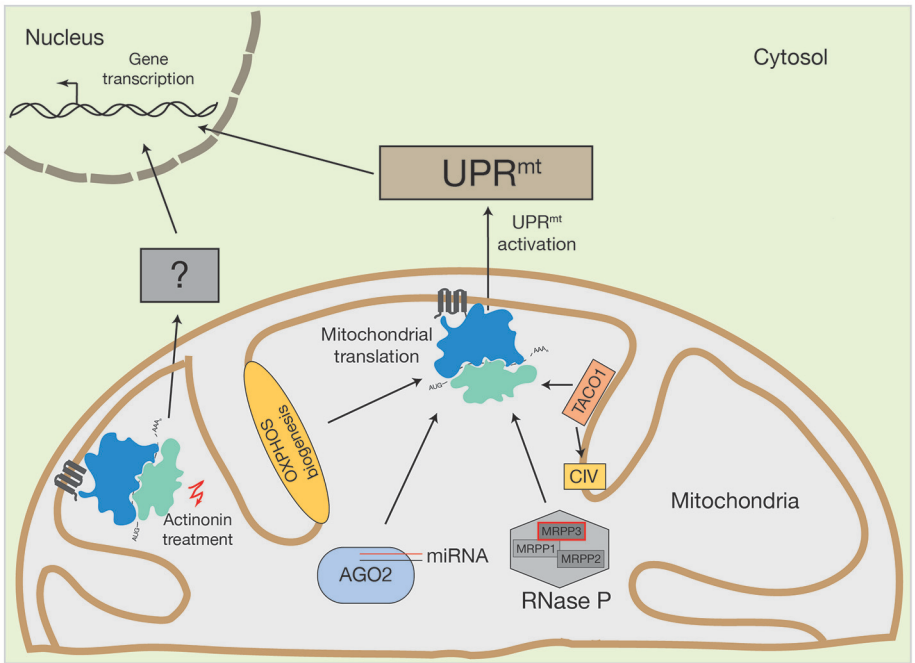

FIGURE 2 | Activation of human UPR^{mt} by altered mitochondrial translation. Conceivable options to initiate UPR^{mt} by mitochondrial translation could be OXPHOS biogenesis, mitochondrial miRNA molecules in association with AGO2, PNPase-mediated mRNA processing or translational regulators, such as TACO1 (specific for cytochrome *c* oxidase (CIV)). Inhibition of mitochondrial translation with different drugs also initiate the UPR^{mt}. However, actinonin treatment can also provoke an alternative stress response.

HOW DO DEFECTS IN MITOCHONDRIAL TRANSLATION INITIATE MITOCHONDRIAL STRESS RESPONSES?

Mammalian mitochondrial gene expression differs to that of the yeast system in various aspects (Meisinger et al., 2008; Richter-Dennerlein et al., 2015; Ott et al., 2016; Timón-Gómez et al., 2017). For example, yeast mitochondrial DNA (mtDNA) contains introns that are missing in higher eukaryotes (Foury et al., 1998). Especially the use of mouse models has significantly contributed to our understanding of mammalian mitochondrial gene expression (Trifunovic et al., 2004; Park et al., 2007; Metodiev et al., 2009; Cámara et al., 2011; Almajan et al., 2012; Gustafsson et al., 2016). Mammalian mtDNA has a size of ∼16 kb, contains no significant introns and two noncoding regions; the D-loop, which harbors the origin of heavy-strand (H-strand) replication and both strands transcription, and a second smaller 30 nt region in which resides the origin of replication of the light-strand (L-strand) (for a comprehensive overview see Gustafsson et al., 2016). Human mtDNA encodes for two rRNAs, 22 tRNAs, and 13 proteins. The latter are all essential components of the OXPHOS machinery. Gene expression is initiated by the generation of two polycistronic transcripts, originating from the light- or heavy-strand promoter respectively and further processed into individual RNA species (Ott et al., 2016). Mammalian mtRNAs do not contain significant 5′-UTRs. Although two mammalian translation initiation factors have been described [mtIF2 (Overman et al., 2003) and mtIF3 (Christian and Spremulli, 2009)] a translation regulation system involving 5′-UTR RNA-binding translational activators that are specific for each mRNA, such as it occurs in yeast, is largely missing (Meisinger et al., 2008; Richter-Dennerlein et al., 2015; Gustafsson et al., 2016; Ott et al., 2016; Timón-Gómez et al., 2017). To this end, it is not clear how mitochondrial translation is regulated at an mRNA-specific level in mammals. However, recent studies in higher eukaryotes are now providing an insight and evidence is emerging that mitochondrial stress response pathways are closely linked to mitochondrial translation regulation.

Initiation of Mitochondrial Signaling Through MicroRNAs

MicroRNAs (miRNA) are non-coding RNA molecules, usually 18–22 nucleotides in length, that are involved in post-transcriptional gene expression (Bartel, 2004; Bartel and Chen, 2004). These miRNAs are encoded in the nuclear genome, synthesized as pre-miRNAs and pre-matured within the nucleus, prior to their export into the cytosol (Bartel and Chen, 2004). Cytosolic miRNAs assemble into the DICER-complex, where they are further processed, and afterwards engage with the AGO2 protein to form the active mRNA-regulating RNA-induced silencing complex (RISC) (Bartel and Chen, 2004). The RISC complex can bind to its target mRNA and induce mRNA degradation and consequently the abrogation of protein products

(Bartel and Chen, 2004). For patients, miRNA applications are now considered as promising targeting strategies against human diseases, among them are cancer (Gabra and Salmena, 2017; Shirafkan et al., 2017), Parkinson's disease (Arshad et al., 2017) and cardiac perturbations (Chen et al., 2017). Cytosolic miRNAs have been linked to mitochondrial function, too. As reviewed by Zhao et al. (2017), cytosolic miRNAs modulate mitochondrial fission and fusion processes, they are involved in oxidative stress and they play major roles in mitochondrial initiated apoptotic pathways. Interestingly, miRNA molecules and the AGO2 protein have also been found inside mitochondria (Zhang et al., 2014) (**Figure 2**). Until now, the AGO2 protein is the only component of the cytosolic RISC complex that has been reported to enter mitochondria (Zhang et al., 2014; Jagannathan et al., 2015). Controversially, mitochondrial miRNAs seem to have an opposing function to that of their cytosolic counterparts. AGO2, together with miR1, increases CYTB, COX3, and ATP8 translation. AGO2 can also associate with miR-499-5p, whereby it can stimulate ND4L and ND1 translation. To date, ∼150 mitochondrial miRNAs have been described (Bandiera et al., 2013; Geiger and Dalgaard, 2017). However, it has been supposed that even more are targeted to mitochondria that have diverse, yet unknown, functions (Bandiera et al., 2013; Geiger and Dalgaard, 2017).

A conceivable function of miRNAs is their involvement in cellular signaling cascades and stress sensing situations. This hypothesis is supported by the fact that miR-1 is induced during the differentiation of myoblasts to myotubes (Zhang et al., 2014). Hence, mitochondrial miRNAs have the potential to directly influence mitochondrial translation, depending on the physiological state of the cell. An alternative hypothesis could be that mitochondrial miRNAs play crucial roles during mitochondrial stress via UPR^{mt} or UPR^{am} activation. Nevertheless, the exact mechanism of how mitochondrial miRNAs regulate mitochondrial gene expression is an ongoing field of research.

Mitochondrial RNA Processing Is Regulated by the UPR^{mt}

The human mitochondrial transcription process generates one short (containing two rRNAs) and two long mRNA and tRNA encoding polycistronic mRNA units that are further processed and modified to mature RNA molecules (Montoya et al., 1983). Human mitochondrial RNA maturation represents a multilayer system that involves several RNases and RNA modifying enzymes (Temperley et al., 2010; Rorbach and Minczuk, 2012; Bruni et al., 2017). However, a unique feature of the mammalian mRNA and tRNA encoding transcript is the distribution of the tRNA genes, which mostly flank the mRNA genes (Ojala et al., 1981). This situation requires an individual release of each tRNA from the polycistron, a process that is facilitated by ELAC2 (contains RNase Z activity and processes the 3′ ends of tRNA) and the RNase P complex (Rossmanith, 2012). The RNase P complex is composed of three subunits (MRPP1, MRPP2, and MRPP3) and matures tRNA molecules at the 5′ region. Mutations in ELAC2 cause an accumulation of mtRNA precursors and impaired mitochondrial translation and have been linked to hypertrophic cardiomyopathy (Haack et al., 2013). Interestingly, MRPP3 has been described as a target of the UPR^{mt} (Münch and Harper, 2016) (**Figure 2**). During UPR^{mt} activation, the stress dependent induction of the LON protease increases MRPP3 turnover, thereby reducing levels of MRPP3 (Münch and Harper, 2016). Consequently, mitochondrial RNA precursors accumulate, which concomitantly leads to impaired mitochondrial translation (Metodiev et al., 2016). Hence, it is tempting to speculate that mitochondrial mRNA processing, facilitated by the RNase P complex, is integrated into, or promotes, mitochondrial dependent stress response pathways.

Can Oxphos Assembly Defects Initiate a Mitochondrial Stress Response?

The mitochondrial OXPHOS machinery is composed of nuclear- and mitochondrial encoded proteins. Since the accumulation of OXPHOS sub-complexes within the inner mitochondrial membrane leads to increased ROS production and subsequent oxidative stress, the supply of subunits from both genetic systems needs to be balanced (Richter-Dennerlein et al., 2015; Dennerlein et al., 2017; Wasilewski et al., 2017). In yeast, mitochondrial protein synthesis is modulated by translational activators that mainly bind specific RNA molecules at defined positions (Mick et al., 2011; Soto et al., 2012; Herrmann et al., 2013; Kehrein et al., 2013; Dennerlein et al., 2017). In contrast, human mitochondria largely lack translational activators. One exception is TACO1, a translational regulator of the cytochrome *c* oxidase core subunit COX1 (**Figure 2**). TACO1 is a soluble protein that resides within the mitochondrial matrix. Its loss causes cytochrome *c* oxidase deficiency and has been implicated in Leigh syndrome (Weraarpachai et al., 2009). Interestingly, TACO1 interacts directly with COX1 mRNA and the mitochondrial ribosome (Richman et al., 2016). Consequently, mutations or loss of TACO1 lead to a reduction in COX1 synthesis (Weraarpachai et al., 2009). It is unknown which cellular stress responses are initiated under such conditions. However, since inhibition of mitochondrial translation can provoke UPR^{mt} activation (as described above), it is conceivable that functional loss of TACO1 could also activate UPR^{mt} signaling pathways.

Richter-Dennerlein et al. (2016) defined three COX1 translation ribosome-nascent chain complexes that contain C12ORF62 (COX14) and MITRAC12 (COA3). Mutations in either protein have been found in patients with cytochrome *c* oxidase deficiency that results from a reduction in COX1 translation (Mick et al., 2012; Szklarczyk et al., 2012; Weraarpachai et al., 2012; Ostergaard et al., 2015). Interestingly, siRNA mediated depletion of C12ORF62 caused a block in COX1 translation, which was released when COX4, the first nuclear-encoded structural cytochrome *c* oxidase subunit, associated with COX1 (Richter-Dennerlein et al., 2016). Hence, mitochondrial ribosomes are able to adapt mitochondrial translation according to the availability of nuclear-encoded subunits. Is this translational plasticity restricted to COX1 in human mitochondria? To date, we lack clear experimental

data to answer this question. However, ribosome profiling data revealed that all human mitochondrial mRNAs are present at defined hotspots during translation (Rooijers et al., 2013). Hence, it can be speculated that the translation and assembly of other mitochondrial-encoded proteins also depends on the supply of nuclear-encoded proteins from the cytosol.

As described above, a block during mitochondrial translation leads to the activation of stress response pathways within mitochondria, which can instigate the UPR^{mt} pathway. The induction of these stress response pathways can also be considered as "checkpoints" for mitochondrial fitness and functionality. Hence, the accumulation of partially translated COX1, stalled in intermediates associated with the mitochondrial ribosome, could potentially initiate the UPR^{mt}. This scenario would directly link mitochondrial OXPHOS assembly, to mitochondrial translation and UPR^{mt} activation, but this hypothesis requires further research.

CONCLUSIONS

The synchronization of mitochondrial translation and OXPHOS assembly with cell metabolic demands is vital for homeostasis. As studies continue to uncover the mechanisms of mitochondrial translation regulation in mammals it becomes increasingly apparent that there exists an important route of communication from mitochondria to the nucleus. Very little is known about these retrograde signaling pathways. The UPR^{mt}, originally identified as a pathway that recognizes internal mitochondrial imbalances, has now been implicated in mitochondrial translation defects. Although evidence is still sparse, it is plausible that due to the tight synchronization between the translation and assembly of OXPHOS components, the UPR^{mt} also senses defects in OXPHOS biogenesis.

These findings drive several key questions; what are the molecular cascades that link mitochondrial translation defects to the UPR^{mt}? Which alternative signaling pathways exist? What is the role of miRNAs in mitochondrial stress signaling? And how are defects in OXPHOS assembly signaled? A number of mitochondrial diseases, that result from either mutations in mtDNA, or in nuclear-encoded mitochondrial genes, cause OXPHOS defects (Suomalainen and Battersby, 2017). An elucidation of how mitochondria communicate translation and assembly defects with the nucleus is particularly imperative to understand how the cell responds in these cases and would ultimately provide novel pathways for targeted treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Mitochondrial Tethers and Their Impact on Lifespan in Budding Yeast

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Tethers that link mitochondria to other organelles are critical for lipid and calcium transport as well as mitochondrial genome replication and fission of the organelle. Here, we review recent advances in the characterization of interorganellar mitochondrial tethers in the budding yeast, *Saccharomyces cerevisiae.* We specifically focus on evidence for a role for mitochondrial tethers that anchor mitochondria to specific regions within yeast cells. These tethering events contribute to two processes that are critical for normal replicative lifespan: inheritance of fitter mitochondria by daughter cells, and retention of a small pool of higher-functioning mitochondria in mother cells. Since asymmetric inheritance of mitochondria also occurs in human mammary stem-like cells, it is possible that mechanisms underlying mitochondrial segregation in yeast also operate in other cell types.

Keywords: mitochondria, lifespan, budding yeast, asymmetric cell division, organelle contact sites

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INTRODUCTION

Mitochondria have emerged as central regulators of lifespan through multiple mechanisms. Mitochondria are the site for generation of intermediary metabolites including acetyl-CoA and NAD^+/NADH, which regulate histone deactylases including the sirtuin family of age modulators (Starai et al., 2002; Hallows et al., 2006). Mitochondria are also the site for biosynthesis of iron-sulfur clusters (Braymer and Lill, 2017). Defects in this process result in nuclear genome instability, one of the hallmarks of aging (Veatch et al., 2009). Moreover, mitochondria serve as signaling platforms that affect lifespan by activation of stress response and quality control pathways. For example, in *C. elegans* and *Drosophila*, mild mitochondrial stress induced by mutation of respiratory chain components results in lifespan extension. These effects are a consequence of activation of the mitochondrial unfolded protein response (UPR^{mt}), a pathway that up-regulates mitochondrial proteostasis, antioxidant defenses and mitochondrial biosynthesis (Durieux et al., 2011; Owusu-Ansah et al., 2013). Mitochondrial stress can also lead to healthspan extension by delaying the age-linked decline in the heat shock response (HSR) pathway that maintains protein quality control within the cytosol and nucleus (Labbadia et al., 2017). Finally, selective autophagy of damaged mitochondria, a process that is driven by ubiquitination of mitochondrial proteins by the E3 ubiquitin ligase Parkin, illustrates the importance of mitochondrial quality control in health- and lifespan. Deletion or overexpression of Parkin shortens (Greene et al., 2003) or extends lifespan, respectively, in *Drosophila* (Rana et al., 2013). In addition, mutation of Parkin or Pink1, a protein that recruits Parkin to mitochondria, is associated with familial Parkinson's disease, an age-associated neurodegenerative disease (Kitada et al., 1998; Valente et al., 2004).

A fundamentally different mechanism whereby mitochondria impact lifespan has been identified in cells undergoing asymmetric cell division, the process in which differential segregation

of cellular constituents generates daughter cells that are not identical. In higher eukaryotes, including humans, asymmetric division is critical for stem cell function. It allows for the simultaneous renewal of stem-cell properties in one daughter cell and the production of a second, differentiating daughter cell that regenerates specific organs and tissues as they age (Ouellet and Barral, 2012). Indeed, it is possible that defects in asymmetric stem cell division contribute to the age-associated declines in stem cell number and function in regeneration of hematopoietic cells (Shaw et al., 2010), mouse forebrain (Molofsky et al., 2006), bone (Gruber et al., 2006), and skeletal muscle (Conboy and Rando, 2012). The budding yeast, *Saccharomyces cerevisiae*, also undergoes asymmetric cell division. One consequence of this is mother-daughter age asymmetry. Yeast mother cells have a finite replicative lifespan: they can produce an average of ∼30 buds. While yeast mother cells continue to age as they replicate, daughter cells are born young, with their full replicative lifespan (Jazwinski, 1990; Kennedy et al., 1994).

Here, we outline the effect of the non-uniform segregation of mitochondria during asymmetric cell division on cell fate and lifespan. In particular, we focus on the role of interorganellar mitochondrial contact sites in several key homeostatic processes in yeast and metazoans and in control of mitochondrial distribution during asymmetric inheritance of the organelle.

SEGREGATION OF MITOCHONDRIA DURING ASYMMETRIC CELL DIVISION

Asymmetric Inheritance of Mitochondria in Human Mammary Stem-Like Cells

Using photoconvertible fluorescent labels to differentially label newly generated and older mitochondria, Katajisto and colleagues identified asymmetric inheritance of mitochondria in human mammary stem-cell-like cells (Katajisto et al., 2015). They found that daughter cells that maintained more stem-like characteristics preferentially inherited newer mitochondria, while daughter cells destined to differentiate and develop epithelial characteristics preferentially inherited older mitochondria (**Figure 1**). Interestingly, maintaining stem cell properties correlates not just with the age of mitochondria but also with mitochondrial function: cells that contain mitochondria with higher membrane potential ($\Delta\Psi$) show increased mammosphere-forming capacity, which is a measure of stem cell function. Stem-like cells also exhibit increased mitophagy, and therefore increased capacity to remove damaged mitochondria, compared to daughter cells destined to differentiate. Consistent with this, inhibition of *Parkin*, a protein that marks mitochondria for mitophagy, reduces asymmetric inheritance of the organelle.

Differential localization is another mechanism for segregation of mitochondria by function in human mammary stem-like cells. Old mitochondria are more likely to localize near the nucleus, while younger ones are distributed more evenly throughout the cytoplasm in asymmetrically dividing stem-like cells. Treatment with an inhibitor of mitochondrial division (mdivi-1), or overexpression of the fission-inducing protein *Drp1*, results in mislocalization of old mitochondria throughout the cytoplasm, decreased segregation of old from young mitochondria and loss of stem cell properties in daughter cells (Katajisto et al., 2015). While it is clear that the asymmetric inheritance of mitochondria in human mammary stem-like cells is affected by the dynamics and localization of the organelle, the mechanism underlying this process is not well understood.

Asymmetric Mitochondrial Inheritance in Budding Yeast

During asymmetric cell division in budding yeast, mitochondria are actively partitioned between the mother and the developing bud, and accumulate at opposite cellular poles: the bud tip and the distal tip of the mother cell. The poleward displacements are achieved by both anterograde movements (toward the bud) and retrograde movements (toward the distal tip of the mother) (Fehrenbacher et al., 2004).

Recent studies exploring mitochondrial quality parameters as a function of subcellular localization found that yeast daughter cells contain mitochondria with less reactive oxygen species (ROS), higher reducing potential and higher $\Delta\Psi$ compared to mitochondria in mother cells (McFaline-Figueroa et al., 2011; Higuchi et al., 2013; Pernice et al., 2016). Thus, fitter mitochondria are preferentially inherited by yeast daughter cells. In contrast, there are some high- and low-functioning mitochondria in mother cells. As a result, the overall function of mitochondria in mother cells is lower than that of mitochondria in buds. Interestingly, the small population of higher-functioning mitochondria that are present in mother cells localize to the tip of the mother cell that is distal to the bud (mother cell tip) (**Figure 2**) (Pernice et al., 2016).

Live-cell imaging revealed a role for mitochondrial motility, dynamics, and region-specific tethering of the organelle in the asymmetric inheritance of mitochondria in budding yeast (**Figure 2**). First, actin cables, the tracks for mitochondrial movement, are dynamic structures that move from the bud toward the mother cell tip (Yang and Pon, 2002; Huckaba et al., 2006). As a result, fitter mitochondria that exhibit higher anterograde motility rates and can overcome the opposing force of retrograde actin cable flow are preferentially transported from mother to daughter cell (Higuchi et al., 2013). Second, fluorescence loss in photobleaching studies revealed that mitochondria in the bud can be functionally distinct from mitochondria in mother cells, in part because they are physically distinct (McFaline-Figueroa et al., 2011). Third, higher-functioning mitochondria are retained at the opposite poles of the yeast cell because they are anchored and immobilized at those sites. Mitochondria at the bud tip are tethered to the cortical endoplasmic reticulum (cER), an ER network that localizes to the cell periphery and is itself anchored to the plasma membrane (PM) (Swayne et al., 2011). Interestingly, deletion of the 5 genes that mediate cER-PM interactions (Manford et al., 2012) has no effect on accumulation of high-functioning mitochondria in the mother cell tip (Pernice et al., 2016). Thus, distinct mechanisms mediate anchorage of high-functioning mitochondria at the opposite poles of the budding yeast cell.

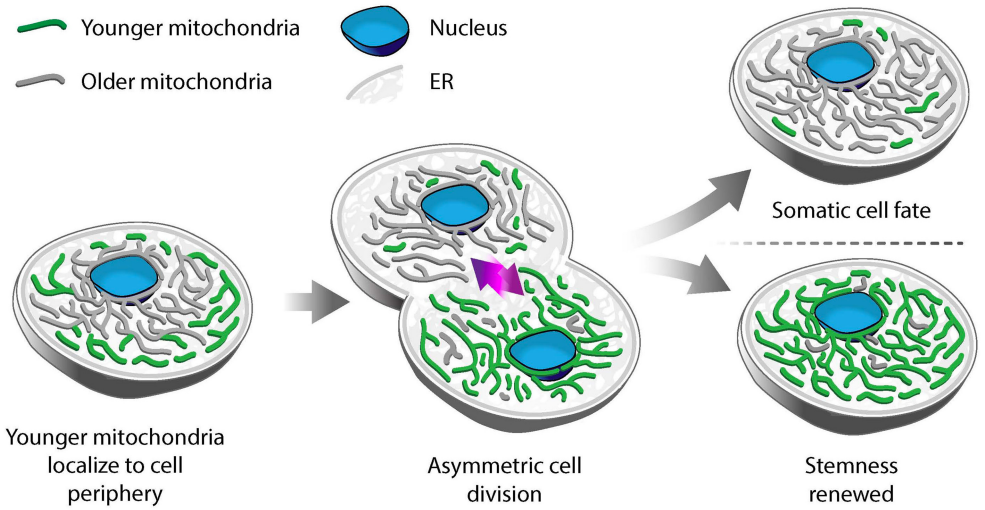

FIGURE 1 | Asymmetric segregation of mitochondria in stem-like cells. In mammary epithelial stem-like cells, a model for asymmetric stem cell division, younger mitochondria (green) localize to the cell periphery, while older mitochondria (gray) are perinuclear. Upon cell division, younger mitochondria are preferentially segregated to the daughter cell that undergoes self-renewal and retains stem-like properties. Older mitochondria are inherited by the daughter cell that differentiates to a tissue progenitor cell.

Below, we describe the role for tethers in asymmetric inheritance of mitochondria and in lifespan control.

TETHERS THAT LINK MITOCHONDRIA TO OTHER ORGANELLES

Organelles were once believed to be physically and functionally distinct subcellular compartments. However, it is now clear that mitochondria interact with other organelles including the ER, PM, vacuoles (the yeast lysosome), and peroxisomes (Murley and Nunnari, 2016). Several of these contact sites appear to function primarily in controlling mitochondrial distribution. Other interorganellar mitochondrial contact sites function in key biosynthetic and signaling pathways.

Mitochondrial Interorganellar Contact Sites that Function in Cell Metabolism and Signaling

One primary function of mitochondrial contact sites with other organelles is the exchange of lipids between the apposed membranes. Mitochondria must import phospholipids, including phosphatidylcholine (PC), and precursors for phosphatidylethanolamine (PE) and cardiolipin (CL) biosynthesis from the ER (Vance, 2015). The ER-mitochondrial encounter structure (ERMES) of *S. cerevisiae* (Kornmann et al., 2009) was the first tether identified that links mitochondria to ER. It consists of mitochondrial outer membrane proteins and an integral ER membrane protein. Three out of four ERMES subunits contain a synaptotagmin-like mitochondrial-lipid binding protein (SMP) domain (Lee and Hong, 2006; Kopec et al., 2010), suggesting that ERMES proteins facilitate lipid transport directly (Schauder et al., 2014). Surprisingly, deletion of ERMES subunits has only subtle effects on the levels of aminoglycerophospholipids in mitochondria, phospholipids that are produced at sites of ER-mitochondrial contact (Kornmann et al., 2009; Nguyen et al., 2012; Voss et al., 2012).

Further analysis of ERMES mutants led to the identification of another mechanism for lipid transport at mitochondria: contact sites between mitochondria and the vacuole (vCLAMPs). Schuldiner and colleagues found that deletion of 2 proteins previously implicated in vacuolar fusion (Vps39 and Vam7) (Price et al., 2000; Stroupe et al., 2006) results in an increase in ERMES (Elbaz-Alon et al., 2014). They also found that Vps39 localizes to vCLAMPs, vCLAMPs expand in ERMES mutants, and repression of ERMES subunits in *vps39Δ* cells results in defects in phospholipid composition of mitochondria (Elbaz-Alon et al., 2014). In complementary studies, Honscher et al. found that deletion of Vps39 results in a decrease in vCLAMPs and that overexpression of Vps39 increases the size of vCLAMPS and rescues growth defects observed in ERMES mutants (Hönscher et al., 2014). Finally, recent studies revealed that Lam6, a conserved protein that contains GRAM lipid-binding domains (Doerks et al., 2000; Gatta et al., 2015), co-immunoprecipitates with multiple ERMES subunits and co-localizes with ERMES (Elbaz-Alon et al., 2015). Interestingly, Lam6 also localizes to vCLAMPs and to nucleus-vacuolar junctions (NVJs) and overexpression of *LAM6* results in expansion of all 3 junctions (Elbaz-Alon et al., 2015). Overall, these studies indicate that vCLAMPs and ERMES have redundant functions in lipid transport to mitochondria and that Lam6 plays a role in regulating the cross-talk between multiple organelle contact sites. Yet to be determined is the mechanism underlying vCLAMP function in lipid transport to mitochondria.

ER-mitochondria contact sites also participate in processes other than lipid transport. For example, they function in calcium ion (Ca^{2+}) transport between the organelles (Rizzuto et al., 1998; Stone and Vance, 2000). Recent studies indicate that the SMP domain-containing protein *Pdzd8* localizes to ER at sites of mitochondrial-ER interaction, mediates interactions between mitochondria and ER in mammalian cells, and is required for synaptically induced Ca^{2+} transport between the two organelles (Hirabayashi et al., 2017). Interestingly, mtDNA nucleoids also localize to mitochondria-ER junctions and undergo DNA replication at those sites (Lewis et al., 2016). Finally, these contact sites contribute to mitochondrial fission. Seminal studies revealed that ER tubules wrap around mitochondria and recruit the actin cytoskeleton to that site. Actin then generates contractile forces at the mitochondria-ER interface, leading to assembly of dynamin-related protein 1 (Drp1) at the site of constriction and further contraction of the organelle (Friedman et al., 2011; Korobova et al., 2013). Thus, mitochondria-ER interactions affect fundamental processes, including lipid biogenesis and transport, calcium homeostasis, and mitochondrial dynamics and genome replication, which ultimately affect cellular fitness.

Mitochondrial Tethers that Control Mitochondrial Distribution during Asymmetric Cell Division and Affect Lifespan

As described above, the mitochondrial motility machinery promotes inheritance of fitter mitochondria by yeast daughter cells, which in turn affects daughter cell fitness and lifespan. Region-specific retention of mitochondria has also emerged as an important mechanism that contributes to the faithful partitioning of the organelle and mitochondrial quality control during yeast cell division. Three major retention mechanisms have been identified in yeast: bud tip-specific tethering by Mmr1; cortical maternal tethering through Num1; and mother tip-specific retention through Mfb1. We here describe how these proteins contribute to mitochondrial quality control and lifespan in yeast.

Mmr1: A Bud-Tip Tether that Affects Quantity and Quality of Mitochondrial Inheritance

Mmr1 was identified in a screen for genetic interactions with the type V myosin motor Myo2 (Itoh et al., 2004). Mmr1 binds to the Myo2 tail and also to unknown factors on mitochondria, suggesting that it might be a receptor for a motor protein that drives mitochondrial movement on actin cables (Eves et al., 2012). However, a more complex picture of Mmr1 function has emerged. *MMR1* protein and mRNA localize to the bud tip (Shepard et al., 2003), which suggests that Mmr1 functions in the bud tip and not in the mother cell where there are high levels of mitochondrial motility. Indeed, Mmr1 localizes to the interface between mitochondria and cER in the bud tip. Moreover, deletion of *MMR1* or failure to localize the protein to the bud tip results in defects in accumulation of mitochondria in the bud tip (Swayne et al., 2011). In addition, cells bearing a temperature-sensitive mutation in *MMR1* release their mitochondria from the bud tip when shifted to restrictive temperature (Higuchi-Sanabria et al., 2016). Thus, Mmr1 appears to be a multifunctional tether that links mitochondria to a myosin for movement, and to cER in the bud tip for inheritance (**Figure 2**). Interestingly, the localization and deletion phenotype of *MMR1* are fundamentally different from those of ERMES mutants. Therefore, available evidence suggests that Mmr1-mediated anchorage of mitochondria at the bud tip occurs through a mechanism that does not rely on ERMES.

Mmr1 also contributes to control of mitochondrial quality during asymmetric cell division, which in turn affects lifespan. Deletion of *MMR*1 results in the generation of two populations of yeast (McFaline-Figueroa et al., 2011). One population of *mmr1Δ* cells has reduced replicative lifespan and elevated ROS levels. The other population of *mmr1Δ* cells are longer-lived and contain less ROS than wild-type cells. Taken together, these observations led to the model that Mmr1-mediated tethering of higher-functioning mitochondria in the yeast bud tip contributes to retention of higher-functioning mitochondria in the bud,

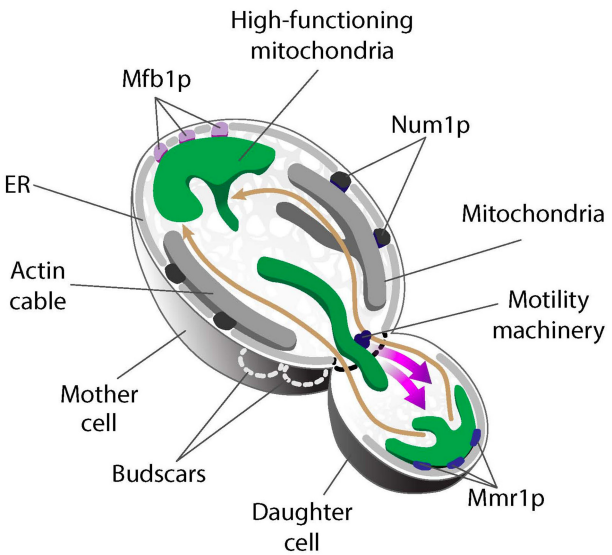

FIGURE 2 | Asymmetric mitochondrial inheritance in yeast. Higher-functioning mitochondria (green) localize to both the bud tip and tip of the mother cell in *S. cerevisiae*. This is achieved by the coordinated effort of the mitochondrial motility machinery and region-specific anchorage of high-functioning mitochondria during cell division. Mitochondria are transported from mother cells to buds along actin cables, dynamic tracks that exhibit retrograde flow (movement from buds to mother cells). High-functioning mitochondria are more motile and therefore able to overcome the opposing force of retrograde actin cable flow and move into buds. These higher-functioning mitochondria are anchored to cER in the bud tip by Mmr1. Two tethers retain mitochondria in mother cells: Num1 mediates mitochondrial tethering throughout the maternal cortex, but does not contribute to mitochondrial quality control during inheritance. In contrast, Mfb1 localizes to and mediates anchorage and retention of high-functioning mitochondria specifically at the mother cell tip. Loss of function of either Mmr1 or Mfb1 results in defects in mitochondrial quality control and altered lifespan.

which in turn affects daughter cell fitness and lifespan (Higuchi-Sanabria et al., 2014).

Maternal Mitochondrial Retention in Budding Yeast

Num1 together with Mdm36 forms the Mitochondria-ER Cortex Anchor (MECA) structure. MECA anchors mitochondria to the PM in yeast mother cells. Specifically, deletion of *NUM1* abolishes cortical anchorage of mitochondria in mother cells and impairs maternal mitochondrial retention, which results in disproportionate inheritance of mitochondria by daughter cells (Klecker et al., 2013; Lackner et al., 2013). Num1 forms punctate structures that distribute throughout the maternal cortex and—as the name implies—both ER and mitochondria are consistently present at Num1 foci. Although MECA does not directly partake in ER-mitochondria tethering (Lackner et al., 2013), it is possible that MECA stabilizes ERMES-mediated tethering of the two organelles. Indeed, Num1 deletion causes severe defects in mitochondrial fission, an event that occurs at mitochondrial-ER contact sites (Cerveny et al., 2007; Klecker et al., 2013).

In contrast to Num1, which controls the *quantity* of mitochondria in yeast mother cells, the mitochondrial F-box-containing protein Mfb1 controls the *quality* of maternal mitochondria (Pernice et al., 2016; Kraft and Lackner, 2017). Mfb1 was originally described as a mitochondrial morphology regulator that is enriched in mother cells (Dürr et al., 2006; Kondo-Okamoto et al., 2006). However, Mfb1 has since been shown to control mitochondrial localization and cell lifespan. Deletion of *MFB1* results in depletion of mitochondria specifically from the mother cell tip. Equally important, there is no accumulation of higher-functioning mitochondria in the mother cell tip in *mfb1Δ* cells. Moreover, overall mitochondrial function is severely compromised in *mfb1Δ* cells (Pernice et al., 2016). Finally, deletion of *MFB1* results in a significant decrease in replicative lifespan. These findings indicate that anchorage of a small population of higher-functioning mitochondria in the mother cell tip is mediated by Mfb1 and that this process affects cellular fitness and lifespan. Since Mfb1 co-localizes with mitochondria that are anchored in the mother cell tip, it is likely that it has a direct role as a tether for mitochondria at that site (**Figure 2**).

What could be the mechanism for mitochondrial quality control by Mfb1? Treatment of yeast with agents that damage mitochondria, including oxidizing agents or small molecules that reduce $\Delta\Psi$, has no effect on Mfb1 localization or function in anchorage of mitochondria in the mother cell tip. Thus, Mfb1 is not actively sensing mitochondrial function (Pernice et al., 2016).

Instead, the association of Mfb1 with a high-functioning mitochondrial population may occur via indirect mechanisms. In particular, this association appears to be linked to patterns of cell polarity. Yeast haploid cells display an axial budding pattern: new buds always form adjacent to the previous bud site. As a result, the bud tip of a daughter cell becomes the mother cell tip in the daughter's next round of cell division (Bi and Park, 2012; Martin and Arkowitz, 2014). Interestingly, during telophase, some Mfb1 localizes to the bud tip where it functions as an anchor for mitochondria. Moreover, Mfb1 that is present at the bud tip remains at that site after cytokinesis, as the cell's polarity reverses and the old bud tip becomes the new mother cell tip. Hence, instead of actively sensing mitochondrial quality, cell cycle-regulated localization of Mfb1 to the bud tip may allow it to capture high-functioning mitochondria at the bud tip and anchor them in the new mother cell tip as the newborn cell begins to replicate (Pernice et al., 2016).

CONCLUSION

Overall, these studies indicate that inheritance of fitter mitochondria by daughter cells during asymmetric division in yeast relies on a tether in the bud (Mmr1) that retains high-functioning mitochondria in the bud by anchorage of mitochondria to cER at that site. It also relies on a tether in mother cells (Mfb1) that retains a small population of higher-functioning mitochondria in mother cells by anchorage to the mother cell tip. Defects in either tether compromise asymmetric inheritance of mitochondria and, in turn, lifespan.

Several outstanding questions remain. Mfb1 is the only known protein in yeast that localizes to the mother tip throughout the cell cycle and to the bud tip in telophase. It may therefore interact with thus far uncharacterized polarity cues in yeast. Moreover, the differential localization of old and new mitochondria in human mammary stem-like cells prompts the speculation that tethers between mitochondria and ER in the nuclear envelope and/or PM may contribute to segregation of mitochondria during asymmetric cell division in this stem cell model, and potentially other mammalian cells. Future studies will explore these questions, identify the mechanisms for Mmr1- and Mfb1-mediated mitochondrial tethering, reveal tethers for mitochondria-PM interactions in mammalian cells, and determine whether these tethers contribute to asymmetric inheritance of mitochondria, lifespan control and/or cellular fitness by localizing the most functional mitochondria to their sites of action.

AUTHOR CONTRIBUTIONS

Each of the authors contributed to writing and editing the mini-review. WP prepared the figures in addition to writing the manuscript.

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Crosstalk between Lysosomes and Mitochondria in Parkinson's Disease

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Parkinson's disease (PD) is the most common motor neurodegenerative disorder. In most cases the cause of the disease is unknown, while in about 10% of subjects, it is associated with mutations in a number of different genes. Several different mutations in 15 genes have been identified as causing familial forms of the disease, while many others have been identified as risk factors. A striking number of these genes are either involved in the regulation of mitochondrial function or of endo-lysosomal pathways. Mutations affecting one of these two pathways are often coupled with defects in the other pathway, suggesting a crosstalk between them. Moreover, PD-linked mutations in genes encoding proteins with other functions are frequently associated with defects in mitochondrial and/or autophagy/lysosomal function as a secondary effect. Even toxins that impair mitochondrial function and cause parkinsonian phenotypes, such as rotenone, also impair lysosomal function. In this review, we explore the reciprocal relationship between mitochondrial and lysosomal pathways in PD. We will discuss the impact of mitochondrial dysfunction on the lysosomal compartment and of endo-lysosomal defects on mitochondrial function, and explore the roles of both causative genes and genes that are risk factors for PD. Understanding the pathways that govern these interactions should help to define a framework to understand the roles and mechanisms of mitochondrial and lysosomal miscommunication in the pathophysiology of PD.

Keywords: alpha-synuclein, LRRK2, autophagy, mitophagy, lysosomes, mitochondria, Parkinson's disease, neurodegeneration

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INTRODUCTION

Parkinson's disease (PD) is a multifactorial and genetically heterogeneous neurodegenerative disorder, characterized by bradykinesia, resting tremor, postural instability, and muscle rigidity. Together with motor symptoms, more variable non-motor symptoms may develop, including anosmia, sleep disorders, depression and, with disease progression, dementia. The primary neurodegenerative pathology features the loss of dopaminergic neurons in the *substantia nigra pars compacta*, while the main histopathological hallmark seen at post mortem is the presence in the surviving neurons of protein aggregates, known as Lewy bodies (LB).

The cause of PD in the vast majority of subjects is unknown—referred to as "sporadic"—or associated with exposure to environmental toxins (Tanner et al., 2011). In about 10% of subjects, it is caused by monogenic mutations and 15 causative genes have so far been identified associated with familial disease, frequently of early onset (**Table 1**). Genome-wide association studies have revealed mutations in many other genes that increase disease risk. Many of the causative or risk

factor genes for PD show an association with mitochondrial quality control pathways, ranging from mitochondrial proteins to proteins that regulate endo-lysosomal function (Verstraeten et al., 2015).

Interestingly, the familial forms of the disease associated with mutations of proteins involved in the autophagic-lysosomal pathway, often show mitochondrial defects (Gusdon et al., 2012; Ramonet et al., 2012; Tang et al., 2015; Wang et al., 2015). Moreover, alpha-synuclein (aS) aggregation, as well as LRRK2 mutations, which cause PD, can be both involved in dysregulation of autophagic and endo-lysosomal pathways through different mechanisms, and are also associated with mitochondrial dysfunction (Niu et al., 2012; Papkovskaia et al., 2012; Nakamura, 2013; Yang et al., 2014). On the other hand, mitochondrial complex I inhibition induced by rotenone [a toxin that cause parkinsonian phenotype (Tanner et al., 2011)] alters the expression of lysosomal genes (Fernández-Mosquera et al., 2017). To be noted is the observation that mitochondrial complex I deficiencies were associated with sporadic forms of PD, making this defect one of the more common features of the pathophysiology of PD (Schapira et al., 1990).

More generally, several studies have shown that lysosomal dysfunction impacts on mitochondria by impairing mitophagy and possibly by changes in other signaling pathways. These mechanisms appear to play a significant role in the pathophysiology of other diseases, especially in lysosomal storage disorders (LSD) (Plotegher and Duchen, 2017). Remarkably, mitochondrial defects have also been shown to impact on lysosomal function (Diogo et al., 2017), suggesting a complex reciprocal relationship between these two compartments (Raimundo et al., 2016).

In this review, we will discuss some of the genes whose mutations are associated with PD and will focus on the crosstalk between mitochondria and endo-lysosomal pathway in the pathophysiology of genetic PD.

FROM MITOCHONDRIA TO LYSOSOMES IN PD: ARE THE POWER HOUSES OF THE CELL CLOGGING THEIR RECYCLE BINS?

Mutations in parkin, PINK1 and DJ-1 cause autosomal recessive parkinsonism, and have come to represent the archetype of PD associated with impaired mitophagy. Both parkin and PINK1 encode proteins involved in the regulation of mitophagy. The clearance of dysfunctional mitochondria can be initiated through several different pathways, among which the parkin/PINK1 pathway is one of the most extensively characterized. The loss of mitochondrial membrane potential leads to PINK1 accumulation on the outer mitochondrial membrane. PINK1 recruits the E3 ubiquitin ligase parkin to the mitochondria (Narendra et al., 2010), and the activation of parkin leads to the ubiquitination of mitochondrial membranes, initiating the removal of dysfunctional organelles (Narendra et al., 2008).

While mutations in PINK1 and parkin apparently affect mitochondrial function because they permit the accumulation of dysfunctional mitochondria, it was also shown that parkin-deficiency in PD patient fibroblasts causes defects in the function of retromer, a trimeric cargo-recognition protein complex responsible for protein trafficking in the endosomal compartment (Song et al., 2016). Moreover, PINK1 depletion inhibits lysosomal function and induces the enlargement of the vacuolar compartment. Defects in the lysosomal compartment also occurred in response to inhibition of the mitochondrial ATP-synthase using oligomycin (Demers-Lamarche et al., 2016) and in T cells from mice with knockout of *Tfam,* the major transcription factor in mitochondrial biogenesis (Baixauli et al., 2015).

Of special relevance for PD is the recent characterization of the effects of rotenone treatment on lysosomal biogenesis. Rotenone is a pesticide that inhibits complex I of the mitochondrial respiratory chain. It induces a parkinsonian phenotype in animal models (Liu et al., 2015) and epidemiological studies suggest that environmental exposure to rotenone may increase the risk of PD in humans (Tanner et al., 2011). Acute exposure of mouse embryonic fibroblasts to rotenone caused a rapid increase in the transcript level of some lysosomal genes, while chronic treatment induced a marked decrease in the expression of these same genes (Fernández-Mosquera et al., 2017). Considering the defects in complex I function described in sporadic PD, it seems important to understand how this affects lysosomal function in these forms of PD.

The PD-related protein DJ-1 localizes to mitochondria and seems to act as an antioxidant and chaperone, although its specific role remains controversial (Junn et al., 2009; Girotto et al., 2014). DJ-1 is involved in both mitochondrial function and autophagy (Thomas et al., 2011): DJ-1 silencing in the M17 neuroblastoma cell line caused a reduction of mitochondrial membrane potential, mitochondrial fragmentation and accumulation of autophagy markers. Overexpression of parkin or PINK1 proteins rescued this phenotype. Also DJ-1 knockout flies showed defects in mitochondrial respiration and reduced ATP production. Interestingly, DJ-1 overexpression can rescue the disease phenotype in PINK1 deficient flies, but not in *parkin*$^{-/-}$ flies (Hao et al., 2010). These results further suggest that DJ-1 plays a role in the control of mitochondrial homeostasis, likely involving the PINK1/parkin pathway. DJ-1 deficiencies also impact on the autophagy pathway, with a less clear mechanism that may depend on mitochondrial defects (Mccoy and Cookson, 2017). This suggest that jeopardizing mitochondrial function at any level (quality control, dynamics, or respiration) can impact on the function of the lysosomal compartment in PD (**Figure 1A**).

FROM THE ENDO-LYSOSOMAL PATHWAY TO MITOCHONDRIA IN FAMILIAL PD: WHEN GARBAGE STOPS ENERGY PRODUCTION

In the last few years, extensive efforts led to the identification of novel genes implicated in the pathogenesis of PD. Among these genes, it is striking that many encode proteins involved in endo-lysosomal trafficking and function. Defects in this pathway can

TABLE 1 | Overview of PD causative genes, their function(s), the clinical features of the PD forms associated with their mutations and the involvement of mitochondrial and/or endo-lysosomal dysfunction.

Gene	Primary function	Endo-lysosomal defects	Mitochondrial defects	Mode of inheritance	PD form and pathology
SNCA	Synaptic vesicles	Yes	Yes	AD	EO or LO; alpha-synuclein accumulation
parkin	Mitophagy	NA	Yes	AR	EO; occasional alpha-synuclein accumulation
DJ-1	Oxidative stress; chaperone	Yes	Yes	AR	EO
LRRK2	Endo-lysosomal trafficking and function	Yes	Yes	AD	LO; alpha-synuclein, tau, and TDP-43 accumulation
PINK1	Mitophagy	Yes	Yes	AR	EO; occasional alpha-synuclein accumulation
ATP13A2	Lysosomal ATPase, cation homeostasis	Yes	Yes	AR	JO; iron accumulation
FBXO7	Adaptor protein in SCF ubiquitin E3 ligase	NA	Yes	AR	JO
PLA2G6	A2 phospholipase (phosphatidylcholine)	NA	Yes	AR	JO; iron accumulation
VPS35	Retromer complex; protein trafficking	Yes	Yes	AD	LO
EIF4G1	Recruitment of mRNA to the ribosome	NA	NA	AD	LO; alpha-synuclein, tau and Aβ accumulation
DNAJC6	Clathrin-mediated endocytosis	NA	Yes	AR	JO
ATP6AP2	Vacuolar ATPase component, lysosomal pH	Yes	Likely	X linked	JO or EO; tau accumulation
COQ2	Coenzyme Q10 biosynthesis	NA	Yes	AR	LO; alpha-synuclein accumulation
SYNJ1	Clathrin coated vesicles disassembly	NA	NA	AR	JO
DNAJC13	Retromer-mediated endosomal protein sorting	Yes	NA	AD	LO; alpha-synuclein accumulation

EO, early onset; LO, late onset; JO, juvenile onset; AR, autosomal recessive; AD, autosomal dominant; NA, unknown.

negatively affect mitochondrial function and can interfere with the removal of defective mitochondria, also through impaired autophagy (**Figure 1B**).

Vacuolar protein sorting-associated protein 35 (Vps35) is part of the retromer, which regulates protein trafficking within the endosomal compartment, and the pathogenic D620N mutation in Vps35 causes alterations in endosomes and trafficking defects (for example, it disrupts the trafficking of cathepsin D, a lysosomal protease) (Follett et al., 2013). Cathepsin D trafficking defects may contribute to lysosomal deficiencies, but most studies have shown that the main victims of pathogenic mutation of Vps35 seem to be mitochondria.

In fact, it was suggested that Vps35 is responsible for mediating transport of mitochondrial derived vesicles (MDVs) from mitochondria to other cellular compartments (Soubannier et al., 2012), therefore being involved in mitochondria quality control. Moreover, Tang et al. showed that in mouse dopaminergic neurons, depletion of Vps35 promotes degradation of Mitochondrial E3 Ubiquitin Protein Ligase 1 (MUL1), thus reducing Mitofusin 2 (Mfn2) stabilization and impeding mitochondrial fusion (Tang et al., 2015). Mitochondrial fragmentation was also observed in cultured neurons, in mice and in fibroblasts carrying the D620N Vps35 mutation (Tang et al., 2015; Wang et al., 2015). The mechanism causing these defects was associated with the increased turnover of the mitochondrial protein dynamin-like protein 1 (DLP1) through increased trafficking of MDVs to lysosomes for degradation.

Vps35 was also linked to the parkin pathway. Malik et al. showed that Vps35 and parkin pathways may interact in flies (Malik et al., 2015). In particular, flies heterozygous for both *vps35*/+;*parkin*/+ were more sensitive to the herbicide paraquat [which, similarly to rotenone, is a toxin linked to PD (Tanner et al., 2011)] and presented degeneration of dopaminergic neurons. Moreover, Vps35 overexpression in *parkin*-mutant fly models rescued many of the phenotypes, but the same was not true for *pink1*-mutant flies.

Another gene whose mutations cause an atypical form of PD, termed the Kufor-Rakeb syndrome, but also a complicated form of hereditary spastic paraplegia (Estrada-Cuzcano et al., 2017) is ATP13A2. The protein encoded by this gene is a lysosomal P-type ATPase that seems to be involved in cation homeostasis (Ramonet et al., 2012). Mutations of ATP13A2 are associated with marked changes in lysosomal and mitochondrial function. Modulation of ATP13A2 expression decrease cytosolic calcium levels, while silencing ATP13A2 induces mitochondrial fragmentation (Ramonet et al., 2012). In fibroblasts from patients carrying mutations in ATP13A2, mitochondrial maximal respiratory rate and membrane potential were reduced (Grünewald et al., 2012). Gusdon et al. showed that ATP13A2 knockout in neurons and SHSY5Y cells increased mitochondrial mass, suggesting decreased turnover (Gusdon et al., 2012), likely associated with impaired autophagic flux, causing failure of clearance of defective mitochondria. However, we cannot exclude that other pathways link ATP13A2 deficiencies to mitochondrial dysfunction.

Mutations of the ATPase H^+ Transporting Accessory Protein 2 (ATP6AP2) gene cause a rare X-linked form of parkinsonism with spasticity (Korvatska et al., 2013). ATP6AP2 transmembrane protein is an accessory component of the vacuolar ATPase, essential to keep lysosomal pH acidic. ATP6AP2 deficiencies cause the accumulation of autophagosomes and autophagolysosomes, and p62, suggesting impairment in autophagy and lysosomal clearance (Korvatska et al., 2013). A link between alkalinization of acidic vacuoles and mitochondrial dysfunction was established in yeast, and has aso been described in mammalian cells (Hughes and Gottschling,

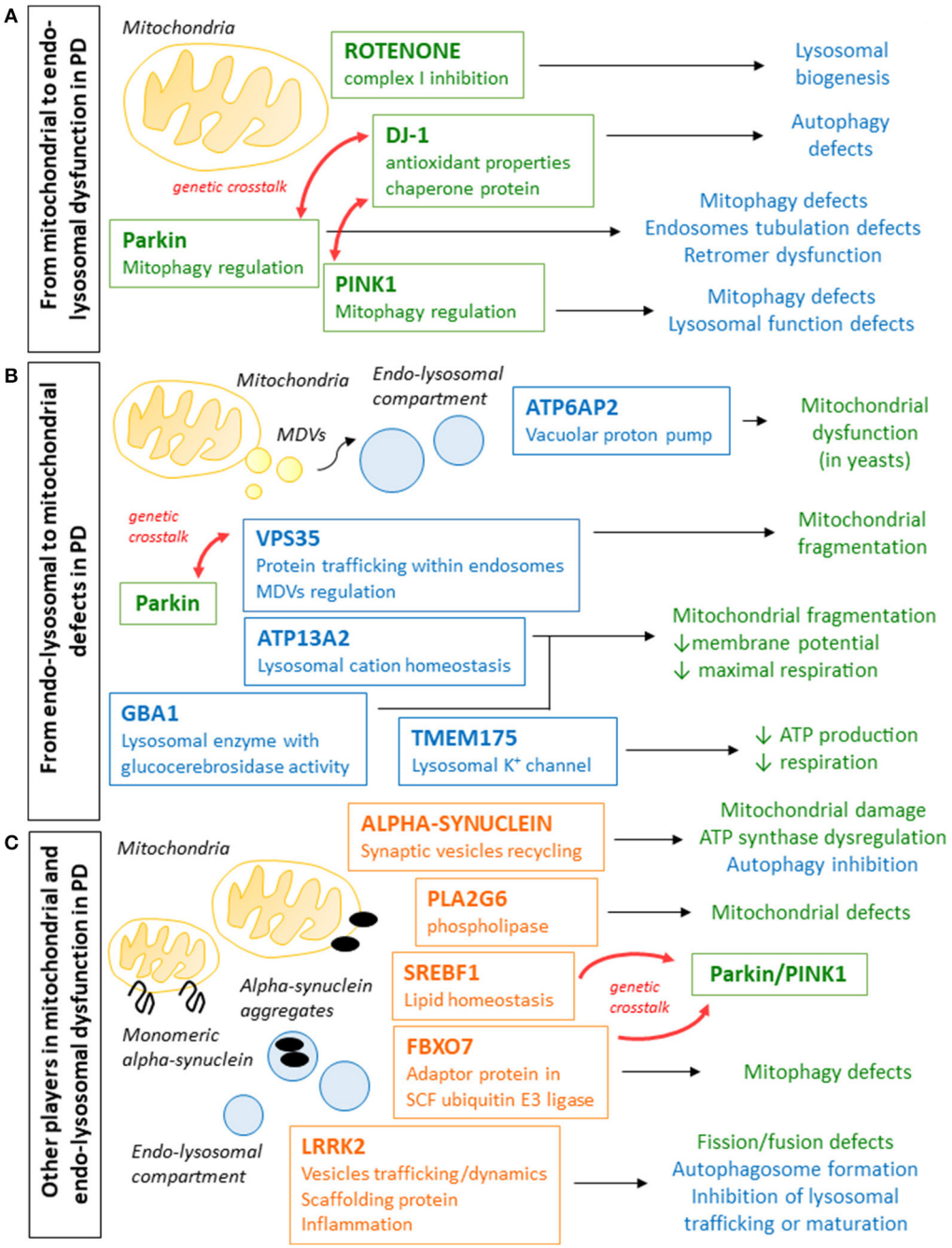

FIGURE 1 | Schematic representation of the different ways in which genes whose mutations are involved in PD pathogenesis cause defects in mitochondrial and endo-lysosomal compartments. **(A)** Parkin, PINK1, and DJ-1 mutations cause PD and are all involved in the regulation of mitophagy and mitochondrial quality control and mitophagy regulation, but can also lead to endo-lysosomal pathway defects and autophagy impairment. Rotenone, which inhibits mitochondrial complex I and was associated to parkinsonism in animal models and in epidemiological studies, also impacts on lysosomal compartment. **(B)** Genes whose mutations cause PD (VPS35, ATP6AP2, and ATP13A2) or increase the risk (GBA1 and TMEM175) are often involved in endo-lysosomal pathway function. Defects in these pathway can have effects on the mitochondrial function, leading reduced membrane potential, defective mitochondrial respiration and reduced ATP production. **(C)** Mutations in alpha-synuclein and LRRK2, whose functions have not been fully elucidated yet, cause autosomal dominant PD and were shown to affect both mitochondrial and endo-lysosomal function. Other genes whose mutations were associated to PD (PLA2G6, FBXO7, and SBERF1) and whose functions are not directly related to mitochondria or lysosomal compartment, were shown to affect mitochondrial clearance and to interact with other genes relevant to PD, and it cannot be excluded that the endo-lysosomal pathway play a role also in these cases.

2012). It was recently shown that lysosomal pH alkalinization impairs autophagy (Trudeau et al., 2016), while lysosome re-acidification rescued the phenotype.

More studies are needed to understand whether impairment of the autophagy-lysosomal pathway is associated with mitochondrial defects because they permit the accumulation of defective mitochondria through failed mitophagy or whether other pathways are involved.

ROLES OF ALPHA-SYNUCLEIN AND LRRK2 IN ENDO-LYSOSOMAL PATHWAY DEFECTS AND MITOCHONDRIAL DYSFUNCTION

aS is localized at the presynaptic terminal in the mammalian brain and is involved in synaptic vesicle recycling and docking (Burré et al., 2010). Several single point mutations and gene multiplications cause autosomal dominant PD. Moreover, aS fibrils are the main constituent of Lewy Bodies and aS oligomers and aggregates play a key role in neuronal death (Plotegher et al., 2014b).

The accumulation of aS oligomers and fibrils was shown to be involved in both impaired impairment (Xilouri et al., 2016) and mitochondrial dysfunction (Nakamura et al., 2011; Plotegher et al., 2014a; Pozo Devoto et al., 2017). Moreover, impaired autophagy itself was shown to be associated with aS accumulation, further amplifying this detrimental mechanism (Xilouri et al., 2016) and likely affecting also the removal of dysfunctional mitochondria.

Mutations of LRRK2, a large kinase that also shows GTPase activity, account for some 40% of genetic cases of PD. LRRK2 has been shown to play a role in many different pathways, including the endo-lysosomal (Roosen and Cookson, 2016), but its exact functions are still unclear. LRRK2 interacts with many proteins in the endo-lysosomal compartment, such as proteins from the Rab-family, and it can play a role in both autophagosome formation but also in the maturation or in the trafficking of lysosomes.

LRRK2 mutations associated with PD were shown to alter mitochondrial fusion/fission by interfering with the mitochondrial fission factor DLP1 (Niu et al., 2012; Yang et al., 2014). Interestingly, DLP1 mediated defects in mitochondrial dynamics were also seen in association with Vps35 mutations (Wang et al., 2015). Another study suggested that LRRK2 mutations impact on mitochondrial function by causing reduced membrane potential and ATP production (Papkovskaia et al., 2012), which could be associated with defective fusion and fission reported by others.

In both aS and LRRK2-associated PD, both mitochondria and lysosomal compartments are affected, but it is still unclear in which organelles the damage starts and how it is then extended to affect the other (**Figure 1C**). One idea is that an impaired autophagic-lysosomal pathway associated with LRRK2 mutations or due to aS clumps causes the accumulation of dysfunctional mitochondria; another is that mitochondrial damage induced by aS aggregates or LRRK2 defects impact on lysosomal function and biogenesis, as observed for other models showing defective mitochondria. It is possible that both pathways may be activated at the same time, making it even more difficult to understand the pathophysiological cascade.

NEW PLAYERS, KEY LINKS TO MITOCHONDRIAL DEFECTS?

Mutations of several other genes have been shown to cause PD and even if the pathological mechanism(s) are not yet well-understood, a few examples may provide new hints to illuminate the interplay between the endo-lysosomal compartment and mitochondrial network in the pathogenesis of PD.

PLA2G6 mutations have been associated with a variety of neurological disorders collectively termed PLA2G6-associated neurodegeneration (PLAN) among which there is an autosomal recessive form of dystonia-parkinsonism (Paisan-Ruiz et al., 2009). The gene encodes a calcium-independent phospholipase, which localizes to mitochondria and generates free fatty acids by catalyzing the hydrolysis of glycerophospholipids (Gadd et al., 2006). For infantile neuroaxonal dystrophy, which is one of the PLAN disorders, Beck et al. have shown that mitochondria were involved in the neurodegeneration (Beck et al., 2011), suggesting that axonal degeneration is a consequence of the presence of abnormal mitochondria following axonal transportation. More recently, in flies in which the *Drosophila* homolog of PLA2G6 (iPLA2-VIA) was knocked out and in patient fibroblasts carrying PLA2G6 mutations, mitochondrial function was severely compromised, with reduced mitochondrial membrane potential, respiration, and ATP production (Bartolome et al., 2015).

The gene *FBXO7* encodes an adaptor protein in Skp-Cullin-F-box (SCF) ubiquitin E3 ligase complex responsible for mediating its ubiquitination by the SCF E3 ligase. Mutations in this gene cause a Parkinsonian-pyramidal syndrome. FBXO7 interacts with the ubiquitin E3 ligase parkin (Burchell et al., 2013) and participates in the recruitment of parkin at the mitochondrial membrane following depolarization to initiate mitophagy. Moreover, as for Vps35, overexpression of FBXO7 rescued the disease phenotype in parkin-deficient flies, but not in PINK1 mutants. It is especially interesting that Vps35 and FBXO7 act in the same way in terms of the interaction with the Parkin/PINK1 pathway although the two proteins have completely different primary functions.

How defects in the endo-lysosomal compartment contribute to the neurodegeneration in these forms of PD and how and whether this may be associated with the observed mitochondrial defects remain to be fully elucidated (**Figure 1C**).

HINTS FROM GENES FOUND TO BE RISK FACTORS FOR PD INVOLVING LYSOSOMAL-MITOCHONDRIAL COMMUNICATION

Over 25 genes associated with an increased risk of developing PD have been identified through genome-wide association studies (Verstraeten et al., 2015). Among them, some are of

particular relevance for this review. First of all, the most common genetic risk factor for PD is represented by heterozygous mutations in *GBA1*, a gene encoding for the lysosomal enzyme glucocerebrosidase (Sidransky et al., 2009). *GBA1* homozygous mutations cause the LSD known as Gaucher's disease (GD) and some GD patients and their (heterozygous, carrier) relatives show parkinsonian manifestations (Tayebi et al., 2001).

In neurons cultured from a GBA1 knockout mouse, a model for a severe neurological form of GD, autophagy was impaired upstream of the lysosomes, and at the same time mitochondrial function was profoundly compromised, with a reduced membrane potential, severely impaired respiration and mitochondrial fragmentation (Osellame et al., 2013). Mitochondrial function was also impaired in fibroblasts from GD patients (de la Mata et al., 2015). Autophagy defects have also been documented in iPSC-derived neurons from PD patients carrying GBA1 mutations (Schöndorf et al., 2014). Moreover, in many models and patients, GBA1 deficiencies were associated with aS accumulation and aggregation (Choi et al., 2011; Cullen et al., 2011; Osellame et al., 2013), establishing a solid link between the two genes and involving aS as a key element in GBA1-associated neurodegeneration in PD, likely through impairment of the autophagic-lysosomal pathway and mitochondrial dysfunction, possibly attributable to accumulation of dysfunctional mitochondria as a consequence of defective mitophagy.

Mutations in the lysosomal K^+ channel TMEM175 (Jinn et al., 2017) also represent significant risk factors for PD. TMEM175 depletion in SHSY5Y cells was associated with increased lysosomal pH and with reduction in lysosomal degradative capacity. At the same time, mitochondrial respiration was impaired and ATP production reduced. Therefore, a primary lysosomal defect in PD impairs autophagy and mitochondrial function through a mechanism that has yet to be fully elucidated.

Mutations in the sterol regulatory element binding transcription factor 1 (SREBF1), a transcription factor necessary for lipid homeostasis, also increase the risk of developing PD. SREBF1 was also identified as one of a group of genes with a conserved role in mitophagy, favoring parkin translocation to mitochondria to initiate the process (Ivatt et al., 2014).

Overall these risk factor genes for PD point toward a dysregulation in endo-lysosomal compartment that impacts on mitochondria, or toward mitochondrial defects that signal to lysosomes affecting their function (**Figure 1B**). Further studies will be needed to better understand the role for these genes in sporadic PD.

CONCLUSIONS

A remarkable number of genes associated with PD, either causing disease or increasing risk, are associated with endo-lysosomal or mitochondrial defects. However, what is more striking is the fact that a primary defect in either of the two compartments usually leads to damage of the other, suggesting a strong reciprocal relationship between them, and placing the interconnection between the two center stage in the pathogenesis of the disease.

While in the past the link mainly referred to the fact that dysfunctional autophagy also implied dysfunctional mitophagy and therefore accumulation of defective mitochondria, now new avenues are emerging, suggesting that a more complex web of signaling pathways may link the two and give rise to disease.

On the mitochondrial side, strong evidence points to a role for impaired mitochondrial function in signaling to lysosomal pathways, affecting lysosomal biogenesis and function. When this leads to lysosome defects, it can also impact the autophagy-lysosomal pathway upstream, inducing impaired autophagy.

More efforts will be needed to understand how the interplay between mitochondrial and lysosomal dysfunction play a role in PD pathogenesis, and whether these pathways represent a potential generalized therapeutic target for sporadic and familial PD.

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NP and MD conceived and wrote the manuscript.

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REVIEW
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Protein Localization at Mitochondria-ER Contact Sites in Basal and Stress Conditions

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Mitochondria-endoplasmic reticulum (ER) contacts (MERCs) are sites at which the outer mitochondria membrane and the Endoplasmic Reticulum surface run in parallel at a constant distance. The juxtaposition between these organelles determines several intracellular processes such as to name a few, Ca^{2+} and lipid homeostasis or autophagy. These specific tasks can be exploited thanks to the enrichment (or re-localization) of dedicated proteins at these interfaces. Recent proteomic studies highlight the tissue specific composition of MERCs, but the overall mechanisms that control MERCs plasticity remains unclear. Understanding how proteins are targeted at these sites seems pivotal to clarify such contextual function of MERCs. This review aims to summarize the current knowledge on protein localization at MERCs and the possible contribution of the mislocalization of MERCs components to human disorders.

Keywords: mitochondria-ER contact sites, protein targeting, post-translational modifications, lipid rafts, ER stress

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INTRODUCTION

The term "synapse" refers to a site at which two neurons are close enough to communicate to each other. Here, electrical or chemical signals are integrated to determine specific responses, such as the generation of action potentials. The concept of synapse and synaptic integration can be extended to other cells. For example, immunological synapses have been defined for T cells

Abbreviations: AD, Alzheimer's disease; ASC, apoptosis-associated speck-like protein containing a CARD; ATF4/6, activating transcription factor 4/6; BIM, Bcl2-interacting mediator of cell death; CHOP, C/EBP-homologous protein; CNX, calnexin; DGAT2, diacylglycerol O-acyltransferase 2; eIF2, eukaryotic translation initiation factor 2; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERMES, endoplasmic reticulum (ER)-mitochondria encounter structure; FACL4, fatty acid-CoA ligase 4; GRP75, 75 kDa glucose-regulated protein; IMM, inner mitochondrial membrane; IP3R, inositol triphosphate receptor; IRE1, inositol requiring enzyme 1; MAM, mitochondria-associated membrane; MERC, mitochondria-ER contact site; MFN1/2, Mitofusin 1/2; MPAN, mitochondrial membrane protein associated neurodegeneration; mTOR, mammalian target of rapamycin; OMM, outer mitochondrial membrane; PACS2, phosphofurin acidic cluster sorting protein 2; PC, phosphatidylcholine; PD, Parkinson's disease; PE, phosphatidylethanolamine; PERK, protein kinase R-like endoplasmic reticulum kinase; PI(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PI3K, phosphoinositide 3-kinase; PrP, prion protein; PS, phosphatidylserine; PSD, phosphatidylserine decarboxylase; PSS1/2, phosphatidylserine synthase 1/2; PTEN, phosphatase and tensin homolog; PTM, post-translational modifications; PTP, permeability transition pore; PTPIP51, protein tyrosine phosphatase interacting protein 51; PUMA, p53 up-regulated modulator of apoptosis; REEP1, receptor expression-enhancing protein 1; ROS, reactive oxygen species; RRBP1, ribosome-binding protein 1; S1/2P, site 1/2 protease; SMP, synaptotagmin-like mitochondrial-lipid binding protein; STBD1, starch binding domain-containing protein 1; SYNJ2BP, synaptojanin-2-binding protein; TMX, thioredoxin-related transmembrane protein; TXNIP, thioredoxin-interacting protein; UPR, unfolded protein response; VAPB, vesicle-associated membrane protein associated protein B; VDAC, voltage-gated anion channel; XBP1, X-box binding protein 1.

(Norcross, 1984; Paul and Seder, 1994; Grakoui et al., 1999; Bromley et al., 2001; Viola et al., 2010): these are the sites at which signaling cascades originating from T cell Receptors are ultimately "decoded and integrated" to achieve either activation or tolerance.

This concept can be further extended to contact sites between intracellular compartments such as those amongst mitochondria and Endoplasmic Reticulum (ER). Mitochondria-ER contacts (MERCs) are sites in which the surfaces of the two organelles juxtapose at a constant distance, for several nm in length. These contacts can be isolated through subcellular fractionation procedures and the membrane fraction corresponding to the MERCs is known as MAMs (mitochondria associated membranes; Vance, 1990; Rusiñol et al., 1994). Thus, MAMs are the biochemical counterpart of MERCs (Giacomello and Pellegrini, 2016).

Cues comprising information from cell growth signaling, metabolic, and stress-responsive programmes are integrated at MERCs, determining cell wellbeing/homeostasis. Therefore, it is not surprising that the disruption of MERCs has been associated with an ever-growing number of pathologies, as an element contributing to the propagation of functional imbalances across cellular systems—such as lipid imbalance and insulin resistant states (Arruda et al., 2014).

MERCs-associated functions, composition, and extension seem to be tailored to specialized tissues- further stressing their relevance for the fine tuning and integration of multiple functions. But how are MERCs defined and how is their specific composition dictated? Viola et al. (2010) proposed that integration of different signaling steps may promote the rearrangement of lipids within membranes, thus providing specialized platforms at which signals can be generated, amplified, or even blunted. Whether this principle applies to MERCs is a standing question to explore. The latter have been already shown to have "lipid raft"-like properties (Hayashi and Fujimoto, 2010; Area-Gomez et al., 2012), although this aspect needs further clarification. Besides "raft"-like domains, MERCs are characterized by the presence of proteins that either tether the two organelles together or dictate their biological function. These "molecular bridges" appear as electron dense rods in EM images (Csordás et al., 2006). Among the potential tethers identified to date, the most studied in higher eukaryotes is Mitofusin2 (Mfn2), first discovered as a key factor for mitochondrial fusion (Chen et al., 2003). Its presence at the surface of the ER and the evidence that ER-located Mfn2 binds to the OMM located Mfn2 and Mfn1 (de Brito and Scorrano, 2008; Naon et al., 2016) strongly suggested its involvement in the control of MERCs. Another key protein required for MERCs assembly and activity is PhosphoAcidic Cluster Sorting protein 2 (PACS2; Simmen et al., 2005): its ablation decreases the interaction between the two organelles and the activities of the MAMs resident proteins phosphatidyl serine synthase 1 (PSS1) and long-chain fatty acid acetyl-CoA synthase (ACSL4; Piccini et al., 1998; Simmen et al., 2005). A third tethering complex proposed for higher eukaryotes includes the integral ER protein Vesicle-Associated membrane Protein associated protein B (VAPB) and the OMM Protein Tyrosine Phosphatase Interacting Protein 51 (PTPIP51). Interestingly, the VAPB-PTPIP51 tethering complex negatively controls autophagy and is dysregulated in frontotemporal dementia (De Vos et al., 2012; Stoica et al., 2014; Gomez-Suaga et al., 2017).

Although these (and others, for a detailed list please refer to **Table 1**, and to: Area-Gomez et al., 2012; De Mario et al., 2016) structural components of MERCs have been uncovered, it is nowadays clear that MERCs display cell-specific tissue composition, as highlighted by a number of proteomic analyses (Poston et al., 2011; Horner et al., 2015; Liu et al., 2015; Sala-Vila et al., 2016). While the interest on the biology of MERCs has recently soared, further systematic studies are required to get a complete view of the MERCs toolkit.

Notably, while proteins involved in the maintenance of lipid and Ca^{2+} homeostasis can be retrieved at MERCs in basal conditions, some proteins enrich in these sub-compartments only upon stimulation. Thus, MERCs, similarly to membrane rafts, function as platforms for composite signal transduction complexes. How proteins are recruited to these "biological interfaces" and retained there still needs to be clarified and is fundamental to understand MERCs physiological role. This review focuses on this aspect, and aims to highlight the principles determining protein enrichment/translocation at MERCs.

MERCs FUNCTIONS AT A GLANCE

As stated above, mitochondria-ER contact sites (MERCs) appear in electron microscopy (EM) as the parallel juxtaposition of the ER surface to the Outer Mitochondrial Membrane (OMM), at a distance ranging from 10 to 80 nm (Giacomello and Pellegrini, 2016). The length and width of the cleft separating both organelles and the protein composition of the communicating membranes are strictly bound to the processes in which MERCs are involved (summarized in **Table 1**). A number of recent reviews have already summarized in detail the role of MERCs in different subcellular pathways (Rowland and Voeltz, 2012; De Mario et al., 2016; Eisenberg-Bord et al., 2016; Prudent and McBride, 2017). Here, we will just provide a quick overview of the main MERCs functions.

The most established roles of MERCs pertain to their contribution to lipid and Ca^{2+} handling. Almost three decades ago, (Vance, 1990) highlighted the importance of MERCs for lipid homeostasis. Indeed these contact sites shape the specific route for phospholipid interconversion, allowing for the synthesis of phosphatidylethanolamine and phosphatidylcholine from serine and contributing to the composition of mitochondrial membranes (Rusiñol et al., 1994; Vance, 2014). These MERCs-associated routes may turn essential under restrictive conditions such as ethanolamine deficiency (Flis and Daum, 2013). Notably while the synthesis of cholesterol and its precursors, minoritary components of the OMM, is located at the ER, they can be converted into other molecules such as steroid hormones in MAMs (Bosch et al., 2011b; Sala-Vila et al., 2016). Thus, mitochondria-ER contacts appear as the sites at which coordination among lipid homeostasis and other cell functions occurs.

TABLE 1 | Overview of the main MERCs functions and actors.

MERCs Function	MERCs main players (gene symbol)	References
Ca^{2+} homeostasis	ATP2A1	Chami et al., 2008
	HSPA5	Hayashi and Su, 2007
	HSPA9	Szabadkai et al., 2006
	ITPR	Szabadkai et al., 2006
	MFN2	de Brito and Scorrano, 2008
	PSEN2	Zampese et al., 2011
	PTPIP51	Stoica et al., 2014
	SYGMAR1	Hayashi and Su, 2007
	VDAC	Szabadkai et al., 2006
Lipid homeostasis	ACAT1	Rusiñol et al., 1994
	CAV1	Sala-Vila et al., 2016
	ERLIN 2	Browman et al., 2006
	FACL4	Lewin et al., 2001
	OSBPL	Galmes et al., 2016
	PEMT	Cui et al., 1993
	PTDSS1-2	Stone and Vance, 2000
	REEP1	Cajigas et al., 2012
	SERAC1	Wortmann et al., 2012
	STX17	Hamasaki et al., 2013
	SYGMAR1	Hayashi and Su, 2007
	VAPB	Stoica et al., 2014
Mitochondrial dynamics	DNM1L	Friedman et al., 2011
	FIS1	Iwasawa et al., 2011
	FUNDC1	Wu et al., 2016
	MARCH5	Sugiura et al., 2013
	MFF	Elgass et al., 2015
	MFN2	de Brito and Scorrano, 2008
	MIEF1	Elgass et al., 2015
	MIEF2	Elgass et al., 2015
	PACS2	Simmen et al., 2005
Autophagy/mitophagy	AKT	Betz et al., 2013
	ATG5	Hamasaki et al., 2013
	ATG14L	Hamasaki et al., 2013
	FUNDC1	Wu et al., 2016
	MTOR	Betz et al., 2013
	PARK2	Calì et al., 2013
	PINK1	Cajigas et al., 2012
	STX17	Hamasaki et al., 2013
	ZFYVE1	Hamasaki et al., 2013
Immune response	NLRP3	Zhou et al., 2011
	p66Shc	Lebiedzinska et al., 2009
	PML	Giorgi et al., 2010
	PTEN	Bononi et al., 2013
	PTPIP5	Stoica et al., 2014
	PYCARD	Zhou et al., 2011
	RAB32	Bui et al., 2010
	TXNIP	Zhou et al., 2011
ER homeostasis	ERN1	Mori et al., 2013
	SIGMAR1	Hayashi and Su, 2007
	EIF2AK3	Verfaillie et al., 2012
	CANX	Myhill et al., 2008
	ERO1A	Gilady et al., 2010

MERCs are also the site of Ca^{2+} exchange between the two organelles: they host a protein complex composed of the inositol triphosphate receptor (IP3R), the voltage-dependent anion channel (VDAC) and the chaperone grp75 (Szabadkai et al., 2006), which allows for rapid mitochondrial Ca^{2+} uptake through the Ca^{2+} Uniporter. The efficient shuttling of Ca^{2+} between both organelles depends on the width of the cleft that separates them: an optimal length of 15–25 nm allows both the assembly of the IP3R-grp75-VDAC complex and a fast Ca^{2+} exchange; on the contrary, a distance below 10 nm impedes the formation of the complex due to steric hindrance (Csordás et al., 2006). On the other hand, a distance above 25 nm would decrease the Ca^{2+} diffusion rate and hence blunt mitochondria Ca^{2+} uptake (Giacomello and Pellegrini, 2016). Mitochondral Ca^{2+} levels impinge on the activity of pyruvate, isocitrate, and α-ketoglutarate dehydrogenases (Denton et al., 1972, 1978), and hence on cell bioenergetics (Cárdenas et al., 2010). On the other hand, if pronounced, a sustained mitochondrial Ca^{2+} uptake can be read as a cell death signal able to trigger permeability of the mitochondrial membranes and opening of the permeability transition pore (PTP) (Bernardi et al., 2001; Hurst et al., 2017). Thus, MERCs also contribute to determine the cell fate (Simmen et al., 2005; Bui et al., 2010; Iwasawa et al., 2011; Prudent and McBride, 2017).

More recently, a bunch of additional functions have been ascribed to MERCs. For example they have been proposed as the site of autophagosome formation, thus playing a key role in autophagy (Hamasaki et al., 2013; Martínez-Pizarro et al., 2016). MERCs appear to couple mtDNA synthesis with mitochondrial division, that is also regulated by the interaction between mitochondria and ER (Friedman et al., 2011; Elgass et al., 2015; Lewis et al., 2016), and can behave as a scaffold that ultimately coordinates immune signaling and inflammasome formation (Lerner et al., 2012; Horner et al., 2015).

Finally, in yeast, mitochondria-ER interaction appears also fundamental for appropriate maintenance of cellular iron homeostasis and mitochondrial biogenesis (Wu et al., 2016; Ellenrieder et al., 2017; Xue et al., 2017).

SUBCELLULAR LOCALIZATION: TARGETING SEQUENCES AND MORE

Protein subcellular distribution relies on several mechanisms (**Figure 1**). The most common is the presence of a targeting peptide within the protein, which determines its sorting to specific sites. Two questions must be considered in this case: whether specialized or "consensus"-based mechanisms exist, and whether they are subjected to regulation.

So far a robust consensus motif targeting proteins to MERCs has not been defined. Localization at the OMM or ER surface appears sufficient for a protein to be retrieved at MAMs, in a proportion that varies depending on the cell type, culture conditions, oxidation state, or specific metabolic status of the cell. For example, ∼90% of the chaperone calnexin (CNX) is homogenously spread in the ER in basal conditions, concentrating at MERCs up to 70% under specific (stress) stimuli

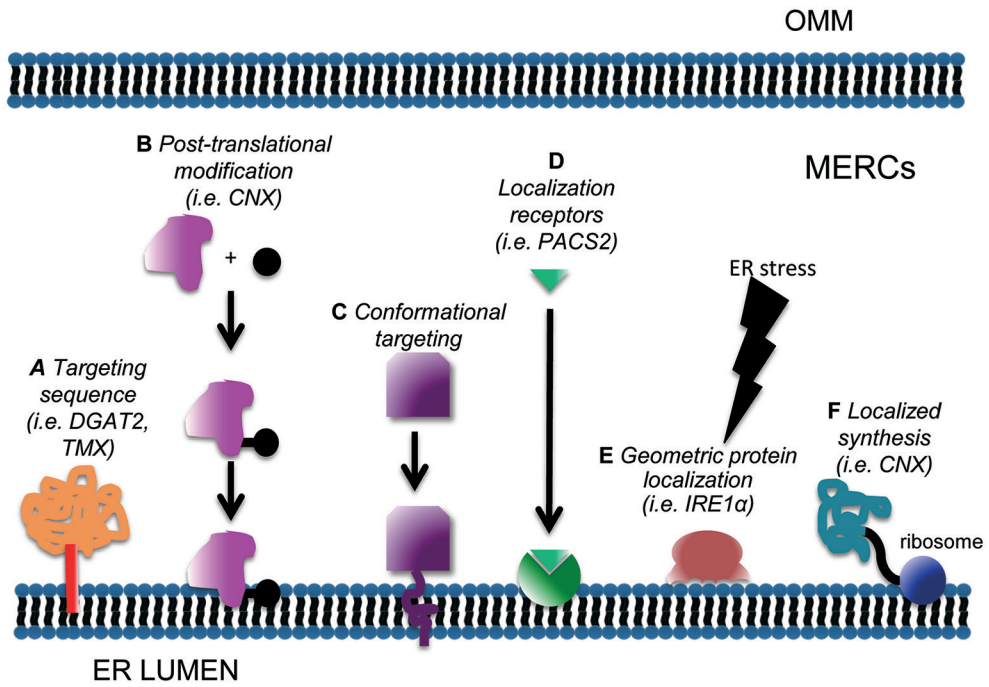

FIGURE 1 | Schematic of subcellular mechanisms for protein targeting at MERCs. Enrichment at mitochondria-ER interface can be achieved through classical targeting sequences **(A)**; post-translational modifications such as phosphorylation, acetylation or sumoylation **(B)**; conformational targeting **(C)**, localization signal receptors **(D)**, geometric protein localization **(E)** and localized synthesis **(F)**.

(Lynes et al., 2012). To date, only a few putative MERCs-targeting signals have been identified: this is the case for example of a stretch of 67 aa in the cytoplasmic N-terminus of DGAT2 (Stone et al., 2009). Another peculiar motif resides in the transmembrane and cytosolic domains of the transmembrane thioredoxin protein TMX, which are "necessary and sufficient" to ensure TMX accumulation at MAMs (Lynes et al., 2012). As stated above, a unique MERCs-targeting motif has not been identified yet. This could depend on the requirement of post-translational modifications (PTMs) and/or conformational determinants. PTMs as mechanisms for the regulated targeting of proteins at MERCs will be discussed separately in the following section, since a number of evidence substantiating this possibility have been already reported. Another mechanism both hampering the identification of "consensus target motifs" and rendering "alternative compartmentalization" possible might reside in mRNA processing. This remains still a hypothesis, since evidence for the existence of alternative splicing programmes dictating protein localization at MERCs is still lacking.

Another hypothetical MERCs-targeting mechanism would be the existence of "conformational" motifs. That is, a domain composed not by a linear stretch of amino acid, but by a functional interaction surface determined by the rearrangement of the 3D structure of the protein. The possibility of a "conformational" domain appears particularly intriguing for MERCs, since it would provide a mean for switchable recruitment of proteins. If this possibility holds true, it would perfectly match with the highly plastic lipid environment of MERCs. In fact, one of the means for PTMs-regulated targeting to MERCs might rely on such conditional conformational state (see below). Molecular threading for "tridimensional alignment" has been classically very challenging in terms of computational power requirements, but recent advances may ease these approaches to study conformational MERCs-targeting domains.

Another widespread mechanism for regulated protein compartmentalization relies on masking of localization domains: interaction with other partner molecules may confine them into the cytosol or other subdomains. This mechanism usually keeps "silent" (i.e., inactive) a certain protein until its release, which induces its re-localization at sites where specific interacting partners and/or target functions are (Bauer et al., 2015). Theoretically the concept of domain masking could be extended also to MERCs: targeting motifs would be exposed only if the specific function of the protein of interest is needed at these sites.

Subcellular targeting relies also on appropriate "localization signal receptors" (Bauer et al., 2015). This term denotes the presence of a sequestering/scaffolding protein able to bind its ligand and restrict its diffusion. Sequestration of a protein implies that the density of binding sites within a subcellular domain is high enough to significantly limit its mobility toward other locations. Interestingly, the affinity of a localization signal for its receptor can be modulated, especially through PTMs such as phosphorylation, lysine acetylation, or SUMOylation of either the receptor or of the ligand. Changes in the affinity of this interaction can either increase or decrease the compartmentalization of the ligand, by unveiling or,

alternatively by masking, any signaling peptide. Thus, PTMs would exert their function not only by modulating the activity of proteins, but also by controlling their localization. As a consequence, they can shape the composition (and hence the function) of subcellular compartments. As to MERCs, an example of localization signal receptor is PACS2, which mediates the localization and enrichment of the CNX at these sites (Myhill et al., 2008). It is interesting to note that not only proteins, but also lipids and phospholipids could behave as localization receptors. For example, it has been shown that phosphatidylinositol (3,5) diphosphate can act as a membrane-targeting molecule, mediating the binding of different proteins to biological membranes (Ferguson et al., 2009; Salminen et al., 2013). Due to the special lipid composition of MERCs, we further elaborate in a separate section on this topic (see below).

Another common protein targeting strategy is "localized synthesis" (Kejiou and Palazzo, 2017). mRNAs encoding for a given polypeptide can be localized to specific subcellular domains where they are either kept silent, waiting for specific stimuli to trigger translation, or efficiently translated if necessary in basal conditions (Kejiou and Palazzo, 2017). Classical examples of spatial protein segregation by localized synthesis can be found in neurons (Rangaraju et al., 2017). Here, localized production is required to quickly shape the response of neurites to the signals coming from synapses. Interestingly, a study conducted to describe the local transcriptome in the synaptic neuropil of CAI Hippocampus (i.e., the mRNA enriched in this specific cell subdomain) highlighted the presence of MERCs resident or regulatory proteins, among which also calnexin, mTOR, Pink1, presenilin2, REEP1, Sigma-1 Receptor, α-synuclein, VAPB (Table S10 of Cajigas et al., 2012). These data support the hypothesis that local translation also contributes to the plasticity of MERCs, that with their activity could in turn match the needs of specialized cell structures.

Finally it's worth mentioning two additional mechanisms, although yet unproven for protein targeting at MERCs. A first one has been termed "geometric protein localization" (Ramamurthi et al., 2009; Updegrove and Ramamurthi, 2017). Mostly studied in bacteria, it relies on the ability of some proteins to "sense" membrane curvature and bind to specific geometric cues. In this case protein localization will be dictated by the shape of the membrane, independently of its composition (Hatzakis et al., 2009; Bhatia et al., 2010). These proteins are often characterized by membrane-binding amphipathic helices (Updegrove and Ramamurthi, 2017) that recognize even small changes in the curvature of membranes and enrich at these special sites. Interestingly, some curvature-sensing proteins have been reported in eukaryotes: this is the case of dynamins (Ramachandran and Schmid, 2008), cytochrome b5 (Taylor and Roseman, 1995), and interestingly, the ER stress-transducer IRE1 (Halbleib et al., 2017; see below). Recently, it was also reported that membrane-anchored proteins can efficiently sense membrane curvature, the latter being an additional mechanism for their efficient clustering (Hatzakis et al., 2009). As to MERCs, so far they have been characterized and described according to specific parameters: the relative length of the ER surface portion that run in parallel to the OMM and the width of the cleft that separates the two organelles (Giacomello and Pellegrini, 2016). Whether they are characterized by a particular membrane curvature range remains, at least to our knowledge, to be defined. The study of "geometric protein localization" at MERCs remains also challenged by the lack of appropriate readouts, compatible with the manipulation of such properties specifically at those sites.

The second mechanism, already shown to operate in the context of ER segregation and partitioning in yeast, pertains to the establishment of "protein boundaries" able to restrict the lateral diffusion of other membrane components (Chao et al., 2014). In this case, cytoskeleton-associated proteins (septins) are the effectors of such compartmentalization. Intriguingly, MERCs constitute in some experimental models points for actin- and microtubule-assisted mitochondrial fission (Ji et al., 2015; Prudent and McBride, 2016). So far, no clear connection has been established between MERC composition and cytoskeletal components, nor septins.

POST-TRANSLATIONAL MODIFICATION: "LOCALIZATION" OR "RELOCATION" AT MERCs?

PTMs are normally used by the cell to modulate the activity, stability, interaction profile and/or subcellular segregation of proteins. MERCs components are no exception: substantial evidence exists for multiple PTMs fine-tuning their properties, including their localization. As such, most MERCs proteins subjected to PTMs are characterized by (at least) a dual localization: either they have a broad subcellular distribution (for example, at the cytosol, or at the ER, or at the OMM), and then they enrich at MAMs, or the reverse (i.e., they appear located at MERCs, and upon PTMs, they redistribute to other subcellular compartments).

The first possibility applies to CNX. Its binding to PACS2 appears not sufficient for its enrichment at MERCs (Myhill et al., 2008), palmitoylation being the additional trigger necessary for its complete relocation at these interfaces (Lynes and Simmen, 2011). Examples have also been described for the second scenario. Impairment of ER oxidizing conditions causes Ero1α to lose its MAMs localization (Gilady et al., 2010), while heme oxygenase-1 relocates from MAMs to rough ER in the absence of palmitoylation (Lynes and Simmen, 2011). Another example is N-myristoylation: when the carbohydrate-binding protein starch binding domain-containing protein 1 (Stbd1) undergoes this modification, it is mostly retrieved on the ER, its wild type form being on the contrary enriched at MAMs "by default" (Demetriadou et al., 2017).

Generally speaking, PTMs also represent a switch for the activity of several enzymes. Thus, PTMs might control MERCs activity in a dual fashion: on one side, through a modulatory function, by directly enhancing or decreasing the activity of a MERCs-resident enzyme; on the other, by enhancing the contribution of MERCs to a subcellular process through re- or delocalization of specific proteins. Transient PTMs-dependent recruitment at MERCs may explain, at least

in part, why changes in the expression levels of some proteins have a profound effect on ER-mitochondria apposition, despite they are not enriched in purified MAM fractions (Wieckowski et al., 2009; Eisenberg-Bord et al., 2016; Naon et al., 2016). Such an example has recently been reported for a novel OMM-ER tethering complex composed by two proteins broadly distributed to the OMM and ER surface: SYNJ2BP and RRBP1 (Hung et al., 2017). These data support the hypothesis of "auxiliary tethers," according to which some proteins would not be strictly necessary to form a (functional) contact site, but they would be able to do so whenever their MERCs-related function was needed (Eisenberg-Bord et al., 2016).

"LIPID RAFT"-LIKE BEHAVIOR AS A MECHANISM FOR COMPARTMENTALIZATION AT MERCs

Another layer for regulating MERCs compartmentalization comes from the distinct lipid composition of the membrane domains delimiting MERCs (**Figure 2**). The concept of dynamic membrane nanodomains or "lipid rafts" initially introduced by Simons and van Meer (1988) almost 30 years ago states that one of the key properties of such membrane patches is the efficient accruing and stabilization of transmembrane proteins and membrane-associated activities (Simons and Sampaio, 2011). Correspondences between mitochondria-ER contacts and generic lipid rafts have been highlighted (Area-Gomez et al., 2012; Annunziata et al., 2013): MAMs have a significantly higher content in cholesterol and sphingolipids as compared with bulk ER membranes (Annunziata et al., 2013; Vance, 2014; Sala-Vila et al., 2016); they seem to have a lower degree on curvature as compared with surrounding ER regions (Rowland and Voeltz, 2012).

The "lipid raft"-like organization of MERCs could help to explain some of their properties and functions. As mentioned above, it would induce the stabilization and limitation of lateral diffusion of ensembles of membrane proteins, or "polarization"—a property that would benefit the initial localization and subsequent sequential assembly of tethering complexes. The full collection of MERCs components in higher eukaryotes remains to be listed, but certain properties of the better-defined tethering complexes in yeast (termed ERMES) support this concept. First, ERMES components exhibit aberrant distribution if expressed in the absence of their partners (Kornmann et al., 2009, 2011; Stroud et al., 2011). Second, analysis of their structure revealed that their membrane-binding domains are fully functional only if they form complexes with appropriate stoichiometry, and only in this case they can recognize organelle contact sites. Thus, it seems that (a) MERCs lipid composition is an essential, but not a

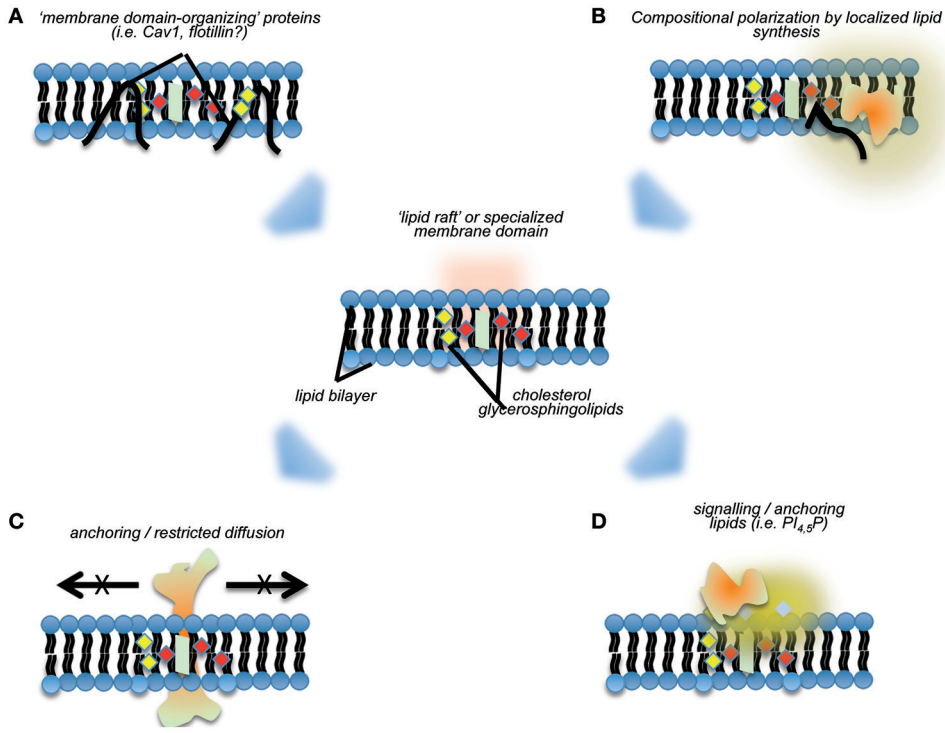

FIGURE 2 | Cartoon summarizing aspects pertaining to the organization of "nanodomains" on membranes, and their potential effects regarding recruitment of specific activities. "Lipid raft"-like domains may be stabilized by specialized membrane-binding proteins, such as caveolins **(A)**, and/or through directional local synthesis and accumulation of specific lipid species **(B)**. The subsequent definition of such membrane nanodomains may preclude lateral diffusion of specific transmembrane proteins **(C)**, and or act as "molecular beacons" for the specific recruitment of proteins **(D)**.

sufficient, feature to ensure MERCs targeting; and (b) cooperative recruitment may ensure the regulated, reversible assembly of tethering complexes. The differential lipid composition of MERCs appears critical for the targeting of specific proteins to this subcellular compartment in higher eukaryotes, a major example being constituted by the Prion protein (PrP^C). While in basal conditions PrP^C localizes mostly at the plasma membrane, pro-apoptotic stimuli can induce its relocalization to the MAMs, transducing a pro-death signal from the surface into the cell (Mattei et al., 2011).

Local synthesis and modification of lipids are hallmarks of lipid rafts. These have been proposed as potential mechanisms for the formation and stabilization of these nanodomains: a directional flux of specific lipid building blocks might favor *per se* the establishment of regions characterized by differential composition (Simons and Sampaio, 2011). A major subset of processes enabled by MERCs comprises lipid anabolism and trans-organelle transport of lipids (a classical example being phospholipids, see above). Furthermore, recent surveys highlight the enrichment of lipid metabolism functions at MERCs, including cholesterol synthesis and modification and fatty acid catabolism (Sala-Vila et al., 2016). A still open question is whether such local lipid metabolism sustains the differential composition of MERCs or not. Intriguingly, and further related to these observations, membrane proteins typically ascribed as lipid raft scaffolds/organizers, such as caveolin-1 (Cav1) are specifically enriched at MERCs. Cav1 is a cholesterol-binding protein famous for its role as an essential scaffold of plasma membrane nanodomains named *caveolae* (Parton and del Pozo, 2013). However, Cav1 also assembles in oligomers in the ER and determines cholesterol trafficking across subcellular compartments-including MERCs (reviewed in Bosch et al., 2011a). Cav1 genetic ablation is associated with increased cholesterol content and altered composition of MAMs derived from hepatocytes: Cav1 absence preferentially affects MAMs components involved in cholesterol and fatty acid metabolism, thus stressing the importance of lipid precursor fluxes for the organization and stabilization of these organelle contacts (Sala-Vila et al., 2016).

Altogether, these evidences favor another emerging function of MERCs, acting as "gauges" for lipid homeostasis in the cell. Similarly to plasma membrane lipid rafts, MERCs are sensitive to conditions disrupting or causing imbalances in lipid metabolism and membrane composition (Zhuang et al., 2005; Vance, 2014). Since MERCs are a platform in which many cell pathways converge, their "design" as elements highly sensitive to changes in lipid homeostasis allows for the integration of virtually all those signaling networks with lipid homeostasis. Thus, their dynamics might contribute substantially to phenomena such as dyslipidemia-associated modulation of proteostasis (see below) or insulin resistance (Arruda et al., 2014; Tubbs et al., 2014). Indeed, key regulators of the Pi3K/AKT/mTOR pathway have been reported to specifically localize at MERCs (Betz et al., 2013; Bononi et al., 2013). It is likely that the lipid raft-like properties of MERCs drive such recruitment and that of additional molecular beacons like phosphatidylinositol phosphate species (Hill et al., 2002; Goswami et al., 2005; Simons and Sampaio, 2011).

UPR AND ER STRESS SIGNALING AT MERCs

Besides being the site for the synthesis, folding, and maturation of secreted and organelle-targeted proteins (Braakman and Bulleid, 2011), the ER also allocates other essential tasks, including lipid homeostasis and mobilization, red/ox control and Ca^{2+} flux regulation. Therefore, the ER constitutes a "hub" through which specific imbalances (i.e., dyslipidemia) can be easily propagated to other cellular systems, underpinning complex pathogenic processes such as obesity-related diseases and cancer. Eukaryotes have evolved a complex surveillance system to cope with functional imbalances in the ER, generally termed ER stress: the Unfolded Protein Response (Ellgaard and Helenius, 2003; Chakrabarti et al., 2011; UPR). UPR regulates either pro-survival programmes, aimed at enhancing ER capacity and/or lowering its functional demand; or pro-death pathways, in case of sustained unresolved ER stress (Ron, 2002; Rutkowski and Kaufman, 2004; Naidoo, 2009).

The UPR includes three signaling branches, associated with three ER-resident transmembrane transducers. The first is the Activation Transcription Factor-6 (ATF6). ATF6 is translocated to Golgi membranes, where it is sequentially cleaved by the S1P and S2P proteases (Haze et al., 1999). This yields an N-terminal fragment which acts as a leucine-zipper transcription factor and drives the expression of adaptive programmes. ATF6 signaling leads to the induction of the turnover system "ER Associated Degradation" (ERAD) and of ER chaperones (for a more detailed overview of UPR and ERAD, please refer to Yoshida et al., 2000; Okada et al., 2003; Galehdar et al., 2010; Tsai and Weissmann, 2010; Smith et al., 2011; Hetz, 2012; Arensdorf et al., 2013). The second branch relies on PRKR-like endoplasmic reticulum kinase (PERK), one of the four eIF2alpha-kinases expressed in higher eukaryotes. Upon "sensing" alterations in ER function or integrity through its luminal domain and its transmembrane segment, PERK oligomerizes and becomes catalytically active, repressing mRNA pools and thus reducing the ER load (Harding et al., 1999). PERK activation also favors translation of the activation transcription factor 4 (ATF4), which controls the expression of master regulators of cell survival and apoptosis (Lu et al., 2004). The third and most conserved UPR transducer is the inositol-requiring enzyme 1 (IRE1, of which two isoforms exist, being IRE1α the essential and most ubiquitous one; Tirasophon et al., 1998). IRE1 catalyzes the unconventional splicing of the X-box binding protein 1(XBP1) mRNA, yielding to a potent transcriptional activator that orchestrates adaptive programmes like the physical expansion of the ER itself (Harding et al., 1999; Yoshida et al., 2001; Calfon et al., 2002; Hetz et al., 2006).

As an integral part of ER, it is not surprising that MERCs function and structure are linked to UPR signaling. IRE1 has been found in MAMs, and in turn the ER-mitochondrial interaction significantly impacts on its activation (Mori et al., 2013). In particular, upon acute ER stress, the MERCs-resident chaperone Sigma-1 receptor (SigR1) stabilizes IRE1α, thus favoring its activation and UPR initiation (Hayashi and Su, 2007; Mori et al., 2013). PERK has also been retrieved in

MAMs (Verfaillie et al., 2012), where it directly associates with Mfn2. This interaction seems to increase the activation threshold of PERK (Muñoz et al., 2013). Further, conditions disrupting MERCs, such as Mfn2 knockdown, are associated with a sustained activation of some UPR/ER stress response transducers even in basal conditions (Ngoh et al., 2012; Sebastián et al., 2012; Schneeberger et al., 2013). Recent evidence suggests that changes of MERCs dynamics can influence not only UPR triggering thresholds and amplitude, but also UPR shutdown dynamics. For example, the MERCs stabilization appears as an essential component to induce IRE1 shutdown during ER stress recovery (Sanchez-Alvarez et al., 2017). Conversely, MERCs also coordinate cell functioning and UPR activation: increased coupling of ER and mitochondria accompanies early phases of ER stress and sustains the metabolic adaptations necessary for the cell to cope with non-physiological conditions (Bravo et al., 2011). Further potential ties between MERCs and adaptive UPR signaling pertain to activities determining red/ox potential in the ER. For example, protein disulfide isomerases such as PDIA6 accrue at MERCs (Vance and Vance, 2009) and regulate IRE1 activation (Eletto et al., 2014, 2016). ER stress associated with ROS dysregulation is likely transduced by the PERK-dependent branch at MERCs too (Verfaillie et al., 2012). Hence, the confinement of UPR transduction at MERCs appears fundamental for the integration of the UPR response with multiple signaling pathways.

Does ER homeostasis surveillance influence MERCs composition? Specific adaptations at MERCs take place during ER stress. In these circumstances expression levels of CNX at the plasma membrane decrease (Wiest et al., 1995; Okazaki et al., 2000), increasing in parallel at MAMs (Myhill et al., 2008; Lynes et al., 2012). Another prominent example are programmes favoring cell apoptosis in the face of unresolved or excessive ER stress. Sustained PERK activation contributes to stabilize MERCs, enabling lipid peroxidation at the mitochondrial membrane-which in turn enhances expression and/or mitochondrial recruitment of proapoptotic regulators such as Bax and Ca^{2+} uptake (McCullough et al., 2001; Puthalakath et al., 2007; Galehdar et al., 2010; Verfaillie et al., 2012). A peculiar case of MERCs targeting regulated by UPR is embodied by IRE1 and PERK themselves. As stated above, they continuously monitor misfolded protein levels in the ER lumen through specialized domains. Deletion mutants of yeast IRE1 and of vertebrate IRE1 and PERK lacking luminal domains appear insensitive to acute protein misfolding, but retain sensitivity to conditions altering ER composition or physical properties, such as increased global acyl chain saturation or cholesterol content (Brodsky and Skach, 2011; Volmer et al., 2013; Volmer and Ron, 2015). At least in the case of IRE1, such "membrane monitoring" relies on the features of its transmembrane domain (Halbleib et al., 2017; Kono et al., 2017). The latter mechanism is likely to determine the segregation of UPR transducers to MERCs, possibly in combination with other regulatory layers such as transient dimerization or conformational changes. Recruitment of these UPR sensors at MERCs further contributes to the integration between UPR signaling and cell metabolism (Walter and Ron, 2011).

DISRUPTED MERCs LOCALIZATION: POTENTIAL IMPACT IN HUMAN DISORDERS

In general, it is well-established that defective subcellular localization can either alter the activity of a protein and/or the subcellular processes in which it is involved. If we extend this concept to MERCs and we take into account that they participate in a myriad of essential process (see above), it seems obvious that their alteration or adaptation to stress conditions could both worsen and propagate imbalances across cellular systems—a phenomenon that appears to be common-place for complex diseases. For instance, the disruption of general mechanisms impacting on MERCs, such as protein palmitoylation, could simultaneously affect their integrity and that of other cellular functions. This could be the basis of phenotypic variability and epistatic effects across many different disorders, ranging from schizophrenia and other neurodegenerative disorders to tumor development (Giorgi et al., 2010; Mórotz et al., 2012; Sander et al., 2015).

MERCs dysfunctions could be caused not only by mutations of proteins that exert their function at these sites, but also by impaired targeting of MERCs-resident proteins. The subsequent pathological conditions associated to MERCs defects will be more evident in tissues where that specific MERCs protein is mostly expressed/active. This is the case, for example, of CNX. CNX acts mostly as a chaperone for glycoproteins, which are key molecules for the development and maintenance of myelin structure (Denzel et al., 2002; Quarles, 2002). Hence, it is predictable that defective CNX would cause myelinopathy: this is actually the case, as confirmed in CNX knockout mice (Kraus et al., 2010). Demyelination has diverse causes, such as for example mutations in myelin basic proteins or altered activity of enzymes responsible for the production of cholesteryl esters: defective MERCs activity should be added to the list of possible mechanisms underlying it.

As reported above, MERCs have been implied in metabolic diseases, like obesity and diabetes (Tubbs and Rieusset, 2017). Hepatocytes from obese mice are characterized by increased coupling between mitochondria and ER, and the consequent mitochondrial Ca^{2+} overload is paralleled by higher mitochondrial reactive oxygen species (ROS) production and abnormal glucose metabolism (Arruda et al., 2014). This phenotype can be ameliorated upon silencing of PACS-2 and IP3R1, leading to lower cell stress and increased glucose tolerance (Arruda et al., 2014).

Another MERCs protein, Mfn2, besides being the genetic cause of an inherited peripheral neuropathy (Charcot Marie Tooth 2a), has also been associated with metabolic dysfunctions (Sebastián et al., 2012; Boutant et al., 2017). A recent study highlighted that metabolic transitions in liver are accompanied by changes in the MERCs structure (Sood et al., 2014), further suggesting that MERCs play an active role in metabolic processes: hence, even mild dysfunction of MERCs could exacerbate a given pathological condition.

Downregulation or mutations of a protein that regulates broad physicochemical properties of MERCs may alter the recruitment

or stability of defined subsets of MERCs components, thus preferentially impacting specific functions or metabolic routes. An example of such scenario is showcased by models of genetic deficiency in Cav1 protein. Quantitative proteomic profiling of MAM fractions purified from livers of Cav1KO mice shows a depletion of steroid metabolism and fatty acid catabolism regulators (Sala-Vila et al., 2016). It remains to be elucidated whether these changes are due to aberrant membrane composition (i.e., high free cholesterol), and which is their contribution to the metabolic phenotypes associated with Cav1 deficiency (i.e., lipodystrophy and metabolic inflexibility; Bosch et al., 2011b; Fernández-Rojo et al., 2013; Parton and del Pozo, 2013). MERCs-associated lipid metabolism might be of relevance not only for metabolic phenotypes: it could add to the pathogenesis of neurodegenerative disorders like Alzheimer's disease (AD). One of the main gene products associated to familial cases of AD (presenilins, PSs) is enriched in MAMS, although the link between MAMs and sporadic AD is less obvious (Zampese et al., 2011). Importantly, a genetic connection exists between phospholipid/cholesterol dyshomeostasis and AD (Mapstone et al., 2014; Chang et al., 2017). Further, inhibition of cholesterol transport impairs PSs localization at the ER, inducing their accumulation in vesicles and enhancing the production of the main component of AD neurofibrillary tangles, Aβ (Runz et al., 2002). Finally, an apolipoprotein E variant associated with higher risk of lipid metabolism-associated disorders (ApoE4) specifically alters MERCs lipid metabolism and favor AD-like changes *in vitro* (Area-Gomez et al., 2012).

Another interesting example of a potentially pathological mislocalization of a MERCs component is C19orf12. Mutations in this protein, whose physiological function is yet unknown, are the genetic cause of Mitochondrial Membrane Protein Associated Neurodegeneration (MPAN, Hartig et al., 2013). This severe, early-onset pathological condition is characterized by optic atrophy, generalized dystonia, neuropathy, and psychiatric symptoms. Interestingly, C19orf12 has been retrieved at mitochondria, ER and MAMs, and its mutated forms appear to mislocalize. The evidence that fibroblasts from MPAN patients are characterized by higher mitochondria Ca^{2+} uptake suggest that this protein somehow regulates MERCs function, its localization likely causing enhanced ER-mitochondria Ca^{2+} transfer and hence increased sensitivity to apoptosis (Venco et al., 2015). Notably, C19orf12 mutations have been linked to Parkinson's Disease (PD), strengthening the possibility that PD is linked to defective MERCs function. This hypothesis is substantiated by a number of additional findings. For example α-synuclein (α-syn), a protein whose mutations are linked to PD, has been also retrieved in MAMs (Eliezer et al., 2001; Jao et al., 2008; Guardia-Laguarta et al., 2014). The group of E. Schon demonstrated that mutated α-syn has lower affinity for MERCs, thus challenging the theory that mutant α-syn are "gain of function", and favoring a "loss of MAMs function" hypothesis (Guardia-Laguarta et al., 2014, 2015). Additional data support defective MERCs contribution to the etiology of PD: mutants for parkin (PARK2), DJ-1 (PARK7), and PINK1 (PARK6), all causing recessive early-onset PD cases, can impact on ER-mitochondria tethering, mitochondrial quality control, and Ca^{2+} transfer between the two organelles (Li et al., 2005; Narendra et al., 2008; Davison et al., 2009; Ziviani et al., 2010; Calì et al., 2013).

Mutated MERCs proteins are not the only reason for the development of MERCs linked diseases or symptoms: being "raft"-like domains, changes in lipid homeostasis could exert deleterious effects on their structure/composition. One of such example could be atherosclerosis. It has been shown that an ER overload of cholesterol in murine macrophages causes prolonged ER stress and UPR activation culminating in apoptosis, substantially contributing to the progression of this disease (Tabas, 2002; Feng et al., 2003). An intriguing question is whether MERCs are modulated in arterial wall cell populations at different stages of atherosclerosis progression, and what their pathogenic impact may be. Notably, MERCs could also take part to the inflammatory response involved in such pathologies, as they have also been involved in the activation of the inflammasome complex and ILβ production, although this needs to be more deeply investigated (Zhou et al., 2011; Lerner et al., 2012; Marchi et al., 2014).

Altogether these findings suggest that defective MERCs localization is likely not only to constitute discrete, primary elements of pathogenesis, but also to be a source of epistatic effects underlying the impact of additive risk factors.

CONCLUSIONS

In the last years the interest on MERCs biology has exponentially grown, due to the evidence that at these interfaces many biological processes integrate and that MERCs defects underlie several pathological conditions. Many proteins have been retrieved in the biochemical counterparts of MERCs (that are, MAM fractions, see above) but so far the mechanisms responsible for targeting at MERCs have yet not been fully elucidated. Interestingly, post-translational modifications such as palmitoylation, miristoylation, and oxidation seem to gain the upper hand over a more canonical targeting signal. Another standing question pertains as to how special conditions, such as ER stress, specifically contribute to determine MERCs composition and hence functional state. It is likely that several independent features of an ER-stressed cell take part to such remodeling. Acute ER stress is frequently associated with alteration (mostly *attenuation*) of signaling pathways which are considered to stabilize MERCs, namely AKT-mTOR signaling (Betz et al., 2013), or with increased ER Ca^{2+} levels, that could in turn enhance (at least in early stress phases) mitochondria-ER proximity as an adaptive response for the maintenance of intracellular Ca^{2+} homeostasis (Bravo et al., 2011). Chronic ER stress as well can induce MERCs remodeling: for example, lipid imbalance associated with obesity might promote connectivity between the two organelles, in an attempt to restore equilibrium among different lipid species and to exert a tighter control on Ca^{2+} homeostasis, which is significantly perturbed in such dyslipidemic states (Fu et al., 2011; Arruda et al., 2014).

Overall, despite a small number of elegant studies on MERCs targeting mechanisms and protein relocation at MAM fractions

have been published (such as for example, Myhill et al., 2008; Lynes et al., 2012), this aspect in the field of MERCs biology appears to be just at its infancy. Exciting findings lie ahead, and their discovery will certainly represent another step forward into the complexity of cellular signal transduction, as well as in the understanding of pathological processes.

AUTHOR CONTRIBUTIONS

MG, MS-Á, and MD: conceived, designed, and wrote the manuscript; NI, MB, and VC: drafted and revised the article; MS-Á and VC prepared figures.

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Mitochondria and Lysosomes: Discovering Bonds

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In the last decade, the traditional view of lysosomes has been challenged by the recognition that lysosomes are not only degradative organelles, but also metabolic sensors that play a key role in the regulation of metabolism and cell growth. Similarly, mitochondria are now seen as crucial metabolic hubs dictating cell fate decisions, not just ATP-producing machines. Importantly, these functions are generally performed as a coordinate response of distinct organelles that are physically and functionally connected. While the association between mitochondria and the endoplasmic reticulum is well known, a similar interaction between mitochondria and lysosomes is now emerging. This interaction could be required to shuttle amino acids, lipids and ions such as Ca^{2+} between the two organelles, thereby modulating their metabolic functions. In addition, a tethering complex linking the two organelles has recently been described in yeast, although the mammalian counterpart has yet to be identified. Here, we discuss the implications of these recent findings.

Keywords: mitochondria, lysosome, TFEB, reactive oxygen species, Ca^{2+}, inter-organelle contact site

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INTRODUCTION

In the last decade, there have been major changes in the way we understand the function of organelles. For example, lysosomes, acidic organelles that contain an array of hydrolases required for the degradation of macromolecules, have historically been known as degradation centers. However, we now know that they also play a key role in nutrient sensing, and act as a reservoir for amino acids and ions such as Ca^{2+} (Reviewed in Appelqvist et al., 2013; Ballabio, 2016). Similarly, mitochondria, the "powerhouse of the cell," are much more than an ATP factory. They perform a diversity of cellular roles (reviewed in Patten et al., 2010; Nunnari and Suomalainen, 2012; Mailloux et al., 2013; Raimundo, 2014), including many of the key reactions of intermediary metabolism, control of apoptosis, reactive oxygen species (ROS) signaling, and control of cellular differentiation during development (Kasahara et al., 2013; Khacho et al., 2016). A second aspect of organelle biology that has come to the fore in recent years is the presence of interorganelle contact sites that physically and functionally connect distinct organelles. While mitochondria-endoplasmic reticulum (ER) contact sites have been known for years, this has now been expanded to other organelles including lysosomes-mitochondria interactions (Elbaz and Schuldiner, 2011; Daniele and Schiaffino, 2014). Direct contact sites between mitochondria and lysosomes have been suggested to be required for lipid transfer between the two organelles (Elbaz-Alon et al., 2014; Honscher et al., 2014), although their exact role remains to be elucidated.

Interestingly, alterations in mitochondria and lysosomes are often present concomitantly in the neurons of patients affected by neurodegeneration, suggesting close functional links between

mitochondria and lysosomes. In fact, a wide range of muscular and neurological disorders are caused by mutations in specific mitochondrial or lysosomal proteins. Many lysosomal storage diseases caused by mutations in lysosomal acid hydrolases exhibit neurodegeneration as a prominent clinical feature. Similarly, mutations in mitochondrial proteins often cause neurological diseases. These mutations directly affect the structure and function of these important organelles, leading to oxidative stress, accumulation of undigested intracellular material and impaired cellular function (reviewed in Vafai and Mootha, 2012; Appelqvist et al., 2013; Raimundo, 2014; Ballabio, 2016; Lloyd-Evans and Haslett, 2016). The functional and physical association between mitochondria and lysosomes is thus emerging as a key determinant of cell function. Although the mechanisms through which these interactions occur remain to be elucidated, we hypothesize that they are required to shuttle important metabolites and ions between the two organelles (**Figure 1**, outstanding questions are represented by boxes 1–3).

FUNCTIONAL CROSSTALK BETWEEN MITOCHONDRIA AND LYSOSOMES

Macromolecules to be degraded reach lysosomes by two main routes: endocytosis and autophagy. During autophagy, cytoplasmic material is packaged into a double membrane vesicle (autophagosome), which then fuses with a lysosome (reviewed in Boya et al., 2013; Shen and Mizushima, 2014; Xu and Ren, 2015). In addition to starvation-induced bulk autophagy, a selective form of autophagy termed mitophagy is required to degrade damaged mitochondria. Disruption of this process is therefore thought to lead to the accumulation of dysfunctional mitochondria, oxidative stress, and cellular damage (reviewed in McWilliams and Muqit, 2017; Mouton-Liger et al., 2017; Rodolfo et al., 2017).

While mitophagy and the consequences of its disruption have been extensively studied, the consequences of mitochondrial dysfunction on lysosomal activity have only been recently reported. Two studies have demonstrated that mitochondrial activity is required to maintain lysosomal structure and function, following the chemical inhibition of the electron transport chain, as well as in *in vitro* and *in vivo* genetic models of mitochondrial dysfunction (Baixauli et al., 2015; Demers-Lamarche et al., 2016). Specifically, disruption of mitochondrial function causes the accumulation of enlarged endo-lysosomal structures, and impairs lysosomal acidification and activity (Baixauli et al., 2015; Demers-Lamarche et al., 2016). These lysosomal alterations were observed in a large array of mitochondrial mutants, including the deletion of TFAM (loss of mtDNA), OPA1 (disrupted mitochondrial fusion and cristae structure) and PINK1 (mitophagy), as well

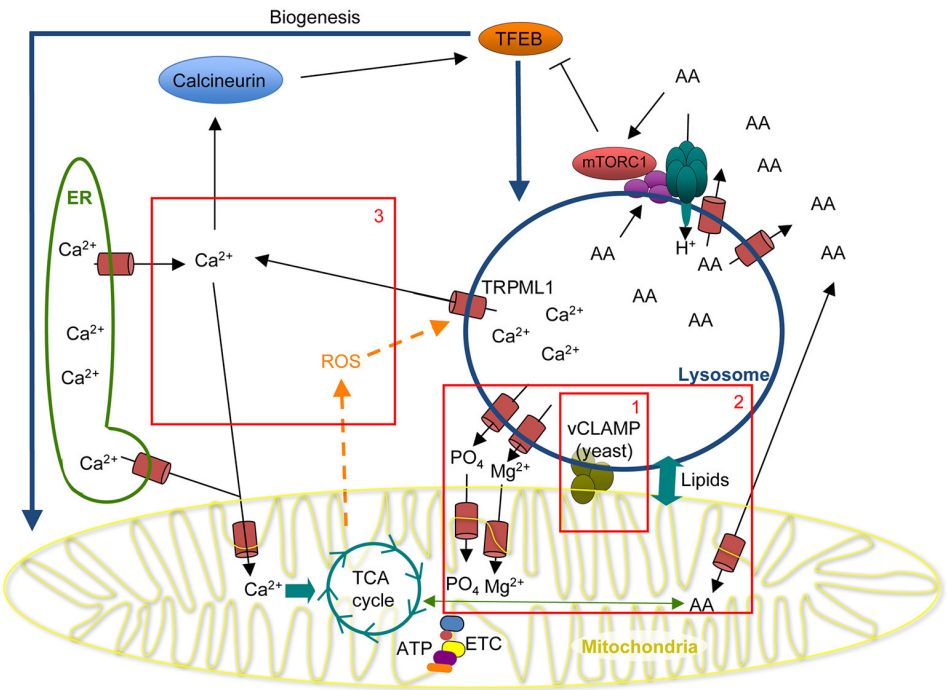

FIGURE 1 | Model for the physical and functional interaction between mitochondria and lysosomes. Boxed areas represent outstanding questions: (1) The physical interaction between mitochondria and lysosomes occurs through vCLAMP in yeast but the identity of the proteins mediating this interaction in mammalian cells remain to be determined. (2) Lipid and amino acid metabolism are controlled by both lysosomes and mitochondria. However, how the interaction between the two organelles affects metabolism is still unknown. Possible mechanisms include lipid and amino acid transfer, as well as exchange of ions that regulate mitochondrial function. (3) Ca^{2+} is a key signaling molecule regulated by both lysosomes and mitochondria, in addition to the ER. Ca^{2+} activates several cellular processes including Calcineurin-dependent activation of TFEB and mitochondrial metabolism. As mitochondrial ROS stimulates lysosomal Ca^{2+} release, the role of Ca^{2+} in controlling lysosome-mitochondria cross-talk needs to be addressed. AA, amino acids; ETC, Electron transport chain; ER, Endoplasmic reticulum.

as in several cell types (neurons, fibroblasts, T cells; Baixauli et al., 2015; Demers-Lamarche et al., 2016). Interestingly, this was associated with the accumulation of disease-associated lysosomal substrates including sphingomyelin, which accumulates in some lysosomal storage diseases (Baixauli et al., 2015), as well as ubiquitinated protein aggregates, a prominent feature of neurodegeneration (Demers-Lamarche et al., 2016). A functional link between mitochondria and lysosomes is also supported by the phenotype of yeast lacking mitochondrial cardiolipin synthase (Cdr1). Cardiolipin is a crucial mitochondrial lipid required for mitochondrial function and thus, deletion of Crd1 disrupts mitochondrial activity. Importantly, Cdr1 mutants also show enlargement of their vacuoles (the yeast equivalent of lysosomes) and impaired vacuolar acidification (Chen et al., 2008), consistent with the lysosomal phenotype of mammalian cells with mitochondrial dysfunction.

The presence of a functional interaction between mitochondria and lysosomes is also supported by the observation that biogenesis of both organelles is controlled by the same transcriptional program dependent on transcription factor EB (TFEB; **Figure 1**). TFEB regulates lysosomal biogenesis by stimulating the expression of proteins involved in lysosomal activity and autophagy regulation (Palmieri et al., 2011). TFEB is activated by an increased need for lysosomal activity (such as during starvation), or when lysosomal function is impaired (Settembre et al., 2012). Interestingly, TFEB is also induced by mitochondrial dysfunction (TFAM deletion, Complex I chemical inhibition, Complex I subunit mutants; Baixauli et al., 2015; Fernandez-Mosquera et al., 2017) and has been reported to regulate mitochondrial biogenesis independent of peroxisome proliferator activated receptor-γ coactivator (PGC1α; Mansueto et al., 2017). Consistent with TFEB regulating mitochondrial biogenesis, loss of TFEB affects mitochondrial complex II activity, increases oxidative stress, and reduces ATP production (Mansueto et al., 2017). Altogether, these results indicate that mitochondria and lysosomes share strong functional links that could play a fundamental role in both normal physiology and pathology. Recent work suggests new clues about the nature of these links, including a direct physical interaction between the two organelles, as well as transfer of ions and metabolites, but their exact nature remains to be elucidated (**Figure 1**, boxes 1–3).

ROLES AND MECHANISMS OF LYSOSOME-MITOCHONDRIA INTERACTIONS

While the mechanisms governing the functional crosstalk between mitochondria and lysosomes remain to be elucidated, the presence of common pathways regulated by both organelles could shed light on the physiological roles of this interaction. In fact, both organelles play a crucial role in amino acid metabolism and Ca^{2+} homeostasis, suggesting that these are key aspects of their interaction. In addition, a physical interaction between mitochondria and lysosomes has been reported in yeast (Elbaz-Alon et al., 2014; Honscher et al., 2014) and more recently in skeletal muscle (Aston et al., 2017). In yeast, this interaction is mediated by the vacuole and mitochondria patch (vCLAMP) that contains Vps39, a protein required for vacuolar transport. Whether the mammalian orthologs of Vps39 play a similar role still remains unknown (**Figure 1**, Box 1), but other proteins are likely involved. For example, the mitochondrial fusion protein MFN2 is required for the interaction between mitochondria and the lysosome-related organelle melanosome (Daniele et al., 2014). Interestingly, MFN2 has also been proposed to regulate ER-mitochondria contact sites (de Brito and Scorrano, 2008), raising the possibility of a three-way communication system that could be involved in Ca^{2+} signaling.

Ion Transfer between Mitochondria and Lysosomes

Ca^{2+} is a crucial signaling molecule with pleotropic roles ranging from vesicle exocytosis to cell death. While the major Ca^{2+} stores are located outside the cell and within the ER, mitochondria also play a key role in Ca^{2+} regulation (Raffaello et al., 2016). Ca^{2+} enters the mitochondrial matrix through a low affinity transporter, the mitochondrial calcium uniporter (MCU), the regulation of which is crucial for proper cell physiology (reviewed in Raffaello et al., 2016). Because MCU is a low affinity transporter, Ca^{2+} uptake by mitochondria is dependent on close contacts sites with the ER where Ca^{2+} levels reach the concentration required to activate the MCU (Raffaello et al., 2016). Ca^{2+} uptake by mitochondria ensures the rapid removal of cytosolic Ca^{2+} but also regulates mitochondrial bioenergetics by activating pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and isocitrate dehydrogenase, thereby stimulating the TCA cycle and ATP production (Denton, 2009). In addition, mitochondrial Ca^{2+} levels can act as crucial deciding factor for cell death by activating either apoptosis or necrosis (Rizzuto et al., 2012).

While the regulation of Ca^{2+} by mitochondria is well known, the role of lysosomes as a Ca^{2+} store has only recently been recognized. Lysosomal Ca^{2+} signaling regulates different cellular process such as autophagy (Medina et al., 2015), membrane fusion (Cao et al., 2015), exocytosis (Jaiswal et al., 2002), and cell death (Mirnikjoo et al., 2009). In addition to these roles, the close proximity of mitochondria and lysosomes suggests that Ca^{2+} release from lysosomes could also play a role in regulating mitochondrial activity. In fact, the activation of the Ca^{2+}-dependent phosphatase Calcineurin by lysosomal Ca^{2+} release has been shown to activate TFEB (**Figure 1**). TFEB then stimulates the transcription of PGC1α and PPARα (peroxisome proliferator activated receptor α), inducing the expression of mitochondrial fatty acid β oxidation enzymes (Vega et al., 2000; Settembre et al., 2013). In addition, TFEB can promote mitochondrial respiration and ATP production in a PGC1α-independent manner in muscle (Mansueto et al., 2017). These observations suggest that lysosomal Ca^{2+} regulates mitochondrial catabolism through a TFEB-dependent transcriptional program.

In addition to TFEB-dependent mitochondrial biogenesis, lysosomal Ca^{2+} signals could have more direct impact on mitochondrial metabolism. This is suggested by the fact that

the uptake of Ca^{2+} by mitochondria stimulates mitochondrial metabolism (Denton, 2009; Raffaello et al., 2016). Thus, similarly to ER-mitochondria contact sites, lysosomes could control mitochondrial activity through Ca^{2+} release at mitochondria-lysosome contact sites, either directly or as a result of ER Ca^{2+} release triggered by lysosomal Ca^{2+} release (**Figure 1**, Box 3). A role for ER Ca^{2+} in this process is supported by the observation that, upon stimulation, the initial lysosomal Ca^{2+} release is followed by ER-dependent cytosolic Ca^{2+} waves (Kilpatrick et al., 2013).

Ca^{2+} is released from lysosomes through two types of channels: two-pore channels (TPCs) and Transient Receptor Potential Mucolipin (TRPML/MCOLN) channels (Raffaello et al., 2016), the activity of the latter being regulated by ROS (**Figure 1**). Given that mitochondria are a major source of cellular ROS (Patten et al., 2010; Mailloux et al., 2013), this adds another potential level of crosstalk between the two organelles. In fact, the TRPML family member TRPML1 has recently been shown to act as ROS sensor, being activated by an increase in ROS to release lysosomal Ca^{2+} to the cytosol (Zhang et al., 2016). In this study, TRPML1-dependent Ca^{2+} release led to the activation of the Calcineurin/TFEB signaling cascade, increased autophagy and subsequent elimination of damaged mitochondria and excess ROS. Metabolic changes beyond autophagy were not investigated. Importantly, while physiological ROS levels likely play a key role in the coordinated regulation of mitochondria and lysosomes, increased ROS production is detrimental. This is evidenced by the observation that mitochondrial dysfunction impairs lysosomal structure in a ROS-dependent manner (Demers-Lamarche et al., 2016) and that loss of TRPML1 induces the accumulation of ROS, which in turn cause loss of mitochondrial membrane potential and fragmentation of mitochondria (Coblentz et al., 2014).

Iron is a second highly regulated ion associated with mitochondria and the endosomal compartment. Within mitochondria, iron is assembled into Iron-Sulfur clusters, inorganic cofactors that participate in a large array of cellular processes including the electron transport chain, metabolic conversion and protein synthesis (Braymer and Lill, 2017). To reach mitochondria, iron first enters the cell associated with transferrin and is subsequently released inside endosomes. Although the classical view is that iron then transit through a cytosolic labile iron pool before entering mitochondria, recent evidence indicates that iron is transferred from endosomes to mitochondria through a "kiss and run" interaction between iron-containing endosomes and mitochondria (Sheftel et al., 2007; Das et al., 2016). This mechanism would prevent the cytosolic accumulation of iron, which can catalyze the formation of damaging ROS.

Metabolic Regulation

A second essential role of lysosomes is the degradation of macromolecules, generating free amino acids, sugars and lipids that can be used in biosynthetic pathways or for energy production. As mitochondria are a major metabolic hub, lysosome, and mitochondria could regulate the function of each other through the production, transfer, or degradation of metabolites. In support of this hypothesis, mitochondria-lysosome contact sites participate in the transfer of phospholipids between the two organelles (Elbaz-Alon et al., 2014; Honscher et al., 2014; **Figure 1**, Box 2). Furthermore, respiratory growth on non-fermentable carbon sources in yeast increased ER-mitochondria contact sites at the expense of mitochondria-lysosome contacts sites (Honscher et al., 2014), indicating that these contact sites are actively involved in metabolic regulation. In addition, several small molecule transporters have been proposed to localize to vCLAMP in yeast (Elbaz-Alon et al., 2014), including a vacuolar phosphate transporter and a vacuolar magnesium channel. Their activation close to mitochondria could increase mitochondrial uptake of these ions, both of which stimulate mitochondrial activity (**Figure 1**, Box 2; Hackenbrock, 1966; Yamanaka et al., 2016). On the other hand, the regulation of lysosomes by mitochondria is independent of cellular ATP levels but can be affected by changes in NAD+/NADH ratio and mitochondrial ROS (Baixauli et al., 2015; Demers-Lamarche et al., 2016).

In proliferating cells, amino acids (especially glutamine/glutamate) provide an important carbon source for the TCA cycle (DeBerardinis et al., 2008). The close proximity of mitochondria and lysosomes could thus similarly provide an easy access to amino acids generated by lysosomal proteolysis, especially during starvation (**Figure 1**, Box 2). However, the role of lysosomes in amino acid homeostasis extends well beyond protein degradation. In fact, lysosomes serve as a platform to sense amino acid contents both outside and inside of the organelle (Bar-Peled and Sabatini, 2014; Lim and Zoncu, 2016). This nutrient-sensing machinery regulates the mammalian target of rapamycin (mTOR), a crucial kinase that acts as a hub for the control of cell growth and metabolism. In the presence of amino acids, mTOR activity stimulates protein translation and promotes cell growth, while inhibiting autophagy and suppressing TFEB activity. When amino acids become scarce, mTOR is inactivated. This relieves its inhibitory effect on autophagy and TFEB-dependent lysosomal biogenesis, thus promoting amino acid recycling (**Figure 1**; Settembre et al., 2012; Zhou et al., 2013). Interestingly, mTOR also regulates the efflux of essential amino acids from lysosomes. During amino acid starvation, mTOR inhibition leads to the selective sequestration of essential amino acids within lysosomes as a preservation mechanism. On the other hand, non-essential amino acids such as glutamine and glutamate are not affected by mTOR and are thus still released under starvation conditions (Abu-Remaileh et al., 2017; Wyant et al., 2017). As a result, they could potentially be imported into mitochondria and used as an energy source. In addition to this direct metabolic regulation, mTOR inhibition also relieves its inhibition of TFEB which, in turn, stimulates lysosomal biogenesis to help with the increased delivery of material to lysosomes caused by increased autophagy.

The metabolic changes caused by amino acid starvation also extend to mitochondria. Starvation promotes mitochondrial

elongation and connectivity, and improves mitochondrial bioenergetics through ATP synthase assembly and changes in inner mitochondrial membrane (cristae) organization (Gomes et al., 2011; Rambold et al., 2011; Patten et al., 2014; Ouellet et al., 2017). While mitochondrial elongation is caused by the PKA-dependent inhibition of DRP1, a Dynamin related GTPase required for mitochondrial fission, other changes are likely controlled more directly by amino acids. For example, the OPA1-dependent narrowing of cristae width caused by starvation requires sensing by mitochondrial solute carriers (SLC25A family), including the glutamate/aspartate transporter AGC (Patten et al., 2014). In addition to these direct changes, the fact that TFEB stimulates mitochondrial biogenesis in addition to lysosomal biogenesis suggests that there is a coordinated metabolic program that is activated by amino acid starvation to promote cellular adaptation to metabolic stress. Interestingly, a recent study indicated that mTOR also regulates mitochondrial structure through a TFEB-independent pathway that relies on MTFP1, a mitochondrial protein promoting mitochondrial fragmentation. In nutrient-replete cells, a key role of mTOR is to repress 4eBP, an important translation inhibitor. However, during starvation, mTOR inactivation relieves this inhibition, thereby decreasing protein translation. As MTFP1 translation is sensitive to 4eBP, mTOR inhibition results in the loss of MTFP1 protein, which leads to mitochondrial elongation and branching, and promotes cell survival (Morita et al., 2017). Overall, these studies indicate that amino acid starvation co-ordinately regulates the function of mitochondria and lysosomes. This metabolic control is driven by mTOR and TFEB, also at a more direct level by the flux of amino acids and fatty acids between the two organelles.

CONCLUSION

In the last decade, the realization that organelles interact in a close physical and functional manner has opened new research areas with important implications for our understanding of several diseases. Recent findings highlighting the physical and functional interaction between mitochondria and lysosomes suggest that this crosstalk plays a major role in metabolic regulation. However, several key questions remain unanswered (**Figure 1**, boxes 1–3). First, the nature of the physical interaction between the two organelles in mammalian cells remains unknown, making it difficult to assess to which extent their functional interaction requires direct physical contact. Second, both mitochondria and lysosomes have independently been studied for their role in the regulation of amino acids and lipids, but how these processes are coordinated and integrated remains an open question. Third, while both mitochondria and lysosomes are now recognized as important for Ca^{2+} regulation, how this participates to the crosstalk between the two organelles remains to be determined. Given the intimate links between mitochondria and lysosomes in disease, especially neurodegenerative diseases, these are important areas that remain to be explored.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Lysosomal and Mitochondrial Liaisons in Niemann-Pick Disease

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Lysosomal storage disorders (LSD) are characterized by the accumulation of diverse lipid species in lysosomes. Niemann-Pick type A/B (NPA/B) and type C diseases Niemann-Pick type C (NPC) are progressive LSD caused by loss of function of distinct lysosomal-residing proteins, acid sphingomyelinase and NPC1, respectively. While the primary cause of these diseases differs, both share common biochemical features, including the accumulation of sphingolipids and cholesterol, predominantly in endolysosomes. Besides these alterations in lysosomal homeostasis and function due to accumulation of specific lipid species, the lysosomal functional defects can have far-reaching consequences, disrupting *intracellular* trafficking of sterols, lipids and calcium through membrane contact sites (MCS) of apposed compartments. Although MCS between endoplasmic reticulum and mitochondria have been well studied and characterized in different contexts, emerging evidence indicates that lysosomes also exhibit close proximity with mitochondria, which translates in their mutual functional regulation. Indeed, as best illustrated in NPC disease, alterations in the lysosomal-mitochondrial liaisons underlie the secondary accumulation of specific lipids, such as cholesterol in mitochondria, resulting in mitochondrial dysfunction and defective antioxidant defense, which contribute to disease progression. Thus, a better understanding of the lysosomal and mitochondrial interactions and trafficking may identify novel targets for the treatment of Niemann-Pick disease.

Keywords: mitochondria, lysosomes, cholesterol, sphingolipids, intracellular trafficking, lysosomal disorders, acid sphingomyelinase

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INTRODUCTION

Niemann-Pick (NP) diseases encompass a group of autosomal recessive lysosomal storage disorders (LSD), characterized by the accumulation of diverse lipid species in lysosomes. While these diseases were initially considered a single entity with overlapping biochemical, pathological and clinical features, developing evidence demonstrated differential etiological causes (Patterson and Walkley, 2017; Schuchman and Desnick, 2017). Niemann-Pick type A and B (NPA and NPB) diseases are caused by deficits in the activity of acid sphingomyelinase (ASMase), an enzyme that regulates lysosomal sphingomyelin (SM) homeostasis, while Niemann-Pick type C (NPC) disease is caused by mutations in *NPC1* and *NPC2* genes, resulting in functional defects in the

lysosomal proteins NPC1 and NPC2, involved in cholesterol efflux from lysosomes.

Although the primary consequence of ASMase inactivation results in the accumulation of lysosomal SM, cholesterol and other lipids types, such as bis(monoacylglycero)phosphate, glucocerebroside, GM2 and GM3 gangliosides and sphingosine also accumulate in lysosomes (Rodriguez-Lafrasse et al., 1994; Vanier, 2013; Schuchman and Desnick, 2014). Similarly, although in NPC disease cholesterol accumulation is the direct consequence of NPC1/NPC2 loss of function, SM and other sphingolipids, such as lactosylceramide, glucosylceramide, GM2 and GM3 gangliosides and sphingosine, accumulate as well (Pentchev et al., 1984; Lloyd-Evans et al., 2008; Patterson et al., 2012). Hence, these findings imply that the trafficking and metabolism of different lipid species through the endocytic pathway are severily affected in these lysosomal diseases, likely contributing to their pathogenesis. However, the molecular mechanisms and signaling pathways responsable for cell death and tissue damage in these diseases are not entirely clear.

Although both Niemann-Pick type A/B (NPA/B) and NPC diseases are caused by defects in lysosomal homeostasis and function, there are significant differences between these diseases in relation to the degree of cholesterol trafficking to mitochondria, with the consequent impact in mitochondrial dysfunction and impairment in antioxidant defense strategies. In this review, we summarize the biochemical and genetic features of both diseases, highlighting their commonalities and differences regarding lysosomal-mitochondrial cholesterol trafficking and communication as the molecular basis to understand the differential involvement of mitochondrial dysfunction in NPC disease. Further understanding the lysosomal and mitochondrial liaisons in NP diseases may thus provide the opportunity to improve and expand the current armamentarium for the treatment of these lysosomal disorders.

NP DISEASES

NPA/B Disease

ASMase deficiency results primarily in the accumulation of SM in affected tissues of NPA and NPB mice and patients (Schuchman and Desnick, 2017). Patients with NPA present developmental delay, hepatosplenomegaly and progressive neurodegeneration, leading to premature death typically between 2 and 3 years of age (Schuchman and Desnick, 2014, 2017). In contrast, NPB disease presents a highly variable phenotype that is usually diagnosed in childhood by the presence of hepatosplenomegaly (Schuchman and Desnick, 2014, 2017). Most NPB patients do not exhibit neurological defects and consequently NPB patients usually live into adulthood. In the more severely affected patients, a major complication of the disease is progressive pulmonary deterioration (Schuchman and Desnick, 2014, 2017). In addition, liver damage with fibrosis and cirrhosis has been recently recognized as a relevant complication of this disease (Moles et al., 2012; Thurberg et al., 2012; McGovern et al., 2013; Lidove et al., 2015; Cassiman et al., 2016). NPA patients have a dramatic reduction in ASMase activity and typically present <5% of its physiological activity (Smith and Schuchman, 2008; Vanier, 2013; Schuchman and Desnick, 2014). In contrast, NPB patients exhibit higher ASMase residual activity, which correlates with their milder phenotype (Smith and Schuchman, 2008; Vanier, 2013; Schuchman and Desnick, 2014). *The clinical diagnosis of NPA and NPB diseases* is mainly based on the presence or absence of neurological symptons (Vanier, 2013; McGovern et al., 2017; Schuchman and Desnick, 2017). Although NPA and NPB are pan-ethnic, NPA is more frequent in individuals with Ashkenazi Jewish ancestry than in the general population, with an estimated carrier frequency close to 1:80 and a disease incidence of 1/40,000 (Schuchman and Desnick, 2014). In other populations, such as in Chile, the carrier frequency for the type B mutation A359D occurs in 90% of patients close to a 1:106 rate, predicting a disease incidence of 1/45,000 (Acuña et al., 2016a,b).

ASMase is encoded by the SMPD1 gene (sphingomyelin phosphodiesterase 1, gene ID 6609), which is located on chromosome 11 locus 11p15.4-p15.1. More than 180 pathogenic mutations in the SMPD1 gene in patients with NPA and NPB have been identified, which are concentrated in exon two (Schuchman and Desnick, 2017). Recently, the ASMase *crystal* structure has been determined in humans (Xiong et al., 2016) and mouse (Gorelik et al., 2016), which may facilitate genotype-phenotype mutation analysis (Acuña et al., 2016b; Zampieri et al., 2016).

Although tissues from mice or patients with NPA and NPB disease accumulate SM primarily in endolysosomes, cholesterol also increases as a secondary consequence (Huang and Feigenson, 1999; Radhakrishnan et al., 2000; Ridgway, 2000). While the underlying mechanism of cholesterol loading is not well understood, it is known that SM and cholesterol exhibit a high affinity for each other. Indeed, SM binds cholesterol with high affinity (Slotte, 1999; Ridgway, 2000), resulting in the sequestration and subsequent decrease in the efflux of cholesterol out of lysosomes, impairing the *esterification* of cholesterol by acyl-CoA:cholesterol acyl transferase in the ER. These events are in line with findings in macrophages from ASMase$^{-/-}$ mice or in macrophages from wild type mice enriched with exogenous SM, which results in increased lysosomal cholesterol content because of decreased cholesterol efflux (Leventhal et al., 2001). Interestingly, it has been described that ASMase is not only active in lysosomes, but it exhibits a secretory form that acts on the plasma membrane to generate ceramide from SM hydrolysis in response to stress (Falcone et al., 2004; Charruyer et al.,

Abbreviations: ACDase, acid ceramidase; ASMase, acid sphingomyelinase; CLAH, congenital lipoid adrenal hyperplasia; ER, endoplasmic reticulum; GSH-EE, GSH ethyl ester; GM2, ganglioside GM2; GM3, ganglioside GM3; HSL, hormone-sensitive lipase; IMM, inner mitochondrial membrane; LD, lipid droplets; LSDs, lysosomal storage disorders; LE/Lys, late endosomes/lysosomes; mGSH, mitochondrial GSH; MAM, mitochondrial associated ER membranes; MCS, membrane contact sites; NAC, N-acetylcysteine; NP, Niemann-Pick diseases; NPA, Niemann-Pick type A disease; NPB, Niemann-Pick type B disease; NPC, Niemann-Pick type C disease; NPC1, Niemann-Pick type C1 protein; NPC2, Niemann-Pick type C2 protein; OMM, outer mitochondrial membrane; SM, sphingomyelin; SMPD1, sphingomyelin phosphodiesterase 1; SREBP2, sterol regulatory element protein 2; StARD1, steroidogenic acute regulatory protein 1; StARD3, steroidogenic acute regulatory protein 1; START, StAR-related lipid transfer domain; VDAC, voltage dependent anion channel; TSPO, translocator protein.

2005). This event, in turn, reorganizes membrane lipid domains, called "rafts," which trigger downstream signaling events. These observations have led to the suggestion that in addition to lysosomal dysfunction produced by SM accumulation, part of the pathogenesis of NPA and NPB is related to alterations in signaling pathways at the plasma membrane.

NPC Disease

NPC is a neurodegenerative visceral disorder with the cardinal characteristic of unesterified cholesterol accumulation in the liver, spleen and central nervous system (Patterson et al., 2012). This disease presents a broad range of symptoms, ranging from a progressive fatal neonatal disorder to a milder form in the adulthood that can evolve to chronic neurodegeneration. Although, in most cases the severity of the disease is determined by neurological deterioration, systemic signs, such as cholestatic jaundice in the neonatal period and/or hepatosplenomegaly in infancy and childhood, usually precede neurological symptoms (Patterson et al., 2012). In the most common form of NPC disease, patients exhibit progressive neurological defects. In the early period of childhood there is a delay in motor development, while in the later period of childhood and in the juvenile form symptoms manifest as gaits, falls, clumsiness, cataplexy, and school problems. In some cases, there are also psychiatric disorders such as progressive dementia (Patterson et al., 2012; Vanier, 2013).

Deterioration of liver function is a common feature of NPC disease and represents one of the most common metabolic causes of neonatal cholestasis (Patterson et al., 2012; Vanier, 2013). Currently, this disease has an estimated incidence of 1 case per 120,000 live births and is a deadly progressive disease that has so far no cure (Patterson et al., 2012).

The main cause of NPC disease is the functional inactivation of NPC1 and NPC2 proteins due to mutations in the *NPC1* and N*PC2* genes, particularly in *NPC1* gene located in chromosome 18q11, which accounts for up to 95% of cases of the disease (Carstea et al., 1993, 1997), while the remaining 5% are due to mutations in the *NPC2* gene located on chromosome 14q24 (Naureckiene et al., 2000).

NPC1 is a multi transmembrane protein located in late endosomes and lysosomes (Davies and Ioannou, 2000), while NPC2 is a relatively small protein located in the lysosomal lumen (Friedland et al., 2003). NPC1 and NPC2 work in tandem in the release of cholesterol from lysosomes. The current model of cholesterol transport in lysosomes posits that NPC2 binds cholesterol and transfers it to NPC1, which then transports cholesterol through the glycocalyx to the endosomal/lysosomal membrane to be released from the organelle by mechanisms that have not yet been determined (Kwon et al., 2009; Klein et al., 2014). The direct consequence of NPC1 deficiency is the accumulation of unesterified cholesterol in endolysosomes, which is accompanied by a secondary increase in glycosphingolipids, including SM. Consistent with the key role of NPC1 in *intracellular* cholesterol trafficking, NPC1 deficiency in mice reproduces many of the deficits seen in NPC patients, including neurological defects and ataxia by 6–7 weeks of age and severe reduction in the maximal life span to about 10–12 weeks. Experimental studies in NPC1 knockout mice have identified promising therapeutic treatment options for NPC disease (see below) and represent a valid model to examine lysosomal-mitochondrial communications that underlie the widespread defects in intracellular lipid transport.

MITOCHONDRIA-LYSOSOMAL LIAISONS IN NP DISEASE

Reciprocal Functional Regulation between Mitochondria and Lysosomes

The endo-lysosome compartment (LE/Lys) constitutes a highly dynamic membrane structure that plays a key role in the maintenance of cellular homeostasis, as well as in the digestion and recycling of cellular components and in lipid metabolism and trafficking (Luzio et al., 2007; Saftig and Klumperman, 2009; Settembre et al., 2013). Alterations in LE/Lys trafficking and lysosomal function are typical features of LSDs, such as NPA/C diseases characterized by the accumulation of specific lipid species in lysosomes, (Futerman and van Meer, 2004; Ikonen, 2008; Lloyd-Evans et al., 2008). Thus, the LE/Lys system functions like a sorting station equipped with specialized molecular devices involved in the degradation and/or recycling of a wide range of cargo. Moreover, the LE/Lys is not only involved in recycling or secretory pathways but can modulate mitochondrial function as well. For instance, Gaucher disease, a LSD caused by mutations in the *GBA1* gene that encodes for *β-glucocerebrosidase*, displays lysosomal dysfunction and defective mitochondrial turnover due to impaired mitophagy (Osellame et al., 2013), illustrating the functional relationship between lysosomes and mitochondria. In line with this notion, pharmacological and genetic models of lysosomal cholesterol accumulation have been shown to sensitize hepatocytes to acetaminophen hepatotoxicity by impairing mitophagy (see below), highlighting the relevance of lysosomes/mitochondria relationship in drug-induced liver injury (Baulies et al., 2015). In addition, mitochondria are dynamic organelles that change their distribution, structure and function in response to metabolic conditions and stress, and the molecular players involved in this process are modulated by proteins involved in intracellular trafficking (Detmer and Chan, 2007; Lapuente-Brun et al., 2013; Acín-Pérez et al., 2014). For instance, mitochondrial homeostasis is finely tuned by specific endocytic proteins, such as the GTPases dynamin-2, Drp1 (Lee et al., 2016) and EHD1, which promote mitochondrial fission (Farmer et al., 2017) or through VPS35 and the retromer complex, which regulate the turnover of the fusion protein Mfn2 (Rowland and Voeltz, 2012; Tang et al., 2015).

While these findings suggest that LE/Lys can regulate mitochondrial function, there is also evidence that mitochondrial respiration regulates the biogenesis and function of the LE/Lys compartment (Baixauli et al., 2015; Daniele and Schiaffino, 2016; Raimundo et al., 2016; Diogo et al., 2017; Elbaz-Alon, 2017). Ablation of mitochondrial oxidative phosphorylation by genetic deletion of mitochondrial transcription factor A in T cells has been shown to regulate lysosomal homeostasis and function. This event modulates T cell responses through enhanced lysosomal proliferation that *results* in defective lysosomal homeostasis, translating in decreased cathepsin B activation and ASMase

inhibition, which in turn causes lysosomal SM accumulation (Baixauli et al., 2015). In line with these findings, SM accumulation has been shown to inhibit the lysosomal TRP calcium channel, impairing endolysosomal trafficking, protein degradation, and macroautophagy (Shen et al., 2012). Thus, these findings suggest that the reciprocal functional regulation of mitochondria and lysosomes may engage in a self-forward loop of potential relevance in the pathogenesis of NPA/NPC diseases, consistent with the impairment in the clearance of dysfunctional mitochondria in LSDs (Lieberman et al., 2012). Whether strategies that improve mitochondrial function (see below) have a significant impact in LSD pathology remains to be established. Therefore, dissecting the intricate communication between mitochondria and lysosomes may be critical not only for understanding essential physiological processes but also for uncovering the impact of mitochondrial dysfunction in the development of human pathologies, including LSD (Taylor and Turnbull, 2005).

Intracellular Cholesterol Trafficking to Mitochondria in NP Disease

Despite its low content in mitochondrial membranes, the mitochondrial pool of cholesterol plays key physiological roles, including the synthesis of steroids in steroidogenic cells, bile acids in hepatocytes and the maintenance of structural and functional properties of membrane bilayers. However, as described below, NPC but not NPA disease is characterized by the accumulation of cholesterol in mitochondria in both neurons and hepatocytes by mechanisms not fully elucidated. StARD1 has emerged as one of the potential key players involved in cholesterol transport to mitochondria, as best characterized in steroidogenic cells. Indeed, StARD1 regulates the rate-limiting step of steroidogenesis, which is determined by the transfer of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone by P450scc (Stocco, 2001). Mutations in the gene coding for StARD1 protein cause human congenital lipoid adrenal hyperplasia (CLAH) (Miller, 1997), highlighting the relevance of this protein in the synthesis of steroids. CLAH is characterized by defective steroidogenesis in most tissues except the placenta, which does not express StARD1 (Lin et al., 1995). StARD1, a 37 kDa protein, contains a C-terminal cholesterol binding domain and an N-terminal mitochondrial targeting sequence common to matrix proteins. The hydrophobic C-terminal binding pocket domain reversibly binds cholesterol in a 1:1 ratio (Stocco, 2001).

The trafficking of free cholesterol to OMM is mediated by several steps that involve various intracellular organelles, including lysosomes and lipid droplets (LD) and specific proteins, such as the translocator protein (TSPO) and voltage-dependent anion channel (VDAC) (Elustondo et al., 2017). Moreover, cholesterol is transferred from the OMM to the IMM by the action of StARD1 and TSPO, which associate with the mitochondrial membrane contact sites (MCS) to drive the intramitochondrial cholesterol transfer to the P450scc for metabolism and subsequent steroid formation (Rone et al., 2009). When the mitochondrial cholesterol decreases at the OMM additional free cholesterol must be moved from intracellular stores to the mitochondria. In spite of data suggesting the potential participation of TSPO in mitochondrial cholesterol trafficking, recent evidence has questioned this role. For instance, genetic models of global TSPO deletion or its specific ablation in Leydig cells showed a minimal impact in steroidogenesis and hence in mitochondrial cholesterol trafficking (Morohaku et al., 2014; Tu et al., 2014).

Interestingly, 15 genes identified by sequence homology with the StAR hydrophobic lipid-binding pocket domain of approximately 210 amino acids have been described in human and mouse (Ponting and Aravind, 1999; Iyer et al., 2001). These proteins are classified as StAR-related lipid transfer (START) domain proteins (Ponting and Aravind, 1999; Iyer et al., 2001), leading to the denomination of the original described StAR as StARD1 to denote it as the founder member of this expanding family (Alpy et al., 2001, 2009; Elustondo et al., 2017). Although, few members of the START family bind sterols (Lavigne et al., 2010; Calderon-Dominguez et al., 2014; Létourneau et al., 2015; Elustondo et al., 2017), StARD1 and StARD3 (also known as MLN64) have been implicated in cholesterol trafficking into mitochondria.

The extramitochondrial source of the cholesterol pool that reaches mitochondria is not fully understood and could originate from LD, ER, the endosomal pathway or the plasma membrane (Rone et al., 2009). In support for the plasma membrane origin of cholesterol trafficking to mitochondria mainly from LDL receptor, hormone-sensitive lipase (HSL) and the StARD1 proteins in steroidogenic tissues are thought to work together in the transport of cholesterol from plasma membrane to mitochondria (Gocze and Freeman, 1993; Freeman et al., 1998; Shen et al., 2003; Lange et al., 2009). HSL is activated upon phosphorylation mediated by cAMP-dependent protein kinase (PKA) and inhibition of HSL results in decreased steroidogenesis (Rone et al., 2009). Moreover, SNARE proteins have been shown to play a crucial role in intracelular trafficking by a cholesterol-mediated mechanism (Enrich et al., 2015; Kraemer et al., 2017). However, whether mitochondrial cholesterol targeting is regulated by SNARE-mediated trafficking remains to be established.

Another source of mitochondrial cholesterol is the ER. To reach mitochondria, cholesterol from the ER is transported by cytosolic proteins, such as the PAP7 protein, which interacts with TSPO and StARD proteins (Liu et al., 2006; Alpy et al., 2013) or through the connection of ER and mitochondria via MAMs. Recently, protein complexes that are involved in membrane contact between ER and mitochondria have been identified, but their role in lipid transport is still unclear (Elustondo et al., 2017).

Due to the relevance of lysosomal-mitochondrial liaisons in NPC, understanding the trafficking of lysosomal cholesterol to mitochondria may be essential for the pathophysiology of the disease. Interestingly, although astrocytes from NPC1 deficient mice exhibit decreased expression of StARD1 protein and mRNA levels (Chen et al., 2007), we have observed increased expression of StARD1 in liver and brain from NPC1 null mice by a poorly understood mechanism independent of ER stress (Torres et al., 2017). Besides the putative involvement of StARD1, as mentioned above, another candidate to mediate mitochondrial cholesterol

trafficking in NPC is StARD3, also known as MLN64. Its N-terminus named MENTAL (MLN64 N-terminal) domain binds cholesterol (Zhang et al., 2002; Alpy and Tomasetto, 2006) and is responsible for the specific localization of the protein in the membrane of late endosomes (Alpy et al., 2001). Overexpression of StARD3 enhances steroidogenesis (Watari et al., 1997) by stimulating the mobilization of lysosomal cholesterol to the mitochondrial P450scc, whereas mutant StARD3 lacking the START domain was reported to induce cholesterol accumulation into lysosomes (Zhang et al., 2002). Interestingly, StARD3 expression increases in NPC1 cells and its overexpression in hepatocytes increases mitochondrial cholesterol and impairs mitochondrial function, reflected by decreased mitochondrial membrane potential (Balboa et al., 2017). Along with StARD3, NPC2 has been shown to contribute to the transport of endosomal cholesterol to mitochondria (Kennedy et al., 2012). In this regard, it is conceivable that the increase of mitochondrial cholesterol observed in NPC1 deficient cells could derived from the action of StARD3 and NPC2, as their expression are induced in NPC1 cells (Blom et al., 2003; Balboa et al., 2017). However, the causal role of the overexpression of these proteins in the stimulation of mitochondrial cholesterol trafficking remains to be elucidated at the molecular level. According to this potential model, the endosomal cholesterol egress mediated by StARD3 involved cholesterol binding by its MENTAL domain in the late-endosomal membranes followed by the cholesterol transfer through the cytoplasmic StART domain to a cytosolic acceptor protein or membrane (Alpy and Tomasetto, 2006).

Despite this evidence for a putative role of StARD3 in mitochondrial cholesterol trafficking, targeted mutation of the StARD3 StART domain has been shown to cause only modest alterations in cellular sterol metabolism and mice homozygous for the *Mln64* mutant allele exhibited minor perturbations in the metabolism and in the intracellular distribution of cholesterol, questioning its contribution in the intramitochondrial trafficking of cholesterol (Kishida et al., 2004). Moreover, global StARD1 deletion in mice induces lipoid adrenal hyperplasia and mice die 10 days after birth (Caron et al., 1997). These findings illustrate that other members of the StAR family cannot functionally replace StARD1, highlighting a *critical* role of this protein in steroidogenesis and hence in the trafficking of cholesterol to IMM for processing. Thus, although the understanding of the pathways of mitochondrial cholesterol trafficking and accumulation in NPC disease still remains elusive, this process is important for the progression of the disease and its further characterization may be key for the design of future therapies. Whether StARD1 in partnership with StARD3 are critical in this process remains to be fully established.

MITOCHONDRIA-LYSOSOMES RELATIONSHIP AND MITOCHONDRIAL QUALITY CONTROL IN NP DISEASES

General Overview

Mitochondria are double-membrane organelles that are essential for energy supply, metabolism, production of reactive oxygen species and apoptosis signaling (Hatefi, 1985). Mitochondrial function is particularly important in tissues with high energy demand, such as the brain (Chan, 2006), which reflects the dependence of neurons on oxidative phosphorylation for energy supply (Almeida et al., 2001). Given the central role of mitochondria in cellular homeostasis, mitochondrial dysfunction has been linked to neurodegenerative diseases, including NPC (Johri and Beal, 2012; Plotegher and Duchen, 2017). Moreover, limited degradation of dysfunctional mitochondria through different mechanisms may contribute to LSD pathognesis. The degradation of damaged mitochondria can occur either by a selective process called mitophagy or non-specifically via macroautophagy (Youle and Narendra, 2011). The degradation of mitochondria is not only dependent on lysosomal function, but also on the formation of autophagosomes and the subsequent fusion of autophagosomes with lysosomes to generate autolysosomes (**Figure 1A**), a step that can be influenced by the lipid composition of lysosomes.

Besides Parkin and PINK1, which play a key role in mitophagy (McLelland et al., 2016), recent findings have shown that the targeting of mitochondrial components to lysosomes involves the formation of novel structures called mitochondrial-derived vesicles (MDVs) that incorporate selective mitochondrial-derived cargo for degradation in lysosomes (Soubannier et al., 2012; Schrader et al., 2015). However, unlike classical mitophagy, the delivery of MDV-containing oxidized mitochondrial components to lysosomes does not require mitochondrial depolarization and is independent of ATG5 and LC3. Thus, this alternative pathway is distinct from mitophagy and can be regarded as a novel vesicle transport route between the mitochondria and lysosomes, emerging as a complimentary mechanism for the quality control of mitochondria of potential relevance in NP disease (Soubannier et al., 2012).

Mitochondrial Quality Control in NP Disease

A considerable body of evidence suggests that impaired autophagy contributes to lysosomal lipid storage in LSDs through the accumulation of ubiquitinated proteins and dysfunctional organelles, including mitochondria (Platt et al., 2012; Osellame and Duchen, 2014). Although the formation of autophagosomes is enhanced in NPC neurons, the fusion of autophagosome with lysosomes is incomplete, translating in the inefficient degradation of autophagosome-containing mitochondria, which leads to the accumulation of defective mitochondria (**Figure 1B**; Elrick et al., 2012; Ordonez, 2012). As neurons are more sensitive to mitochondrial failure than other cells, defects in the mitochondria quality control in neurons may explain the selective neuronal loss observed in NPC disease. In line with this concept, it has been shown that defective mitophagy and increased mitochondrial fragmentation due to abnormal autophagy activation are more severe in neurons from NPC1$^{-/-}$ mice than in NPC fibroblasts (Ordonez, 2012). Moreover, decreased NPC1 function in neurons generated from human embryonic stem cells results in impaired mitochondrial clearance but enhanced mitochondrial fragmentation, phenotypes that can

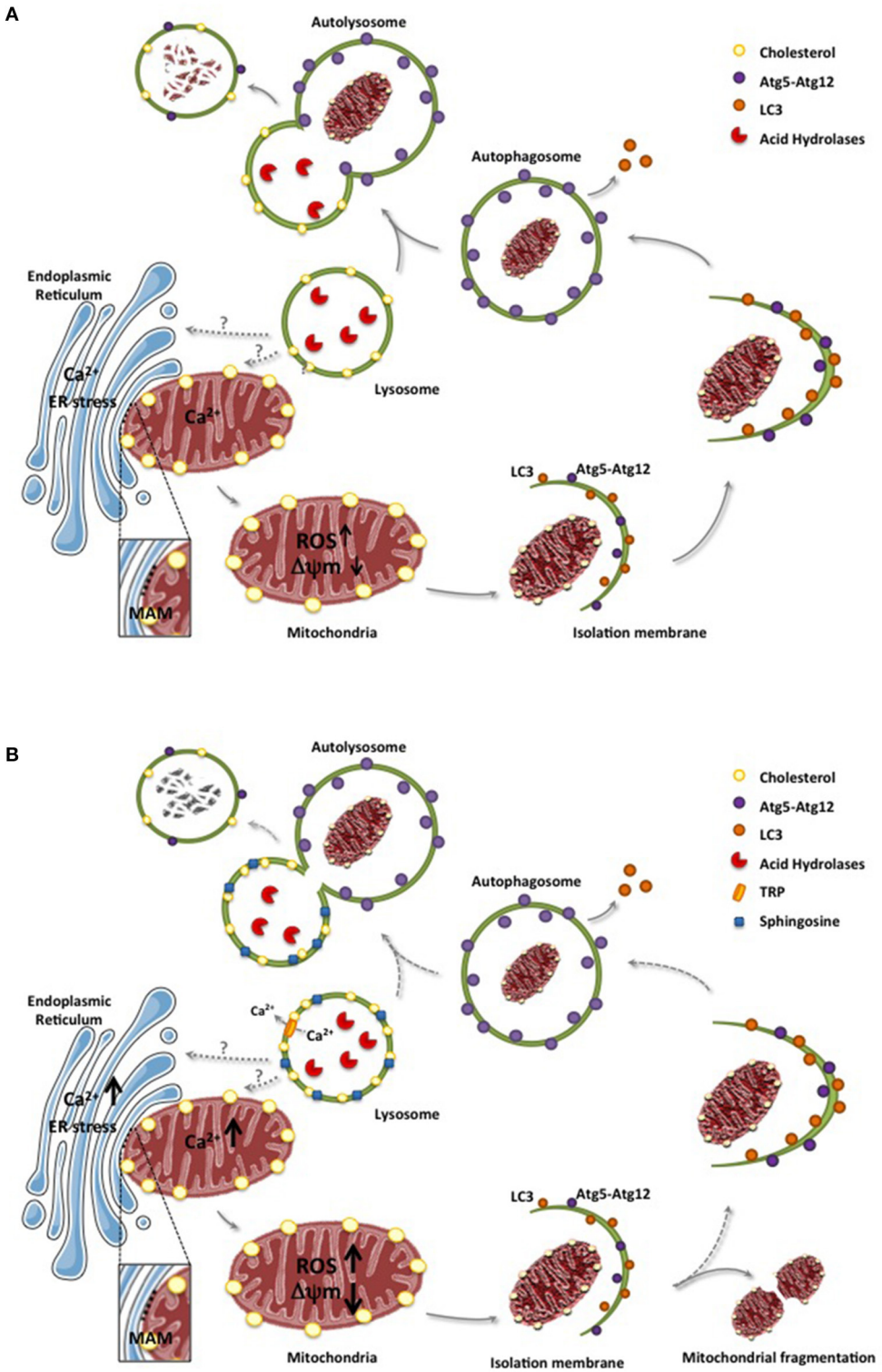

FIGURE 1 | ER and lysosomes in mitochondrial degradation. **(A)**, Mitochondria and ER interaction through MAMs contributes to the uptake of calcium to mitochondria, which in physiological conditions do not cause mitochondrial dysfunction. However, in conditions of ER stress, enhanced flux of calcium from ER to mitochondria can cause oxidative stress, increased generation of reactive oxygen species (ROS) and mitochondrial depolarization. As a mechanism to ensure the elimination dysfunctional mitochondria, altered mitochondria is engulfed in sequential structures originating with the isolation membrane, which evolve to form

(Continued)

FIGURE 1 | autophagosomes. These structures encapsulating dysfunctional mitochondria are fused with lysosomes to form autolysosomes where mitocondria contained in the autolysosomes are degraded by acid hydrolases. **(B)**, In LSD, such as NPA or NPC, lipid species accumulate in lysosomes, including sphingosine and cholesterol, which not only disrupt lysosomal calcium homeostasis through calcium channels (e.g., TRP) but also impair the fusion of lysosomes with autophagosomes leading to defective mitochondrial degradation. Moreover, defective engulfment of dysfunctional mitochondria may occur in earlier steps, which may favor the fragmentation of mitochondria. Of note, while the physical association between ER and mitochondria through MAMs are relatively well defined, the physical apposition between lysosomes and mitochondria is poorly understood and characterized. Dashed lines reflect defective steps in LSD leading to impaired autolysosome formation and defective digestion of dysfunctional mitochondria.

be rescued by inhibition of autophagy with 3-methyladenine or cholesterol extraction with cyclodextrin. These findings suggest that mitochondrial dysfunction in NPC disease may be the consequence not only of defective autophagy induction but also of increased mitochondrial fragmentation mediated by impaired autophagosome formation (Ordonez et al., 2012).

Another factor that can contribute to impaired autophagy and defective clearance of dysfunctional mitochondria is the accumulation of sphingosine in lysosomes, which has been shown to disrupt calcium homeostasis (Lloyd-Evans et al., 2008). While the mechanism of lysosomal sphingosine accumulation in NPC is not completely understood, it has been shown that alteration in the VGEF signaling pathway impairs sphingosine kinase activity, which results in enhanced sphingosine levels and loss of Purkinje neurons via inhibition of autophagosome-lysosome fusion, thus suggesting a link between sphingosine and impaired autophagy in NPC (Lee et al., 2014). Furthermore, NPC2 deficiency impairs autophagy-lysosomal activity, which negatively impacts mitochondrial function in adipocytes (Guo et al., 2016). Interestingly, stimulation of adenosine A2A receptors has been shown to rescue intracellular cholesterol accumulation and mitochondrial abnormalities in cell models of NPC (Ferrante et al., 2016), linking A2A agonism with the improvement of mitochondrial function and pathological phenotype of fibroblasts from NPC patients (Visentin et al., 2013). Therefore, the accumulation of dysfunctional mitochondria can account for the onset of oxidative stress in NPC disease described both in NPC1 knockout mice and NPC patients (Vázquez et al., 2012). Moreover, in addition to impaired mitophagy, defects in mitochondrial motility in neurons and distribution into axons to meet metabolic demands can contribute to the pathogenesis of neurological disorders, including NPC disease (Sheng and Cai, 2012; Woś et al., 2016). However, whether these alterations are relevant for the functional maintenance of Purkinje neurons, whose loss accounts for the characteristic ataxia in NPC disease remains to be established.

In parallel with these findings in NPC, there is also evidence of autophagy defects in NPA/B diseases (Fucho et al., 2014; Baulies et al., 2015; Canonico et al., 2016). For instance, hepatocytes from ASMase$^{-/-}$ mice exhibit impaired autophagy flux determined by the combination of rapamycin with or without chloroquine, an effect that was accompanied by increased LC3BII and p62 levels. In addition, ASMase$^{-/-}$ hepatocytes displayed impaired fusion of autophagosome-containing mitochondria with lysosomes in response to acetaminophen, which translated in increased susceptibility to acetaminophen-mediated liver injury by sustaining mitochondrial damage (Baulies et al., 2015). Although ASMase deficiency results primarily in increased lysosomal SM content, it also causes lysosomal cholesterol accumulation, which accounts for the sensitization to acetaminophen-induced hepatocellular injury. In line with these findings, U18666A, an amphiphilic amino-steroid that induces lysosomal cholesterol accumulation sensitized primary hepatocytes to acetaminophen-induced cell death. Treatment with oxysterol 25-hydroxycholesterol, a ligand for liver X receptors (LXR) that suppresses sterol synthesis, decreases cholesterol accumulation and protects ASMase$^{-/-}$ mice from acetaminophen-mediated liver injury. Moreover, human B lymphocytes from patients with NPB disease exhibit alterations in the rate of autophagic vacuole accumulation, mitochondrial fragmentation and mitophagy induction, indicating impaired clearance of damaged mitochondria (Canonico et al., 2016). Thus, taken together these findings indicate that defective clearance of dysfunctional mitochondria due to impaired lysosomal function or defective fusion of lysosomes with autophagosomes is a common feature of both NPA/B and NPC diseases.

MITOCHONDRIAL CHOLESTEROL ACCUMULATION: A DIFFERENTIAL FEATURE BETWEEN NPA/B AND NPC DISEASES

In contrast to defective autophagy, which is common to NPA and NPC, intracellular cholesterol trafficking and accumulation in mitochondria is a differential feature between NPA/B and NPC diseases (**Figure 2**). Although the primary feature of NPA is the accumulation of SM due to the deficiency of ASMase, cholesterol levels also increase through the regulation of SREBP2. However, the bulk of the intracellular cholesterol accumulation in NPA is sequestered in lysosomes as shown in hepatocytes from ASMase null mice stained with filipin and lysotracker (Fucho et al., 2014). In contrast to this event, there is growing evidence of increased mitochondrial cholesterol loading in both hepatocytes and neurons from Npc1$^{-/-}$ mice (**Figure 2**), and this process mediates, in part, the mitochondrial dysfunction reported in NPC disease (Yu et al., 2005; Marí et al., 2006; Charman et al., 2010; Balboa et al., 2017; Torres et al., 2017). Moreover, while alcohol induced the trafficking and accumulation of cholesterol in hepatic mitochondria, this event was defective in hepatocytes deficient in ASMase, which paralleled the lack of induction of StARD1 in ASMase null mice in response to alcohol feeding (Fernandez et al., 2013). The mechanism underlying the differential increase in mitochondrial cholesterol and expression of StARD1 between NPA and NPC

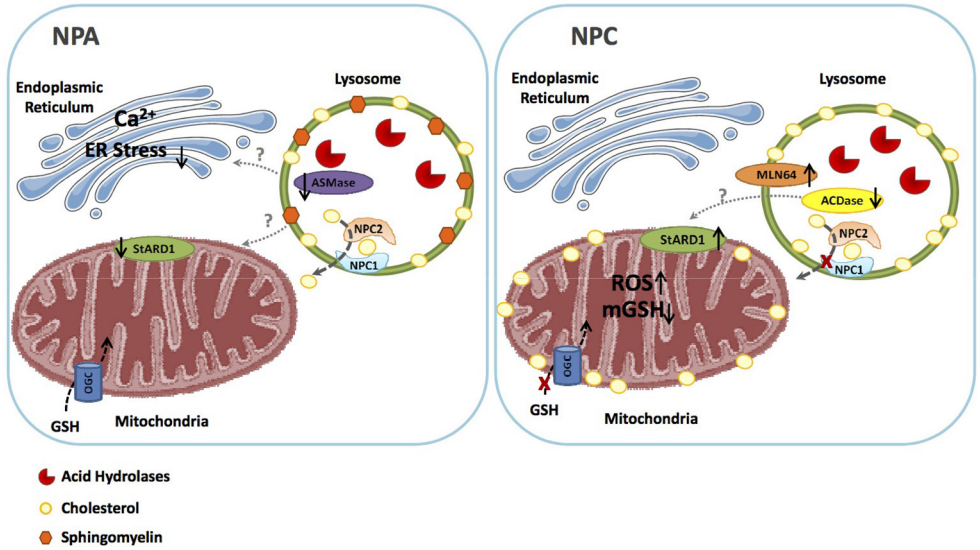

FIGURE 2 | Similarities and differences between NPA and NPC. NPA disease due to ASMase deficiency is characterized by the primary accumulation of shingomyelin in lysosomes, which secondarily lead to the sequestration of cholesterol in these organelles. As ASMase triggers ER stress, the ablation of ASMase impairs ER stress signaling, and consequently the expression of StARD1. Thus, in NPA the bulk of cholesterol accumulation is restricted to lysosomes but not mitochondria, which allows the transport of GSH into the mitochondrial matrix via the 2-oxoglutarate carrier (OGC). In NPC, however, although primarily accumulates in lysosomes, mitochondrial cholesterol transporting polypeptides, such as MLN64 contribute to mitochondrial cholesterol loading. Moreover, besides MLN64, StARD1 is also induced in NPC cells by a poorly understood mechanism independent of ER stress, with the potential involvement of the downregulaton of acid ceramidase, which has been shown to repress StARD1 expression. The subsequent mitochondrial cholesterol accumulation then impairs the transport of GSH into mitochondria, resulting in mitochondrial GSH depletion and subsequent oxidative stress. These findings constitue the basis for the potential beneficial effects of mGSH replenishment by GSH ethyl ester in NPC.

is not currently understood (Torres et al., 2016). As acid ceramidase (ACDase) has been shown to repress steroidogenic factor-1-dependent expression of StARD1 (Lucki et al., 2012), it remains to be established whether decreased ACDase in NPC disease contributes to the upregulation of StARD1 and subsequent mitochondrial cholesterol loading (**Figure 2**), which is currently under investigation. Moreover, as mentioned above, NPC2 has been shown to contribute to the transport of endosomal cholesterol to mitochondria independently of NPC1 (Kennedy et al., 2012). As the loss of function of NPC2 accounts for a minor fraction of NPC cases, it is conceivable that NPC2 may play a role in the trafficking of endosomal cholesterol to mitochondria in most NPC patients (Kennedy et al., 2012).

Increased mitochondrial cholesterol content in NPC cells can lead to important functional consequences, such as decreased mitochondrial membrane fluidity (Colell et al., 2003), reduced ATP generation (Echegoyen et al., 1993; Yu et al., 2005), and decreased mitochondrial GSH (mGSH) import (Marí et al., 2006; Garcia-Ruiz et al., 2009). In addition, mitochondrial membrane potential and the activity of ATP synthase are markedly decreased in NPC1 mouse brains and neurons (Yu et al., 2005; Balboa et al., 2017; Torres et al., 2017) and the inhibition of ATP synthase is reversible upon mitochondrial cholesterol extraction by cyclodextrin (Yu et al., 2005). In line with these findings, increased mitochondrial cholesterol loading triggers metabolic adaptations in NPC models, characterized mainly by enhanced glycolysis (Kennedy et al., 2014). The depletion of mGSH levels has a severe impact in NPC disease, as inferred by its replenishment with GSH ethyl ester (GSH-EE), which restored the mGSH pool in liver and brain of $Npc1^{-/-}$ mice and in fibroblasts from NPC patients, leading to increased median survival and maximal life span of $Npc1^{-/-}$ mice, protection against oxidative stress and oxidant-induced cell death and restoration of calbindin levels in cerebellar Purkinje cells, which improved locomotor impairment in $Npc1^{-/-}$ mice. In addition, high-resolution respirometry analyses showed that GSH-EE treatment improved oxidative phosphorylation, coupled respiration and maximal electron transfer in cerebellum of $Npc1^{-/-}$ mice (Torres et al., 2017). In contrast, the antioxidant and cytosolic GSH precursor N-acetylcysteine (NAC), which increased total GSH levels in liver and brain homogenates from $Npc1^{-/-}$ mice (Fu et al., 2013), failed to restore the mGSH pool (Torres et al., 2017). These results are in agreement with the notion that increased cholesterol content in NPC mitochondria disrupts membrane dynamics and impairs the transport of GSH from the cytosol into mitochondria (Marí et al., 2009), contributing to the activation of apoptotic pathways seen in NPC mutant cells (Klein et al., 2011). In line with the role of oxidative stress, it has been shown that vitamin E supplementation in the diet delayed weight loss, improved coordination and locomotor function and increased the survival of $Npc1^{-/-}$ mice (Marín et al., 2014). Moreover, vitamin E supplementation preserved Purkinje neurons and reduced levels of astrogliosis, nitrotyrosine

and apoptotic signaling mediated by the c-Abl/p73 pathway (Marín et al., 2014). Part of this beneficial effect could be related to increased levels of α-tocopherol in mitochondria, which has been shown to quench ROS production, especially in hepatic mitochondria (Chow et al., 1999), thus preventing GSH depletion (Stojkovski et al., 2013).

In addition to these events, mitochondrial cholesterol loading may impair mitochondrial dynamics reflected in the balance between fusion and fission events. Disruption of appropriate mitochondrial fluidity following cholesterol accumulation can prevent the fusion of mitochondria with adjacent healthy mitochondria (Baker et al., 2014), leading to increased predominance of fragmented mitochondria. In line with these events, we have recently reported that mitochondria from NPC cells exhibited a more rounded and smaller morphology compared to wild type cells, paralleling the decrease in mitochondrial membrane potential and increased mitochondrial superoxide production (Balboa et al., 2017). Finally, disruption of lysosomal-mitochondrial interplay can also have an impact in the Ca^{2+} buffering capacity of mitochondria (Kiselyov and Muallem, 2008) in LSDs, in line with the effect of suppressing lysosomal function and autophagy in mitochondrial Ca^{2+} homeostasis (Jennings et al., 2006). As mentioned above, it has been recently shown that NPC disease is characterized by the disruption of lysosomal Ca^{2+} homeostasis preceded by the accumulation of sphingosine that impairs the endocytic pathway (Lloyd-Evans et al., 2008). Whether this event is linked to altered mitochondrial Ca^{2+} content and function remains to be established. In addition, further work is needed to demonstrate a causal role for the disruption of Ca^{2+} homeostasis in the death of the Purkinje neurons in NPC disease, as shown in other related diseases (Girard et al., 2012; Kasumu and Bezprozvanny, 2012).

THERAPEUTIC APPROACHES AND CONCLUDING REMARKS

Although the genetic causes of NPA and NPC disease are well understood, the consequences at the level of disruption of intracelular trafficking and interorganelle communication is still incomplete, thus limiting the availability of effective therapy. Enzyme replacement therapy for ASMase and NPC1 deficiency is expected to successfully treat peripheral non-neuronal symptoms of both diseases (Schuchman and Desnick, 2017). However, as this approach may be inefficient to replenish the defective enzymes in the brain due to the blood brain barrier, the expectations for the correction of the neurological symptoms may be disapointing. Therefore, there is the urgent need for alternative possibilites to target both diseases exploiting the fact that several intracelular compartments are involved in the pathogenesis of NPA and NPC diseases (Saffari et al., 2017). In the case of NPC, in which mitochondrial dysfunction and cholesterol accumulation have been well established, strategies targeting mitochondrial cholesterol accumulation and/or downstream consequences may be of potential relevance. In this scenario, recent data indicated that the replenishment of mGSH levels by permeable GSH prodrugs have shown promising results in fibroblasts from NPC patients, correcting the mitochondrial dysfunction and ameliorating oxidative stress, while extending the survival of NPC1 null mice (Torres et al., 2017). Quite intriguingly, the efficacy of mGSH replenishment by GSH-EE was not potentiated by parallel treatment with cholesterol extraction with cyclodextrin, suggesting that both processes are related to each other. This outcome raises the possibility that the efficacy of mGSH restoration by GSH-EE could be explored in combination with other therapies targeting differential pathways. For instance, since curcumin has been shown promising results by restoring intracellular sphingosine balance and cytosolic calcium homeostasis (Lloyd-Evans et al., 2008), it may be worth investigating the combination of curcumin with GSH-EE in NPC disease. Moreover, recent evidence has shown a beneficial role for histone deacetylase (HDAC) inhibitors (i.e., vorinostat or valproic acid) as a novel line of therapy for NPC disease, resulting in the reduction of cholesterol accumulation, improvement of neurodegenerative symptoms and visceral alterations, which culminate in the extension of mouse life span (Kim et al., 2007; Pipalia et al., 2011; Alam et al., 2016; Contreras et al., 2016; Munkacsi et al., 2017). As HDAC can regulate mitochondrial function, it may be worth to test whether combined treatment with HDAC inhibitors and GSHEE would exhibit greater therapeutic benefit in NPC1 mutant mice with respect to either agent alone, supportingh this approach as a promising combination therapy for NPC.

AUTHOR CONTRIBUTIONS

ST, EB, SZ, CE, CG-R, and JF-C discussed findings, analyzed literature and wrote the manuscript. ST, CG-R, and JF-C designed the schematic Figures.

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Interorganelle Communication between Mitochondria and the Endolysosomal System

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The function of mitochondria and lysosomes has classically been studied separately. However, evidence has now emerged of intense crosstalk between these two organelles, such that the activity or stress status of one organelle may affect the other. Direct physical contacts between mitochondria and the endolysosomal compartment have been reported as a rapid means of interorganelle communication, mediating lipid or other metabolite exchange. Moreover, mitochondrial derived vesicles can traffic obsolete mitochondrial proteins into the endolysosomal system for their degradation or secretion to the extracellular milieu as exosomes, representing an additional mitochondrial quality control mechanism that connects mitochondria and lysosomes independently of autophagosome formation. Here, we present what is currently known about the functional and physical communication between mitochondria and lysosomes or lysosome-related organelles, and their role in sustaining cellular homeostasis.

Keywords: lysosome, exosomes, autophagy, quality control, proteostasis, aging

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INTRODUCTION

Eukaryotic cells contain membrane-confined organelles that enable them to compartmentalize specialized biochemical reactions like ATP production, lipid breakdown or protein degradation, performing them at specific cellular locations. To fully benefit from the clear advantages of such specialization, organelles within a cell must communicate, and exchange information and metabolites. Indeed, it is now well accepted that such interactions are necessary to maintain cell homeostasis. Communication between organelles does not necessarily require physical contacts, as information between organelles can be transmitted through transfer of specific metabolites. However, membrane contact sites (MCSs) represent a fast and efficient way to communicate. Such interorganelle physical contacts are currently the focus of much study and they have been associated to many cellular processes, such as signaling, the regulation of calcium homeostasis, lipid metabolism, and organelle localization and dynamics (Helle et al., 2013; Daniele and Schiaffino, 2014). Interactions between organelles are also very important for protein quality control, since misfolded, oxidized and obsolete proteins are dangerous to the cell, and they must be handled suitably to maintain correct proteostasis. This problem is resolved in the cell by organelles working together in a complex and coordinated network (Baixauli et al., 2014; Gottschling and Nyström, 2017).

Mitochondria and lysosomes are at the center of devastating genetic diseases. Research into mitochondrial and lysosome-originated diseases has focused on trying to understand the molecular pathology of the affected organelle. However, it was recently discovered that genetic defects in

one type of organelle may lead to an impairment of the other. Specifically, genetic disruption of mitochondria produces a secondary impairment of lysosomes, and genetic defects in lysosomes can lead to mitochondrial dysfunction, highlighting the functional connection between these two organelles.

Mitochondria are fundamental metabolic organelles in cells, but they also participate in iron and calcium homeostasis, as well as in the regulation of apoptosis, and they are increasingly recognized as key signaling platforms (Friedman and Nunnari, 2014; Chandel, 2015). Like the rest of organelles, mitochondria need to interact with other organelles to carry out their functions, exchanging material and transmitting signals responsible for regulating metabolism, intracellular signaling and cell maintenance (Murley and Nunnari, 2016). This communication can be established in different ways, such as vesicular transport (as initially revealed for organelles within the secretory pathway), the exchange of metabolites or signaling molecules by diffusion, or via direct physical contacts.

The best characterized MSCs are the physical contacts established between the mitochondria and endoplasmic reticulum (ER). These contacts are named mitochondrial-associated membranes (MAMs) and they are critical for many intracellular processes, particularly the regulation of calcium homeostasis, lipid metabolism, mitochondrial fission or regulation of mitochondrial DNA synthesis and division (Friedman et al., 2011; Bravo-Sagua et al., 2014; Lewis et al., 2016). These contacts allow the flux of calcium between the two organelles, coordinating ATP production and facilitating mitochondrial calcium buffering. Under specific conditions, the distance between the mitochondria and ER may vary, affecting their communication and organelle function. For example, tight junctions between mitochondria and ER can cause mitochondrial calcium overload, and mitochondrial membrane permeabilization, driving the cell into an apoptotic programme (Csordás et al., 2006).

Interestingly, defects in MAMs, or in their homeostasis, have been associated with certain diseases, highlighting the importance of the contacts between organelles for controlling both cell and organism homeostasis. Obesity induces an increase of MAMs in the liver, which compromises mitochondrial activity and drives oxidative stress (Arruda et al., 2014; Ma et al., 2017). In obese animals, experimental induction of MAMs deteriorates metabolic homeostasis, while their disruption improves the oxidative capacity of mitochondria (Arruda et al., 2014). MAMs have also been linked to inflammation and there is evidence suggesting that MAMs are involved in triggering an inflammatory response to oxidative stress. Inducers of reactive oxygen species (ROS) activate the inflammasome, driving its localization to MAMs (Zhou et al., 2011). MAMs have been related to neurodegenerative diseases (Manfredi and Kawamata, 2016; Paillusson et al., 2016), such that their frequency and activity are enhanced in both familial and sporadic Alzheimer's disease patients (Area-Gomez et al., 2012). Reduced numbers of ER–mitochondria associations and alteration in Ca^{2+} exchange between these two organelles have been observed in two different amyotrophic lateral sclerosis (ALS) mouse models (Paillusson et al., 2016). The loss of MAM homeostasis has also been linked to cancer, diabetes and pulmonary arterial hypertension (Sutendra et al., 2011; Giorgi et al., 2015; Ma et al., 2017; Rodríguez-Arribas et al., 2017).

The endolysosomal system is a highly dynamic membrane compartment that is critical in the maintenance of cellular homeostasis. The endolysosomal system is made up of several membrane-bound organelles, such as early endosomes, multivesicular bodies (MVBs), lysosomes, lysosome-related organelles (LROs) and other specialized organelles (Klumperman and Raposo, 2014). During endosome maturation, inward budding of the limiting membranes forms small intraluminal vesicles (ILVs) in organelles referred to as MVBs. Endosomes, MVBs and lysosomes regulate signaling, secretion, and the degradation of receptors and other cellular components. MVBs can fuse with the plasma membrane and release their ILVs into the extracellular environment as exosomes, or alternatively, they can fuse with lysosomes for their content to be degraded. Besides degradation, lysosomes carry out many other critical functions, acting as acidic calcium stores that release calcium in response to different stimuli, secreting their content through exocytosis, or repairing the plasma membrane (Perera and Zoncu, 2016). Furthermore, lysosomes can sense the metabolic state of the cell, as it occurs in conditions of starvation or stress, in which mTORC1 is activated and released from the lysosomal membrane, triggering a transcriptional response to increase lysosome biogenesis and activity (Roczniak-Ferguson et al., 2012; Settembre et al., 2013; Ballabio, 2016).

This review will explore some of the more recent findings regarding the communication between mitochondria and the endolysosomal system. Physical contacts between lysosomes and mitochondria are a recent discovery due to the advances in microscopy techniques and molecular biology, and they have mainly been studied in yeast. Beside these physical contacts, mitochondrial derived vesicles (MDVs) represent another means of achieving molecular transfer between mitochondria and the endolysosomal system. Although different modes of communication between these organelles have been identified, their biological meaning is often not yet clear.

MITOCHONDRIA AND VACUOLE INTERACTIONS IN YEAST

Yeast has been a powerful model to study the connections between organelles. The vacuole is the equivalent of the lysosome in yeast, and the first description of a functional connection between the vacuole and mitochondria supported a role in lipid metabolism. Indeed, altered cardiolipin synthesis caused vacuolar damage (Chen et al., 2008). Cardiolipin is an anionic phospholipid predominantly found in the mitochondrial inner membrane and its dissociation from mitochondria leads to mitochondrial dysfunction. Deleting cardiolipin synthase, the enzyme responsible for cardiolipin synthesis in mitochondria, causes several defects in vacuoles and it affects cell survival (Chen et al., 2008). Yeast carrying a mutation in cardiolipin synthase have defective vacuolar morphology, decreased v-ATPase activity and vacuolar basification. Hence, cardiolipin plays a key role

in the functional crosstalk between mitochondria and vacuoles. Although the mechanism underlying this crosstalk is not clear, it seems to involve a perturbation of ion homeostasis since deletion of the Na^+/H^+ exchanger ameliorated the vacuolar defects.

Another functional correlation between the vacuole and mitochondria was discovered during yeast aging. Aged yeast exhibit mitochondrial fragmentation, loss of mitochondrial DNA in their progeny and increased levels of mitochondrial ROS (Lam et al., 2011; McFaline-Figueroa et al., 2011). Two genes have been identified that are related to aging: *VMA1*, encoding a subunit of the v-ATPase, and *VPH2,* an integral membrane protein of the ER required for v-ATPase assembly. Overexpression of *VMA1* and *VPH2* decreases the vacuolar pH, delaying the mitochondrial dysfunction associated with aging. This pro-longevity effect is related to the capacity of the vacuole to store neutral amino acids rather than to its degradative function (Hughes and Gottschling, 2012). Reduced vacuolar acidity in aged yeast also remodels the mitochondrial proteome (Hughes and Gottschling, 2012). Upon loss of vacuolar acidity, specific membrane proteins from mitochondrial membranes are sorted into a mitochondrial-derived compartment (MDC) (**Figure 1**). This membrane compartment is then released by fission and transported to the vacuole for protein degradation by autophagy. Proteins are selectively incorporated into the MDC and this requires the mitochondrial import receptors Tom70 and Tom71. This mechanism represents a protective pathway to preserve mitochondrial integrity in times of stress (Hughes et al., 2016).

Two parallel studies demonstrated that physical contacts exist between mitochondria and the yeast vacuole, known as the vacuole and mitochondria patch (vCLAMP) (**Figure 1**). By screening yeast mutants, two genes were found that affect the formation of the physical contact between the ER and mitochondria, the endoplasmic reticulum-mitochondria encounter structure (ERMES) (Elbaz-Alon et al., 2014). Deletion of the two proteins encoded by these genes, Vps39 and Vam7, that are involved in fusion at the vacuole (Ungermann, 2015), causes an increase in the number of ERMES, although they do not act directly on these structures. Vps39 was previously identified as part of the homotypic fusion and protein sorting (HOPS) tethering complex and it was localized to contact sites between the vacuole and mitochondria (Elbaz-Alon et al., 2014). In a parallel study, physical contacts between mitochondria and vacuoles were discovered by electron microscopy and while Vps39 was implicated in this interaction, Ypt7 was identified in the vacuolar part of the contact (Hönscher et al., 2014) (**Figure 1**). In both cases, a relationship between ERMES and vCLAMP was seen to exist, as confirmed by an increase in the number of ERMES when the vCLAMP is impaired. Conversely, there are more vCLAMPs when ERMES are disrupted. Notably, yeast mutants for both mitochondrial contacts are lethal, demonstrating that both vacuolar and ER contacts with mitochondria contribute to mitochondrial function, possibly by carrying out partially redundant functions (Elbaz-Alon et al., 2014; Hönscher et al., 2014). These connections are necessary to mediate the transport of phospholipids between the mitochondria and endomembrane system during phospholipid synthesis, a process that requires both organelles. Moreover, vCLAMPs are also enriched for specific ion and amino acid transporters, such that the activity of vCLAMPs may extend beyond lipid transport to nutrient sensing and utilization (Elbaz-Alon et al., 2014). Interestingly, the formation of these contacts depends on the metabolism of the cell. Yeast grown on glucose have more contact sites between mitochondria and vacuoles, and this number is reduced when they are grown in glycerol (Hönscher et al., 2014). Hence, ERMES and vCLAMPs are regulated in response to the metabolic status of cells, suggesting

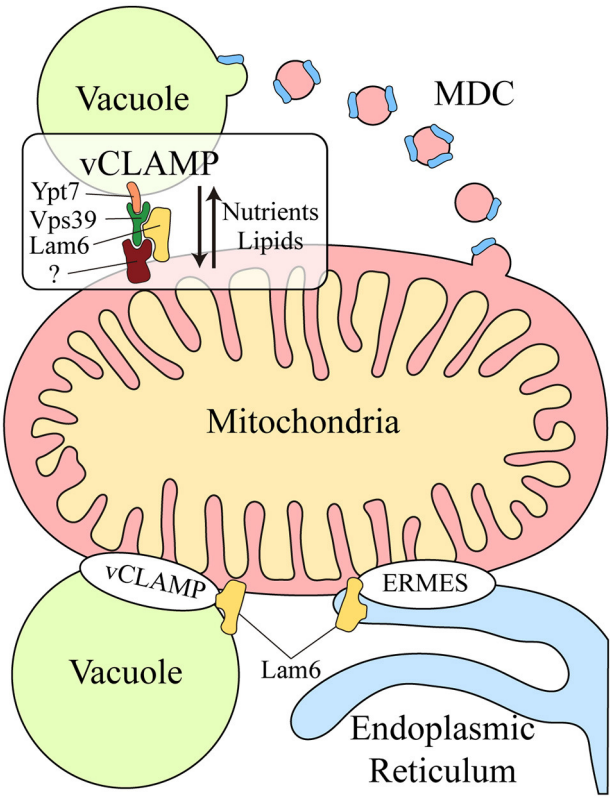

FIGURE 1 | Crosstalk between the mitochondria and lysosomal vacuole in yeast. Two different mechanisms exist for molecular exchange between the mitochondria and lysosomal vacuole: vesicular transport and physical contacts. Mitochondrial membrane proteins can be transported from the mitochondria to the vacuole through the formation of the mitochondrial derived compartment (MDC), particularly for their degradation by autophagy. This mechanism is a protective pathway to preserve mitochondrial integrity in times of stress. In addition, mitochondria and the lysosomal vacuole establish physical contacts, the vacuole and mitochondria patch (vCLAMP), which involves the Vps39, Ypt7, and Lam6 proteins and an unidentified mitochondrial component. This connection participates in the exchange of nutrients and lipids between these organelles. Lam6 is also present in endoplasmic reticulum-mitochondria encounter structure (ERMES), the physical contact between mitochondria and the endoplasmic reticulum. The presence of Lam6 in both mitochondrial contacts makes their co-regulation possible. Hence, vCLAMP is more extensively distributed in an ERMES mutant and conversely, there are more ERMES when vCLAMP is impaired. MDC, Mitochondrial derived compartment; vCLAMP, vacuole and mitochondria patch; ERMES, endoplasmic reticulum-mitochondria encounter structure; (?), unidentified mitochondrial component of vCLAMP.

that both contacts are implicated in similar activities but under different conditions, i.e., respiratory vs. fermentative metabolism.

The coordination of these different contact sites is further revealed by studying Lam6, a novel protein that regulates both contact sites in yeast (Elbaz-Alon et al., 2015). Lam6 is present at both ERMES and vCLAMP contact sites (**Figure 1**), although it is not essential for the formation of these contacts and its absence does not affect their integrity. Rather, Lam6 can extend the contacts between organelles and indeed, vCLAMP is extended in an ERMES mutant but this enlargement does not occur when Lam6 is deleted. Likewise, there are no more ERMES when vCLAMP is impaired in the absence of Lam6, proof of that Lam6 mediates the crosstalk between different organelle contacts in yeast, and in particular that of ERMES and vCLAMP (Elbaz-Alon et al., 2015).

MITOCHONDRIA-ENDOLYSOSOMAL SYSTEM CROSSTALK IN MAMMALS

The molecular mechanisms mediating the communication between mitochondria and lysosomes in mammalian cells remain unclear. An initial mode of communication was discovered when mitochondria were shown to form specific vesicles targeted to the endolysomal system (Soubannier et al., 2012). The protein cargo of MDVs is selectively incorporated, and it may be limited to elements at the outer membrane or it might also include inner membrane and matrix components (**Figure 2**). The molecular mechanism that drives MDVs formation is different from mitochondrial fission, since MDVs are formed in Drp1-silenced cells, Drp1 being a key protein in mitochondrial fission (Soubannier et al., 2012). Parkin induces the PINK1-dependent formation of MDVs under conditions of mitochondrial stress, which are enriched for mitochondrial specific proteins like Tom20 that are targeted to endolysosomal system for degradation (McLelland et al., 2014). These MDVs are originally targeted to the MVB and they finally fuse with lysosome. Such vesicle formation may be a rapid response to mitochondrial stress, shuttling oxidized cargo to lysosomes in order to preserve the integrity of the organelle (McLelland et al., 2014; Sugiura et al., 2014). MDVs have also been reported to be involved in mitochondrial quality control upon acute stress in the cardiac system (Cadete et al., 2016).

Autophagy is the process of removing cellular components through their encapsulation in a double-membrane structure followed by degradation in lysosomes. In particular, the selective removal of damaged mitochondria by autophagy is known as mitophagy. Mitophagy, like MDVs formation, is parkin and PINK1 dependent (Youle and Narendra, 2011; Roberts et al., 2016). However, MDVs are also formed when essential components of autophagy are silenced like Atg5, Beclin-1 or Rab9. Moreover, MDVs do not co-localize with LC3, an autophagosome marker, demonstrating that they are independent of a canonical autophagy process (Soubannier et al., 2012; McLelland et al., 2014). Both MDVs and mitophagy are quality control mechanisms to handle damaged mitochondria. Nevertheless, given that MDVs formation is a faster and less drastic process and it is independent of the classical mitophagy machinery, it could be the first response to mitochondrial stress preceding mitophagy (McLelland et al., 2014; Sugiura et al., 2014).

Mitochondrial dysfunction provokes lysosome impairment, representing a functional connection between the mitochondria and lysosome (**Figure 2**). Deletion of mitochondrial transcription factor A (TFAM) has been used as a model of mitochondrial dysfunction (Vernochet et al., 2012; Viader et al., 2013), as TFAM is essential for the replication and transcription of mitochondrial DNA (mtDNA) (Larsson et al., 1998). In the absence of TFAM, there is less mtDNA, fewer transcripts encoding mtDNA genes and an impairment of the electron transport chain (Gustafsson et al., 2016). Upon deletion of TFAM in T cells, the impairment of mitochondria augments the number of lysosomes. However, lysosomal activity is profoundly impaired, provoking an accumulation of sphingomyelin and autophagy intermediates, and triggering inflammatory responses (Baixauli et al., 2015). The increased lysosomal mass was caused by the activation of the transcription factor TFEB (Transcription Factor EB), a master transcription factor controlling lysosome biogenesis. Increasing the cellular levels of NAD^+ by addition of a NAD^+ precursor improved lysosomal function in this model, evidence that the reduced NAD^+ levels in cells with impaired mitochondrial function causes lysosome dysfunction. Indeed, disturbing mitochondrial function in neurons by genetic deletion of AIF, OPA1 or PINK1, or with specific mitochondrial inhibitors, provokes morphological alterations to lysosomes (Demers-Lamarche et al., 2016). Upon such mitochondrial alterations, lysosomes become larger and they accumulate lysosomal vacuoles, although neurons appear healthy and viable. A higher lysosomal pH, dampened activity of lysosomal enzymes and an accumulation of substrates of autophagy were also registered in these conditions. Antioxidant treatment partially rescued these lysosomal defects, suggesting that excess of mitochondrial ROS triggers lysosome dysfunction (Demers-Lamarche et al., 2016).

More recently, another functional connection between mitochondria and lysosome was discovered. Pharmacologically impaired mitochondria affect lysosome biogenesis, enhancing the expression of certain lysosomal proteins: Lamp1, a lysosomal membrane protein; and lysosomal enzymes for degradation, such as acid alpha-glucosidase and cathepsins. This effect on lysosome activity is dependent on the duration of the mitochondrial damage. Accordingly, some lysosomal genes are upregulated after 1 week of treatment but their expression is downregulated after 4 weeks of treatment, a pattern that is also observed in fibroblasts obtained from patients with complex I deficiency and in mouse embryonic fibroblasts from a mouse model of mitochondrial malfunction (Fernández-Mosquera et al., 2017). The induction of lysosomal biogenesis in early mitochondrial damage is TFEB-dependent and it requires AMPK signaling, representing a response to mitochondrial stress, increased autophagy flux and the capacity to remove damaged mitochondria (Fernández-Mosquera et al., 2017).

Besides this functional and vesicular connection, direct interorganelle contacts between mitochondria and the

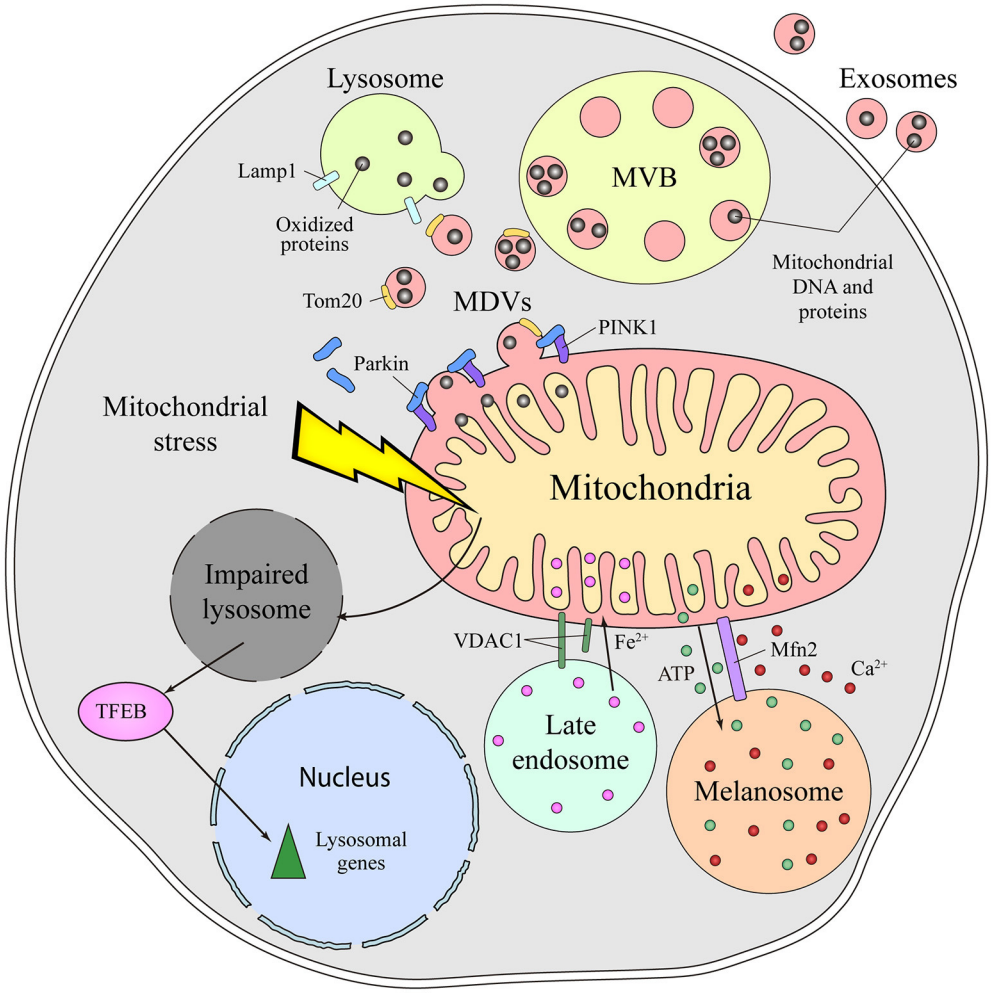

FIGURE 2 | Different means of communication between the mitochondria and lysosome in mammals. Emerging evidence supports the existence of intense crosstalk between the mitochondria and the endolysosomal compartment in mammals. Functional stress or dysfunction of one organelle affects the other. Thus, mitochondrial stress induces a secondary lysosomal dysfunction, which produces activation of TFEB and a transcriptional response associated with lysosomal biogenesis. Additionally, under conditions of stress, mitochondrial derived vesicles (MDVs) are formed in a process dependent on parkin and PINK1. These MDVs traffic obsolete mitochondrial proteins into the endolysosomal system for their degradation, a fast response established to remove oxidized proteins. Once in the endolysosomal route, the mitochondrial content can be degraded by lysosomal enzymes or released to the extracellular milieu via exosomes. Physical connections between the mitochondria and lysosome or melanosome are required for local ATP supply, Ca^{2+} homeostasis, Fe^{2+} transport and to process VDAC1. Mfn2 regulates the mitochondria-melanosome physical connection. MVB, Multivesicular body; MDVs, Mitochondrial-derived vesicles; PINK1, PTEN-induced kinase 1; VDAC1, mitochondrial voltage-dependent anion channel isoform 1; Mfn2, Mitofusin2; TFEB, Transcription Factor EB.

endolysosomal compartment have been identified in mammals, specifically in hypoxic cells and erythrocytes (Sheftel et al., 2007; Hamdi et al., 2016). In conditions of hypoxia, mitochondria are hyperfused and they establish physical contacts with late endolysosomes (**Figure 2**). Such interorganelle contact may be important for the cleavage of VDAC1 by a cysteine protease mainly located in endolysosomes. Cleaved VDCA1 has a different conformation, which protects the mitochondria from autophagy and increases metabolic efficiency (Brahimi-Horn et al., 2015). In erythrocytes, interactions between the mitochondria and late endosomes facilitate the transport of iron between these two organelles (Sheftel et al., 2007; Hamdi et al., 2016).

PHYSICAL CONNECTIONS BETWEEN MITOCHONDRIA AND MELANOSOMES

Lysosome related organelles (LROs) represent a heterogeneous set of organelles that possess some features common to lysosomes but that are cell type-specific. Consistent with their distinct functions, LROs vary in composition and morphology, and they can be distinguished by the primary source of their membrane and their intralumenal content. Some of the LRO's contents are derived from the endolysosomal system, and most of them contain lysosomal proteins and have a low lumenal pH (Raposo et al., 2007).

Melanosomes are LROs of pigmented cells devoted to the synthesis, storage and transport of melanins. Melanosomes are considered biological models of organelle biogenesis and motility. They originate from endosomal precursors and subsequently, they mature through the progressive deposition of melanin and they are transported toward the cell periphery (Raposo et al., 2001). Contacts between mitochondria and melanosomes are visible by electron microscopy and tomography, and these connections affect ca. 1% of melanosomes (Daniele et al., 2014). The formation of these contacts is not based on membrane fusion but on the establishment of fibrillar bridges between the two organelles (**Figure 2**). Mitofusin 2 is thought to play an essential role in these contacts, a surprising new function of this protein that was originally identified as part of MAMs (de Brito and Scorrano, 2008). Mitochondria-melanosome contacts are more abundant in the perinuclear area where new melanosomes are generated and, indeed, stimulating melanosome biogenesis was found to enhance the presence of these contacts. Moreover, these contacts could mediate the ATP synthesis necessary for the maturation and acidification of melanosomes, as well as influencing other events in melanosomes, like melanin synthesis and the exchange of small molecules. Melanosomes are considered acidic calcium stores, indicating that they may also be important in calcium signaling. Due to the antioxidant and free radical scavenging roles of melanin, this connection might be involved in controlling the redox-status of melanocytes.

MITOCHONDRIAL MOLECULES ARE SECRETED TO THE EXTRACELLULAR MEDIA BY EXOSOMES

Exosomes are extracellular vesicles that are secreted by all cell types and that are derived from MVBs upon their fusion with the plasma membrane. The presence of mitochondrial molecules in exosomes represents further indirect evidence of the crosstalk between mitochondria and the endolysosomal system (Torralba et al., 2016). Before being exported to the extracellular milieu, toxic, obsolete or damaged mitochondrial material is loaded into endolysosomes for degradation or extracellular export. Exosomes contain genetic material, mostly non-coding RNA, but also mtDNA (Guescini et al., 2010a,b) (**Figure 2**). Moreover, mesenchymal stem cells and astrocytes can produce even larger vesicles that contain entire mitochondrial particles and mtDNA (Phinney et al., 2015; Hayakawa et al., 2016). The physiological function behind the transport of mitochondrial DNA and proteins out of the cell remains unclear, as do the molecular events responsible for their loading into vesicles. MDVs or direct interorganelle contacts could represent a rapid mechanism to relieve mitochondrial stress in order to maintain the cell fit, perhaps being employed when other degradation pathways are compromised (Desdín-Micó and Mittelbrunn, 2017).

CONCLUDING REMARKS

The study of all facets of interorganelle connections is possible due to new super-resolution imaging and molecular biology techniques, approaches that have shed more light on this emerging and promising field of research. The crosstalk and dependency between organelles could be fundamental to better relate currently misunderstood cell processes. Indeed, secondary effects on related organelles are important in disease pathophysiology and they may represent a starting point to identify novel therapeutic targets to treat human disease. This is especially critical in the case of mitochondrial and lysosomal diseases, devastating pathologies that mostly represent unmet medical needs. Mitochondria and lysosomes (or LROs) interact at different levels and in distinct ways to control cell homeostasis, and these connections are essential for the correct functioning of both organelles. In mammals, communication between organelles has mainly been described at a functional level, observed as secondary damage to lysosomes or LROs when mitochondria are altered, particularly when cells are under stress. Due to the role of lysosomes in degradation, this communication could be a rapid way to firstly reduce mitochondrial stress and preserve mitochondrial integrity. Although physical contact between mitochondria and the endolysosomal compartment has been mainly studied in yeast, recent evidence suggests that this is preserved in mammals. The connection between these organelles could coordinate processes that require the participation of both organelles, such as metabolic adaptation, phospholipid metabolism, regulation of calcium signaling and the control of cellular homeostasis.

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Presenilin 2-Dependent Maintenance of Mitochondrial Oxidative Capacity and Morphology

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Mitochondrial dysfunction plays a pivotal role in the progression of Alzheimer's disease (AD), and yet the mechanisms underlying the impairment of mitochondrial function in AD remain elusive. Recent evidence suggested a role for Presenilins (PS1 or PS2) in mitochondrial function. Mutations of PSs, the catalytic subunits of the γ-secretase complex, are responsible for the majority of inherited AD cases (FAD). PSs were shown to be present in mitochondria and particularly enriched in mitochondria-associated membranes (MAM), where PS2 is involved in the calcium shuttling between mitochondria and the endoplasmic reticulum (ER). We investigated the precise contribution of PS1 and PS2 to the bioenergetics of the cell and to mitochondrial morphology in cell lines derived from wild type (PS+/+), PS1/2 double knock-out (PSdKO), PS2KO and PS1KO embryos. Our results showed a significant impairment in the respiratory capacity of PSdKO and PS2KO cells with reduction of basal oxygen consumption, oxygen utilization dedicated to ATP production and spare respiratory capacity. In line with these functional defects, we found a decrease in the expression of subunits responsible for mitochondrial oxidative phosphorylation (OXPHOS) associated with an altered morphology of the mitochondrial cristae. This OXPHOS disruption was accompanied by a reduction of the NAD^+/NADH ratio. Still, neither ADP/ATP ratio nor mitochondrial membrane potential ($\Delta\Psi$) were affected, suggesting the existence of a compensatory mechanism for energetic balance. We observed indeed an increase in glycolytic flux in PSdKO and PS2KO cells. All these effects were truly dependent on PS2 since its stable re-expression in a PS2KO background led to a complete restoration of the parameters impaired in the absence of PS2. Our data clearly demonstrate here the crucial role of PS2 in mitochondrial function and cellular bioenergetics, pointing toward its peculiar role in the formation and integrity of the electron transport chain.

Keywords: presenilin, mitochondria, glycolysis, oxidative phosphorylation, cellular bioenergetics, Alzheimer's disease

INTRODUCTION

Metabolic dysfunction is central in Alzheimer's disease (AD) since it appears at very early stage of the disorder, even before clinical symptoms (Chen and Zhong, 2013). It is evidenced in patients by a decrease in glucose utilization in temporoparietal association areas, together with cognitive decline and a severe failure in mitochondria oxidative metabolism (Herholz, 2012). Mitochondria are known as the powerhouse of the cell for their capacity to supply energy, but they are also critical in other cellular processes such as apoptosis, reactive oxygen species (ROS) production (Paradies et al., 2014), and calcium homeostasis (Osellame et al., 2012). All these processes turn out to be affected in AD pathology (Hroudova et al., 2014). Studies carried out either on AD patients' tissue samples or AD transgenic mice models reported an array of mitochondrial dysfunctions, including a morphological shift toward fission (Wang et al., 2009), a disrupted motility (Wang et al., 2009; Xie et al., 2013), an impairment of the electron transport chain (ETC) (Bosetti et al., 2002), and an increase in ROS production with deleterious effects on mitochondrial DNA integrity (Onyango et al., 2006). In cytoplasmic hybrid (cybrid) cell lines, generated by insertion of platelets' mitochondria collected from sporadic AD (SAD) patients into human neuroblastoma cells (SHSY5Y) depleted of mitochondria, bioenergetics dysfunctions such as oxidative phosphorylation (OXPHOS) and glucose utilization defects have been observed (Silva et al., 2013). These metabolic perturbations found in AD raised a chicken and the egg issue, namely to define whether mitochondrial dysfunction is a cause or a consequence in this pathology.

Recent studies suggested that Presenilins are involved in the control of mitochondrial functions (Behbahani et al., 2006; Filadi et al., 2016). PS1 and PS2 are two homologous polytopic aspartyl proteases identified as the catalytic subunits of the γ-secretase complex. PSs are directly involved in AD since the production of Aβ is generated after the sequential cleavage of the Amyloid Precursor Protein (APP) by the β and the γ-secretase (Zheng and Koo, 2011). Aβ is the major component of senile plaques found in the brain of AD patients (Miller et al., 1993). Beside their key role in γ-secretase activity and Aβ production, mounting evidence indicate additional roles for PSs in cell physiology, for which their catalytic activity seems less evident. PSs are involved in calcium homeostasis (Zhang et al., 2013), autophagy (Lee et al., 2010), neurotransmitters release (Zhang et al., 2009), synaptic plasticity and memory (Saura et al., 2004). Moreover, although PSs have different localization in the cell with PS1 mainly located at the plasma membrane and PS2 in the trans-Golgi network and endosomal compartments (Meckler and Checler, 2016; Sannerud et al., 2016), they are both enriched in the mitochondria-associated membranes (MAM) (Area-Gomez et al., 2009). MAM are specific membrane domains connecting endoplasmic reticulum (ER) and mitochondria. They are involved in lipid metabolism, calcium and cholesterol homeostasis (van Vliet et al., 2014; Filadi et al., 2017). PS2 has been reported to regulate the formation of ER/mitochondria contacts (Filadi et al., 2016) and calcium cross-talk between these two organelles (Zampese et al., 2011a). It is hence of particular interest to define (i) the precise contribution of PSs to the multiple aspects of mitochondrial function and (ii) to understand the respective contribution of PS1 or PS2 to these processes. Unraveling the role of PSs in cell metabolism is crucial to get insight in their physiological function and to understand how PS gain- or loss-of-function can create a pathological context, related for instance to AD. We performed an array of metabolic measurements on Mouse Embryonic Fibroblasts cell lines (MEFs) derived either from wild type (PS+/+), PS1/2 double knock-out (PSdKO), PS2KO, and PS1KO mice embryos. We found a key role for PS2 (and not PS1) in cell metabolism and especially in OXPHOS and glycolysis. Indeed, the absence of PS2 altered the OXPHOS capacity and integrity but increased glycolytic flux to support energy needs. These pathways are crucial for energy-intensive consumer cells, like brain cells. Such defects might set the basis for further investigation of the metabolic impairments observed in AD.

Abbreviations: ΔΨ, mitochondrial membrane potential; 1R1, PS1 knockout rescued PS1; 2R2, PS2 knockout rescued PS2; AD, Alzheimer disease; APP, amyloid precursor protein; CI-CV, complexes I-V; DTAB, dodecyltrimethylammonium bromide; ETC, electron transport chain; ER, endoplasmic reticulum; FAD, familial AD; FC, flow cytometry; FCCP, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone; HPLC, high pressure liquid chromatography; MAM, mitochondria-associated membranes; MEF, mouse embryonic fibroblast; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PS, presenilin; ROS, reactive oxygen species; SAD, sporadic AD; TEM, transmission electron microscopy; TMRM, tetramethylrhodamine methyl ester; WB, western blotting.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Mouse Embryonic Fibroblasts cell lines (MEFs) derived from wild type (PS+/+), PS1/2 double knock-out (PSdKO), PS2KO and PS1KO mice embryos were previously described (Hebert et al., 2006; Marinangeli et al., 2015). Rescued cell lines refer here to MEFs stably re-expressing human PS1 or PS2 in the corresponding single KO background: PS2KO rescued by human PS2 (2R2) and PS1KO rescued by human PS1 (1R1). Cells were maintained in DMEM low glucose (5.5 mM) (Sigma-Aldrich, St Louis, USA) supplemented with penicillin/streptomycin solution (10 units–10 μg) and 10% fetal bovine serum (FBS) (Thermo Scientific, Rockford, USA). MEF 1R1 and 2R2 cell lines were generated by subcloning the human PSs cDNA sequences in the lentiviral backbone vectors plentiCMV/TOpuro and pTMtm898neo, respectively. Lentiviruses were produced in human embryonic kidney 293T cells. Supernatants containing the lentiviruses were concentrated with the Lenti-X Concentrator kit according to the manufacturer's instructions (Clontech Laboratories; California, USA). MEFs PS1KO and PS2KO were transduced by lentiviral particles followed by puromycin (2.5 μg/ml) selection (1R1) and G418 (500 μg/ml) selection (2R2), respectively. Stable expression of human PSs was monitored by western blotting (WB).

Western Blotting

WB was performed on cell lysates as previously described (Stanga et al., 2016). Primary antibodies used were Anti-OXPHOS

Cocktail (1:1,000; Abcam, Cambridge, United Kingdom); Anti-TOM20 (1:1,000; Proteintech, Rosemont, USA); anti-Presenilin (1:1,000; Cell Signaling, Danvers, USA); anti-Actin (1:3,000; Abcam, Cambridge, United Kingdom). Secondary antibodies used were anti-mouse (1:10,000; GE Healthcare, Little Chalfont, United Kingdom) and anti-rabbit (1:10,000; GE Healthcare, Little Chalfont, United Kingdom).

Metabolic Measurements

Oxygen consumption rate (OCR) measurements were performed with a Seahorse XF96 bioenergetic analyzer (Seahorse Bioscience; Massachusetts, USA). Cells were plated at 80% cell confluence onto a Seahorse 96 well plates 24 h before the assay. According to manufacturer's instructions, cell medium was replaced by the conditional medium (culture medium without FBS and sodium bicarbonate) and incubated at 37°C without CO_2 for 1 h before completion of probe cartridge calibration. Inhibitors targeting the different mitochondrial complexes (Cell Mito Stress Test kit, Seahorse Bioscience) have been added sequentially to the cells during the experiment to measure the basal respiration, the coupling and the spare respiratory capacity: Oligomycin (1 μM); FCCP (1 μM); Rotenone and antimycin A (0.5 μM). Results were normalized to the total amount of protein measured by the Bradford assay kit (Bio-Rad Laboratories, California, USA).

Mitochondrial Membrane Potential ($\Delta\psi$)

Fluorescent cationic probe tetramethylrhodamine methyl ester (TMRM) (Sigma-Aldrich, St Louis, USA) was used to evaluate the $\Delta\Psi$. The uncoupling agent Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) served as control (Sigma-Aldrich, St Louis, USA). Cyclosporin H (2 μM; Abcam, Cambridge, United Kingdom), an inhibitor of multidrug resistance pump activity was used to limit the efflux of TMRM. MEFs were incubated for 30 min at 37°C with TMRM (30 nM), cyclosporine H with or without FCCP (10 μM) diluted in KREBS medium. For the flow cytometry (FC) analysis we trypsinized cells seeded in 6 well plates and harvested them in PBS/5% FBS. After centrifugation at 300 g for 5 min, cells were resuspended in KREBS medium for the FC analysis performed on a BD FACSCanto™ flow cytometer (Biosciences; San Jose, USA). Data were analyzed with the FlowJo software (FlowJo,LLC; Oregon, USA).

Adenine Nucleotides Analysis

24 h after seeding in 10 cm culture dishes, cells were harvested and lysed for 30 min on ice with $HCLO_4$ 1N (Sigma-Aldrich, St Louis, USA) and then centrifuged for 20 min at 10,000 g at 4°C. The pellets were re-suspended in NaOH 0.5 M for protein quantification and the supernatants were adjusted to pH 6–8 using a solution of $KOH/KHCO_3$ 3 M (Sigma-Aldrich, St Louis, USA). Precipitated salts were separated from the liquid phase by centrifugation at 10,000 g at 4°C for 20 min. Samples were stored at −80°C. Nucleotides were separated by high-performance liquid chromatography (HPLC) on 125 × 4.6-mm PartiSphere 5 SAX anion-exchange column (Whatman, Maidstone, UK). Nucleotides were separated with a gradient from 100% buffer A (0.01 M $NH_4H_2PO_4$, pH 3.7) to 100% buffer B (0.48 M $NH_4H_2PO_4$, pH 3.7) over 27 min at a flow rate of 2 ml/min according to the method of Hartwick and Brown (1975). UV detection of nucleotides was performed at 254 nm. Quantification of nucleotides was achieved by peak integration of the area under the curve, validated by the use of external standards (ADP and ATP).

NAD^+/NADH Ratio

Cells were seeded in 96 well plates at 80% confluence 24 h before the assay. NAD^+/NADH ratio was measured by using the bioluminescent NAD^+/NADH-Glo™ assay kit (Promega, Wisconsin, USA) according to the manufacturer's instructions. Briefly, total NAD^+ and NADH were extracted from cell pellets with the basic solution 1% dodecyltrimethylammonium bromide (DTAB). Samples were divided in two for both acid and basic treatments and heated at 60°C for 15 min. The oxidized form is selectively decomposed in the basic solution while the reduced form is decomposed in the acidic solution. For the luminescent reaction, samples were mixed with 100 μl of NAD^+/NADH-Glo™ detection reagent and incubated for 45 min before reading on the GloMax® 96-well plate luminometer (Promega, Wisconsin, USA).

Glucose and Lactate Measurements

Cells were seeded at 80% confluence in 6 well plates. 24 h after, 500 μl of medium per well was collected and deproteinized. Glucose and lactate concentrations were measured using specific enzymatic assays on a CMA600 microdialysis analyzer (CMA Microdialysis AB, Solna, Sweden). Cells were collected for protein quantification and data were normalized to the amount of protein measured by BCA assay (Thermo Scientific, Rockford, USA).

Glycolytic Flux Measurement

Glycolytic rate was evaluated by measurement of the detritiation rate of [3-^{3}H] glucose. Briefly, tritiated glucose (0.2 μCi/ml; Perkin-Elmer; Massachussets, USA) was added to the medium (including 5.5 mM glucose) for 30 min. After medium removal, the tritiated water resulting from detritiated glucose was separated from the non-transported tritiated glucose by column chromatography and measured with the Tri Carb 2,810 liquid scintillation analyzer (Perkin Elmer; Massachussets, USA) as described previously (Marsin et al., 2002). Data were normalized to the amount of protein measured by BCA assay (Thermo Scientific, Rockford, USA).

Complex I (CI) Enzyme Activity Assay

The activity of complex I of the mitochondrial respiratory chain was evaluated with the Complex I Enzyme Activity Dipstick Assay kit (Abcam, Cambridge, United Kingdom). Cells were scraped in PBS and centrifuged for 5 min at 4°C at 500 g. 10 volumes of extraction buffer were added to the pellets prior to 20 min of incubation on ice. After centrifugation, pellets were discarded and supernatants were used to determine protein concentration (BCA assay). Samples (corresponding to 30 μg of proteins) were added to a microplate with blocking buffer. The dipsticks (containing an antibody capturing the CI) were

immersed in the samples to capture the CI. The NADH and NBT were added to allow the oxidation of NADH by the complex I, which in turn reduce NBT to form a purple precipitate. Colorimetric signals were quantified with the Gel Doc 2,000 coupled with Quantity One software (Bio-Rad; California, USA).

Transmission Electron Microscopy (TEM)

Cells were fixed with 2.5% glutaraldehyde in phosphate buffer and kept in the fixative during 1 h at room temperature. They were washed and postfixed with 1% osmium tetroxide in the same buffer containing 0.8% potassium ferricyanide at 4°C. The samples were dehydrated in acetone, infiltrated with Epon resin during 2 days, embedded in the same resin and polymerised at 60°C during 48 h. Ultrathin sections were obtained using a Leica Ultracut UC6 ultramicrotome (Leica Microsystems, Vienna) and mounted on Formvar-coated copper grids. They were stained with 2% uranyl acetate in water and lead citrate. Sections were observed in a Tecnai Spirit electron microscope equipped with an Eagle CCD camera (FEI, Eindhoven, The Netherlands).

Statistical Analysis

The number of experiments for each experimental condition is indicated in the figure legends. Data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) by ANOVA followed by Bonferroni's multiple comparison tests. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Absence of PS2 Results in Decreased OXPHOS without Alteration of the $\Delta\Psi$

PSs expression profile was measured by WB in MEF cell lines with antibodies directed against PS1 and PS2 CTFs (**Figures 1A,B**). The expression profile indicated the restoration of PSs expression in 2R2 (PS2 KO stably expressing human PS2) and 1R1 (PS1KO stably expressing human PS1). We evaluated the OXPHOS capacity by measurement of the OCR. The overall profile of OCR and the parameters related to the OXPHOS activity measured before or after drug addition, that are basal respiration, coupling (oxygen consumption devoted to ATP synthesis under resting conditions) and spare respiratory capacity (maximal uncoupled rate of respiration minus the basal rate) were all impaired in PSdKO and PS2KO cells (**Figures 1C–F**). Stable re-expression of PS2 in the PS2KO background (2R2) restored all these parameters to the levels measured in control cells (PS+/+). Importantly, the absence of PS1 did not affect mitochondrial respiration. We next measured the $\Delta\Psi$ with the TMRM probe by FC (**Figure 2A**) and by fluorescence measurement in microplates (Figure S1A). FC analyzes were performed on gated homogenous populations that accounted for more than 90% of the total population (Figure S1B). FCCP, an uncoupling agent abolishing $\Delta\Psi$ was used as a control. In both approaches (FC and direct fluorescence measurements), no significant differences in $\Delta\Psi$ were observed between the different populations of MEFs, although oxygen consumption was altered in PS2KO and PSdKO cells.

Increased Anaerobic Glycolysis Sustains the Energy Production in PSdKO and PS2KO MEFs

The observed PS2-dependent OXPHOS capacity decrease without impairment of $\Delta\Psi$ led us to evaluate the total ADP and ATP cellular levels. This was achieved by HPLC. No differences were observed in the ADP/ATP ratio between the cell lines (**Figure 2B**). Considering that we detected a significant decrease in coupling in PSdKO and PS2KO cells, we suspected that ATP production in PSdKO and PS2KO cells could result from an increased glycolysis (**Figures 2C,D**) that would counteract the defects observed in OXPHOS. We measured the levels of lactate secreted and glucose consumed by cells, as marker of glycolysis (**Figure 2C**). Both were indeed significantly higher in PSdKO and PS2KO cells. No changes were observed in PS1KO cells. Lactate secreted and glucose consumed measured in 2R2 cells were comparable to those measured in PS+/+ cells. These data were in line with the significant increase of the glycolytic flux (measured by detritiation of [3-^{3}H] glucose) observed in PSdKO and PS2KO cells, that was also restored in 2R2 to the levels measured in PS+/+ cells (**Figure 2D**).

Absence of PS2 Impairs the ETC

So far, our results showed that mitochondrial oxidative capacity was impaired in the absence of PS2. This defect can be compensated by the increased glycolytic flux we observed. We further investigated the mitochondrial defects by profiling the expression of the five mitochondrial complexes. This was performed by WB with a cocktail of antibodies targeting representative subunits of the five mitochondrial complexes. Results showed that the expression of some complexes of the ETC was perturbed in PSdKO and PS2KO cells: CI and CII subunits were significantly decreased in both cell lines, and CIV was more specifically decreased in PS2KO cells. The expression profile of CI, CII, and CIV was restored in 2R2 cells (**Figures 3A,B**). To confirm these observations, we measured by an enzymatic assay the activity of the CI, which was significantly decreased in PSdKO and PS2KO cells but totally restored in 2R2 cells (**Figure 3C**). Since CI uses NADH as an electron donor, we assessed whether the redox state was also affected by measuring the NAD^{+}/NADH ratio. We measured the NAD^{+}/NADH ratio using a luminescence assay. Consistent with the measurement of CI subunits by WB, NAD^{+}/NADH ratio was decreased in PSdKO and PS2KO cells and restored in 2R2 cells (**Figure 3D**).

Mitochondrial Morphology in PSs-Deficient Cells

We measured by WB in cell lysates the expression level of the mitochondrial import receptor subunit TOM20 (**Figures 4A,B**) as a marker of mitochondrial mass (Whitaker-Menezes et al., 2011). No significant difference in TOM20 levels was observed between the different cell types suggesting a comparable mass of mitochondria in all the cell lines tested, including PSdKO and PS2KO cells. Mitochondrial mass is

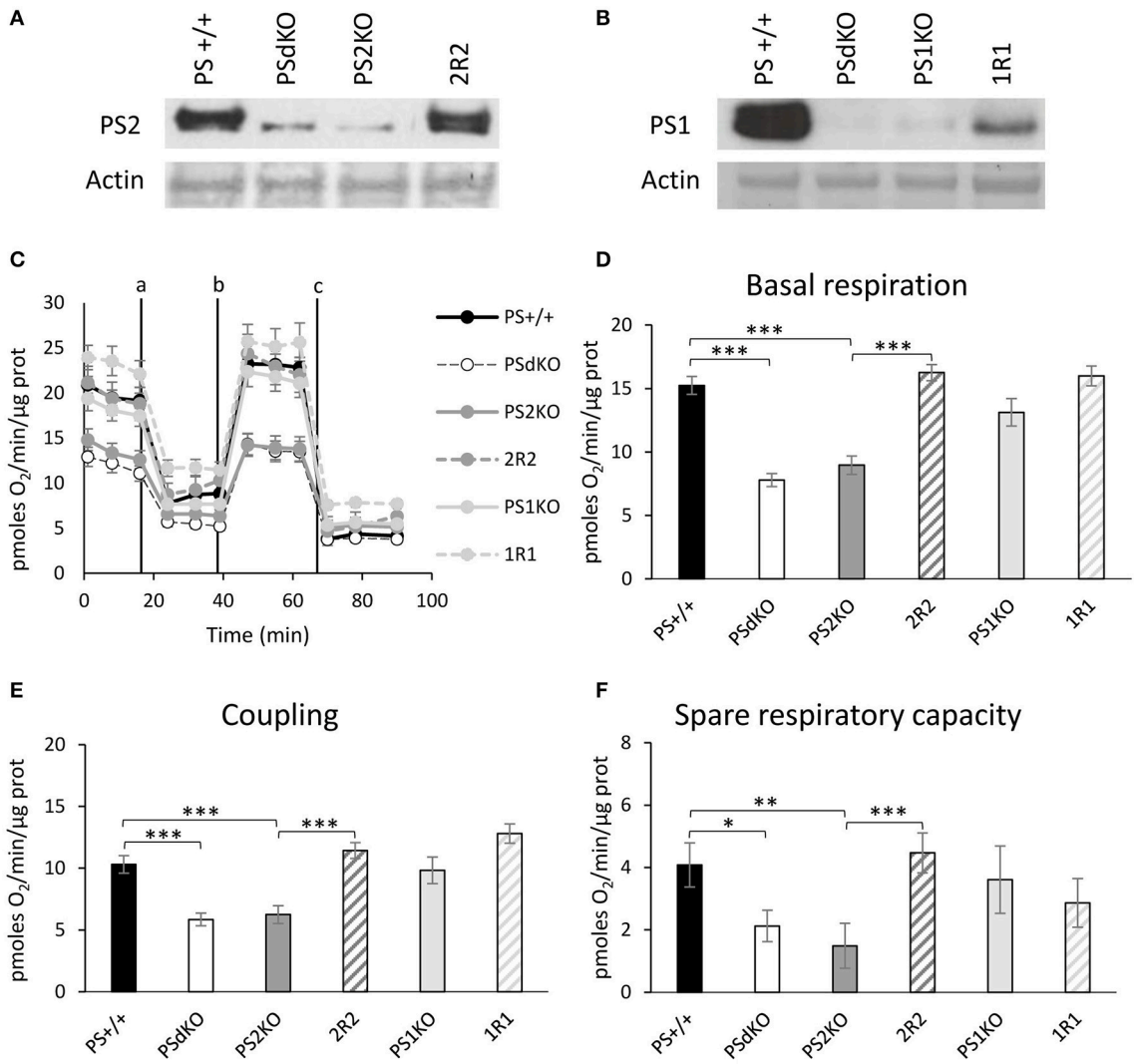

FIGURE 1 | Assessment of the OXPHOS capacity by measurement of the OCR. Experiments were carried out in MEF cell lines wild type (PS+/+), PS1/2 double KO (PSdKO), PS2KO, PS1KO and rescued cell expressing human PS1 and PS2 in PS1KO and PS2KO backgrounds, respectively (2R2 and 1R1). PSs expression **(A,B)** was analyzed by WB in cell lysates. Actin was used as a loading control. **(C)**. OCR was determined using the Seahorse XF96 bioenergetic analyzer. Vertical lines indicate the time point at which the different compounds have been added: a. Oligomycin (CV inhibitor) b. FCCP ($\Delta\Psi$ uncoupler) c. Rotenone (CI inhibitor) and antimycin A (CIII inhibitor). The basal respiration **(D)**, the coupling ratio **(E)** and the spare respiratory capacity **(F)** were calculated according to the Cell Mito Stress Test kit's recommended protocol. Values (means ± sem) are given in pmol O_2/min/µg protein.*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ ($n = 18$ from 3 independent experiments).

a gross indicator that could be only hardly related to the changes in mitochondrial function we observed. Thus, general mitochondrial morphology was evaluated by immunofluorescent staining targeting TOM20 (Figure S2), and no significant changes were observed about the shape and distribution of the mitochondrial network. Morphology and structure of single mitochondria were evaluated by TEM. Strikingly, we observed defective cristae (**Figure 4C**) in PSdKO and PS2KO cells when compared to PS+/+ cells. Cristae were less defined and less numerous in MEFs PSdKO and PS2KO and, importantly, these morphological defects were not observed in PS1KO cells.

DISCUSSION

The physiological function of the Presenilins 1 and 2 is far from being fully elucidated. Over the two last decades, the seminal observation identifying PS1 as the catalytic core of the γ-secretase (De Strooper et al., 1998; Kimberly et al., 2003) launched a considerable research effort aimed at understanding the exact role of PSs in γ-secretase activity and Aβ production. However, an increasing number of studies focused on γ-secretase independent PSs functions such as regulation of calcium homeostasis (Zhang et al., 2013) or neurotransmitter (glutamate) release (Zhang et al., 2009). Since PSs were shown to be enriched

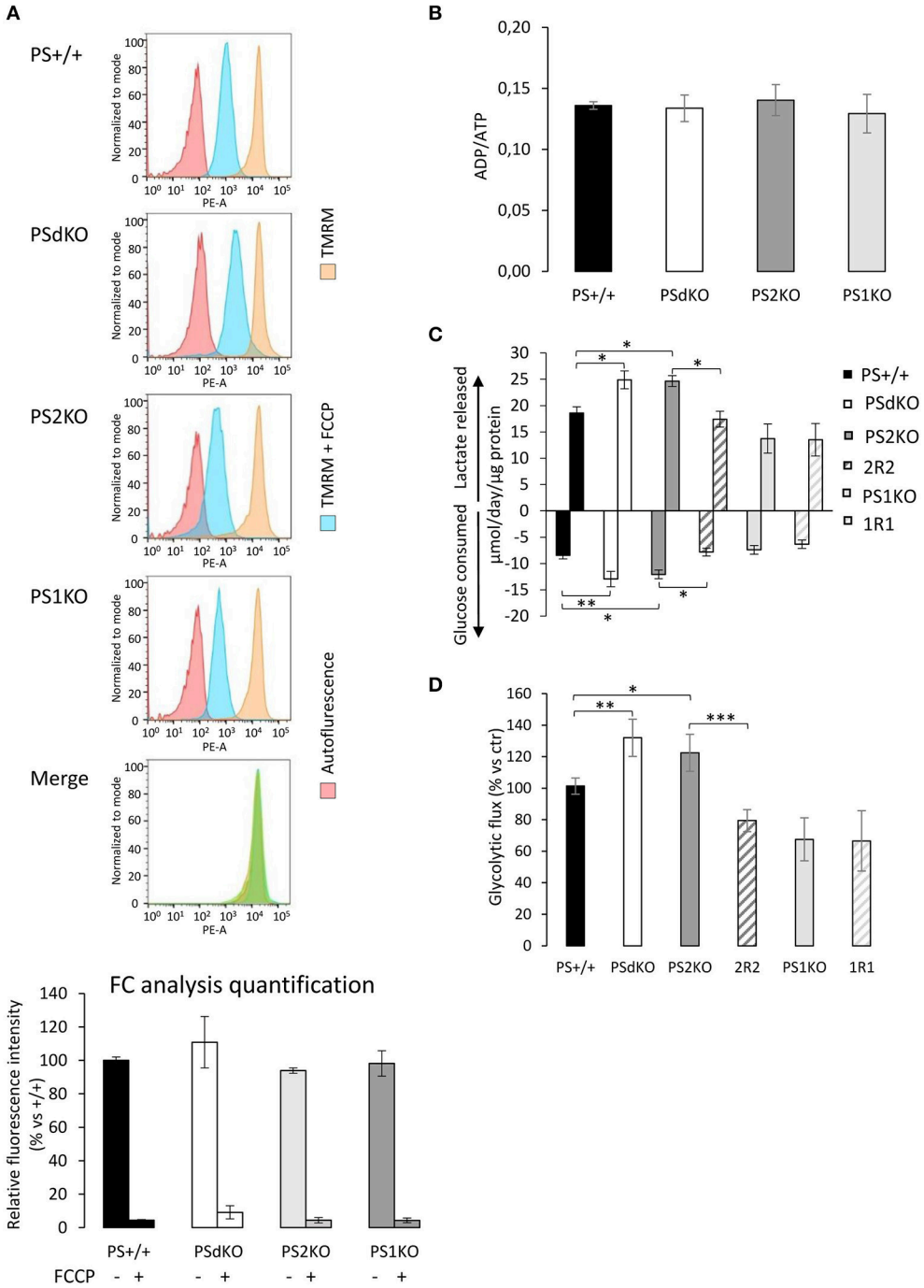

FIGURE 2 | Evaluation of the $\Delta\Psi$, ADP/ATP ratio and glycolysis. $\Delta\Psi$ was evaluated in MEF cells with the TMRM probe in the presence or absence of the $\Delta\Psi$ uncoupling agent FCCP and analyzed by FC ($n = 4$ from 2 independent experiments) **(A).** Histogram peaks represent (from left): autofluorescence; relative fluorescence of cells treated with TMRM+FCCP and of cells treated with TMRM only. The merge of histograms (in green) plots the overlapping signal obtained for the 4 cell lines in the TMRM only condition. Signals obtained were quantified (bottom) and results are expressed as the percentage of relative mean fluorescence intensity measured in PS+/+ cells. (**B**). ADP and ATP were measured by HPLC 24 h after seeding and data, expressed as ADP/ATP ratio, were normalized to protein content ($n = 6$ from 3 independent experiments). **(C)**. Glucose consumption (negative columns) and lactate release (positive columns) were measured in supernatant from fresh medium after 24 h of culture. Data were normalized to protein content and expressed as µmol/day/µg prot. $^{*}p < 0.05$, $^{**}p < 0.01$, ($n = 16$ from 6 independent experiments). **(D)**. Glycolysis rate was determined by the detritation rate of [3-^{3}H] glucose after a 30 min incubation. Data were normalized to protein content. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ ($n = 12$ from 6 independent experiments).

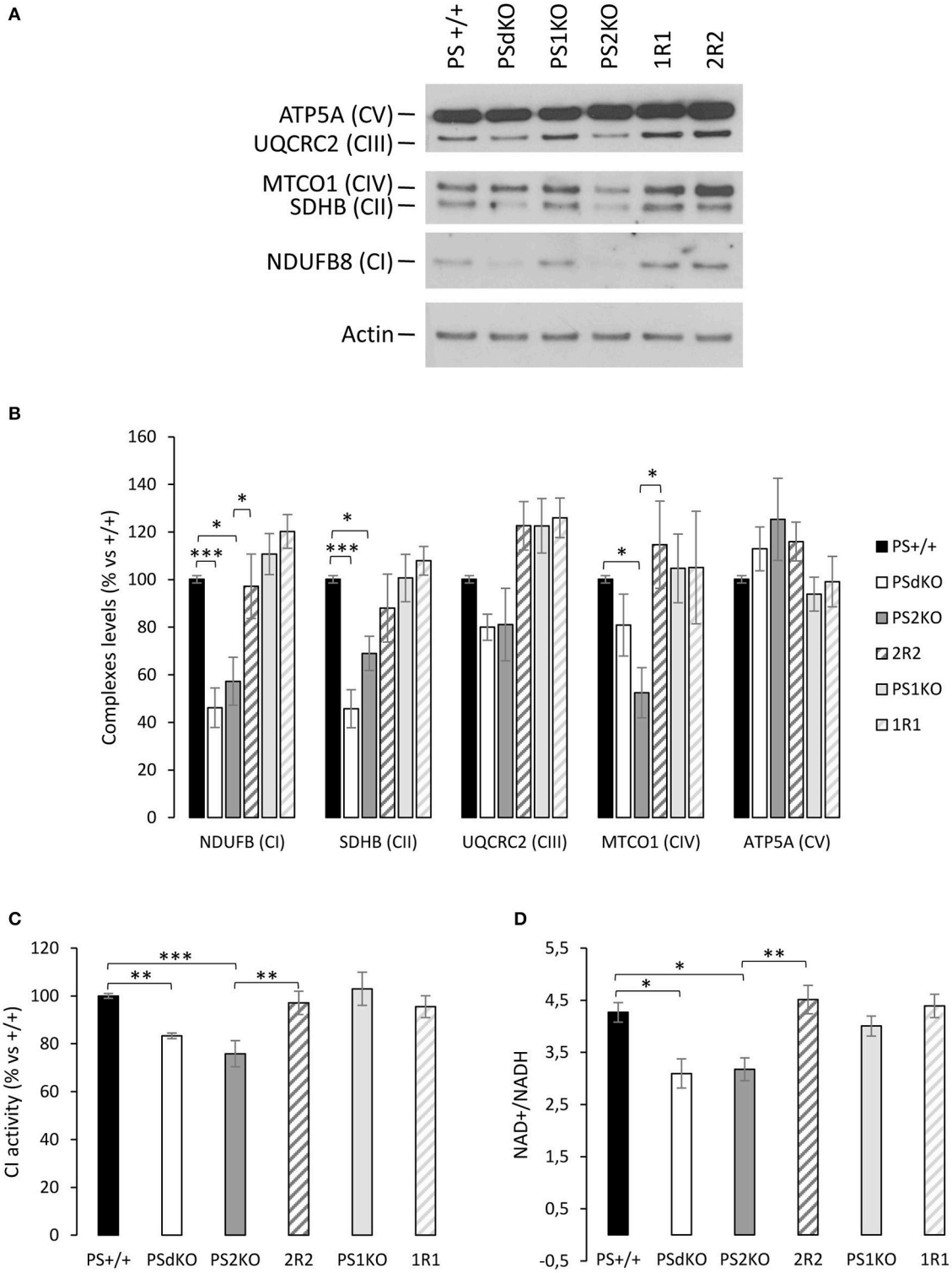

FIGURE 3 | Mitochondrial respiratory chain complexes expression profile, NAD^+/NADH ratio and CI activity. **(A)**. The expression level of representative protein subunits from each of the five mitochondrial complexes (NDUFB8 for CI; SDHB for CII; UQCRC2 for CIII; MTCO1 for CIV; ATP5A for CV) was analyzed by WB on cell lysates. Actin was used as a loading control ($n = 5$ from 5 independent experiments). **(B)**. WB quantifications (means ± sem) are given as percentage of signal measured in PS+/+ cells.*$p < 0.05$, ***$p < 0.001$. **(C)**. Mitochondrial CI activity was evaluated by the enzyme activity dipstick assay. **$p < 0.01$, ***$p < 0.001$ ($n = 12$ from 6 independent experiments). **(D)**. NAD^+/NADH ratio was quantified by a bioluminescent kit *$p < 0.05$, **$p < 0.01$ ($n = 24$ from 8 independent experiments).

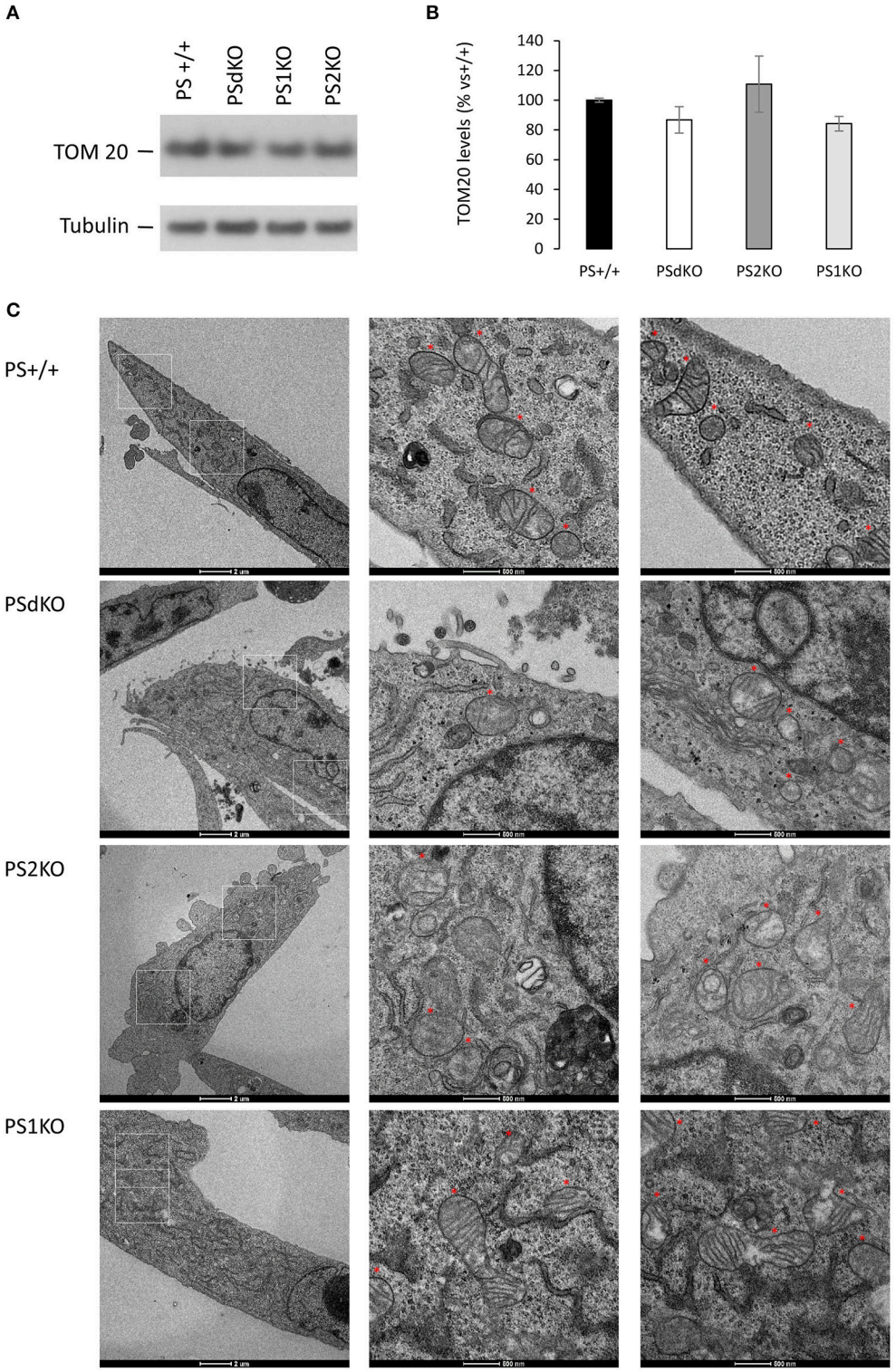

FIGURE 4 | Characterization of mitochondrial mass and morphology. TOM20 expression was analyzed by WB on cell lysates **(A)**. Tubulin was used as a loading control. TOM20 quantification **(B)** (means ± sem) is given as the percentage of the signal measured in the PS+/+ cells ($n = 6$ independent experiments). **(C)** TEM's of cell section. Higher magnification regions (right columns) are boxed in white (left column). Red stars are indicating the position of the mitochondria on the micrograph. Scale bars are indicated on the bottom of the pictures.

in MAM, a domain involved in different pathways related to ER and mitochondria functions (van Vliet et al., 2014; Filadi et al., 2017), we investigated the potential role of PSs in the control of cellular metabolism by measuring an array of parameters related to mitochondrial activity in PSs-deficient MEF cell lines. We observed in the absence of PS2, alterations in OXPHOS capacity and integrity associated with a decrease NAD^+/NADH ratio. Along with these functional defects, the integrity of the mitochondrial cristae was also affected. These defects occurring in absence of PS2 were compensated by an increased glycolysis. Mitochondrial function was fully restored by stable re-expression of PS2 in our model.

PS2 in OXPHOS and $\Delta\Psi$

We measured the OCR as an indirect measurement of the OXPHOS. Our results indicate a significant decrease in PSdKO and PS2KO of the OCR. All the parameters we measured (basal respiration, coupling and spare respiratory capacity) were decreased in PSdKO and PS2KO cells, supporting a general defective OXPHOS capacity. One could have expected here an impact on the $\Delta\Psi$ and ATP production, since the ATP synthase uses the electrochemical gradient formed by pumping protons through the inner membrane to produce ATP. With the TMRM probe, we observed that the $\Delta\Psi$ measured is stable in all the cell types. Previous studies reported a $\Delta\Psi$ decrease in PSdKO and PS2 KO cells (Behbahani et al., 2006). This discrepancy could be due to different procedures as for instance the use of a different probe (JC-1) at high concentration (Perry et al., 2011). Behbahani et al. used like us FC analysis as a readout for $\Delta\Psi$, but it should be noted that in their case the use of the JC-1 probe to measure $\Delta\Psi$ changes implies a shift in the emission wavelength of the probe (with overlapping spectra) that renders the analysis by FC more difficult to interpret. With the TMRM probe, by using FC or direct fluorescent measurement in microplates (see Figure S1) as a readout we observed that the $\Delta\Psi$ is stable in all the cell types analyzed. $\Delta\Psi$ is also known to be one of the most stable parameter in the cell; even in the case of a deficit, the ATP synthase will work in reverse to keep it stable (Uechi et al., 2006).

To note, the main source of ROS production is the ETC, and their implication in AD could rise the question about their production in our model. We could expect a decrease of ROS production in PSdKO and PS2KO regarding of the OXPHOS deficit. A previous study showed a ROS increase in PSdKO compared to PS+/+, in serum deprivation conditions (Boo et al., 2009) that we do not modelize here. We performed few pilot ROS measurement (data not shown) in basal state but no significant message came up. It would be of interest, given the implication of ROS in AD, to further study the PSs-dependent and ROS production in relevant models.

PS2 and ATP Production

Surprisingly, the ADP/ATP ratio we measured by HPLC was not impaired while the coupling (oxygen dedicated to ATP production) was significantly decreased in PSdKO and PS2KO cells. This is consistent with the increase of glycolytic flux and lactate production we observed in PSdKO and PS2KO cells. We used two distinct approaches to estimate glycolysis in our model: (i) we measured the levels of glucose consumed and lactate released in cell media; (ii) we measured the glycolysis rate by detritiation of [3-^{3}H]glucose. These combined approaches showed that the glycolysis is increased significantly in PSdKO and PS2KO, providing the fuel to compensate their OXPHOS deficit. It should be noted that, similarly to undifferentiated stem cells (de Meester et al., 2014), MEF cell lines use anaerobic glycolysis even in the presence of oxygen to produce their energy. It would be of prime interest to analyze if such compensatory capacity exists in non-proliferative and oxidative cells like primary neurons. The metabolic phenotype resulting from altered PS2 function could be more drastic in neuronal cells. This would be very important in the context of the Alzheimer pathology, in which PSs play a central role. Zampese and collaborators highlighted in SH-SY5Y neuronal cells overexpressing a FAD PS2 mutant an enhanced Ca^{2+} transfer between ER and mitochondria (Zampese et al., 2011b). On the other hand, SH-SY5Y treated with siRNA specific for PS2 showed a decreased contact between mitochondria and ER, which induced a decrease in calcium flux. The calcium crosstalk is known to be an important messenger for Krebs cycle enzymes stimulation and kinetics of the mitochondrial complexes. The mitochondrial calcium sequestration capacity is also important in the excitotoxicity and mitochondrial oxidative stress related to AD. It is therefore possible that a lack of calcium flux could be one cause of the OXPHOS defect observed in our PS2KO cells.

PS2 in ETC and Mitochondrial Network and Morphology

We found that the bioenergetics defects observed were neither due to a decrease in the mitochondrial mass nor associated with a compensatory induction in mitochondrial mass. This favors the hypothesis of a direct disruption of the ETC activity in absence of PS2 rather than an overall defect in mitochondria biogenesis. This would be interesting to further address in postmitotic cells, such as neurons, where one might expect a proliferation of mitochondria as an attempt to compensate for the OXPHOS deficits. Such a compensatory induction was seen in heart and muscle (and to a lesser extent in brain) of mice deficient in the adenine nucleotide translocator-1 (ANT1), on which OXPHOS depends since it provides the ADP substrate (Esposito et al., 1999)

In order to understand the origin of the OXPHOS defect, we assessed the expression profile of the 5 mitochondrial complexes (CI-CV) of the ETC with a cocktail of antibodies. Even if we did not evaluate the expression of the entire complexes by this approach, we found the expression of several complexes of the ETC (CI, CII, and CIV) to be decreased in PSdKO and PS2KO cells. This was consistent with a functional analysis indicating a decreased CI activity in the same cells. The most important electron donor produced by the Krebs cycle and used by the CI is NADH and NAD^+ is an important co-factor regulating metabolic homeostasis (Canto et al., 2015). We therefore assessed their redox state by measuring the NAD^+/NADH ratio and observed a significant decrease of the ratio in PSdKO and PS2KO cells. This can result either from an accumulation of NADH not used by the dysfunctional CI or from a decrease in NAD^+

associated to metabolic pathways. NAD^+ and NADH could also both be affected and synergistically influence pathways regulating metabolism homeostasis (Canto et al., 2015).

The perturbation of the integrity of the ETC in PSdKO and PS2KO correlates with an altered structure of their cristae as evidenced by TEM. Cristae are the seat of the ETC, it is thus logical that the OXPHOS capacity and complexes expression are affected when their structure is altered. It has been demonstrated that cristae shape could regulate respiratory chain supercomplexes assembly and stability, impacting the respiratory efficiency (Cogliati et al., 2013). One of the key regulators of cristae shape is OPA1 and it would be interesting to further study the relationship between PS2 and OPA1. Alternatively, considering MAM as an important compartment for the homeostasis of lipids such as for example cardiolipin, one of the most prominent lipids in cristae (Paradies et al., 2014; Ikon and Ryan, 2017), we suggest that the morphological deficit of the cristae could be related to a dysregulation of MAM function in absence of PS2. To note, the MAM hypothesis has been proposed in AD since all the pathways (cholesterol, lipid and calcium homeostasis, mitochondrial OXPHOS stimulation) controlled by this compartment are altered in the pathology (Area-Gomez and Schon, 2017).

In conclusion, we reported here a specific physiological role of PS2 in cellular metabolism. Indeed, absence of PS2 results in defective mitochondrial cristae correlating with an impaired OXPHOS capacity and a modified redox state (NAD^+/NADH ratio). MEF cell lines, which are glycolytic cells, increase this glycolytic capacity to sustain their energy need in absence of PS2. This implies that impairment of these PS2-dependent processes could be involved in the progression of pathologies like AD in which PS2 play a key role. Investigation in an oxidative model and particularly in neurons are required to further explore this hypothesis.

AUTHOR CONTRIBUTIONS

SC, SS, and PK-C designed the research study; SC and SS conducted experiments with fundamental input of PEP, MB, and LB; all the authors analyzed data. SC, SS, and PK-C wrote the paper; all the authors have read and approved the final manuscript.

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REVIEW
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Role of Cardiolipin in Mitochondrial Signaling Pathways

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The phospholipid cardiolipin (CL) is an essential constituent of mitochondrial membranes and plays a role in many mitochondrial processes, including respiration and energy conversion. Pathological changes in CL amount or species composition can have deleterious consequences for mitochondrial function and trigger the production of reactive oxygen species. Signaling networks monitor mitochondrial function and trigger an adequate cellular response. Here, we summarize the role of CL in cellular signaling pathways and focus on tissues with high-energy demand, like the heart. CL itself was recently identified as a precursor for the formation of lipid mediators. We highlight the concept of CL as a signaling platform. CL is exposed to the outer mitochondrial membrane upon mitochondrial stress and CL domains serve as a binding site in many cellular signaling events. During mitophagy, CL interacts with essential players of mitophagy like Beclin 1 and recruits the autophagic machinery by its interaction with LC3. Apoptotic signaling pathways require CL as a binding platform to recruit apoptotic factors such as tBid, Bax, caspase-8. CL required for the activation of the inflammasome and plays a role in inflammatory signaling. As changes in CL species composition has been observed in many diseases, the signaling pathways described here may play a general role in pathology.

Keywords: Barth-Syndrome, cardiolipin, mitochondria, respiratory chain

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INTRODUCTION

The adult human body hydrolyses 64 kg adenosine triphosphate (ATP) per day. Mitochondria are the primary source of the energy in most tissues and their contribution to energy production is particularly important for tissues with high-energy demand, like neuronal or cardiac tissue. Besides their role in energy conversion, mitochondria have multiple functions in the metabolism, like the citric acid cycle, the urea cycle, the metabolism of amino acids and lipids. Mitochondria also play a role in the biogenesis of heme and iron-sulfur clusters (DiMauro and Schon, 2003). Dysfunctional mitochondria, which cannot provide the required energy particularly affect neuronal tissue and the heart (Wallace, 1999). Cardiomyopathies are frequently associated with defects in respiratory chain subunits and their assembly factors, but also with defects in mitochondrial translation and mtDNA maintenance (Schwarz et al., 2014).

Mitochondria are surrounded by the outer membrane (OM), which allows selective transport of small metabolites and connects mitochondria to other cellular organelles like the endoplasmatic/sarcoplasmatic reticulum (ER/SR), the lysosome and the plasma membrane (Gray, 1963; Elbaz-Alon et al., 2014; Ellenrieder et al., 2017). The inner membrane (IM) separates two compartments, the intermembrane space (IMS) and the matrix. The inner boundary membrane

and the cristae structures are distinct functional areas of the IM. The inner boundary membrane is formed by segments of the IM, which approximate the OM in close apposition. The cristae structures are invaginations into the matrix and harbor the respiratory chain. The phospholipid cardiolipin (CL) is a hallmark lipid of mitochondria and almost exclusively found in mitochondrial membranes (Pangborn, 1945). CL is predominantly located in the inner membrane and associated to many mitochondrial functions (see below). Mitochondrial dysfunctions caused by changes in the CL pool are associated to a large number of cardiac diseases (Paradies et al., 2004; Han et al., 2005; Petrosillo et al., 2005; Sparagna et al., 2007; He and Han, 2014; Mulligan et al., 2014). Barth syndrome (BTHS) is an inherited cardiomyopathy, associated with skeletal myopathy, growth retardation and neutropenia and occurs at an estimated frequency of about 1 case per 300,000–400,000 births (Barth et al., 1996; Cantlay et al., 1999; Steward et al., 2010). BTHS is caused by a mutation in the TAZ gene, encoding for Tafazzin, a mitochondrial acyltransferase, involved in the biogenesis of CL (Bione et al., 1996; Dudek and Maack, 2017).

Signaling pathways monitor the physiological state of mitochondria, and trigger a cellular response to various stress conditions. In this review, we describe how alterations in the CL pool cause mitochondrial dysfunction and trigger retrograde signaling pathways. We will discuss the requirement of CL for cellular signaling pathways, such as protein kinase C (PKC) signaling. Upon mitochondrial stress, CL is externalized on the outer mitochondrial membrane forming a binding platform for the specific recruitment of signaling molecules. CL microdomains play a role in autophagy, apoptosis and inflammasome signaling.

CL IS ESSENTIAL FOR MITOCHONDRIAL FUNCTIONS

CL Species Composition and Biosynthesis

Mitochondrial membranes have a characteristic lipid composition, with a high CL content in the IM, where it contributes up to 20% of total lipids (Gebert et al., 2009; Schlame and Greenberg, 2016; Tatsuta and Langer, 2016). The outer mitochondrial membrane also contains CL, where it constitutes up to 3% of the total lipid content (de Kroon et al., 1997). Peroxisomes are the only other organelles, in which a significant amount of CL (2–4% CL of the total phospholipids) was identified (Wriessnegger et al., 2009). CL is composed of a glycerol head group and two phosphatidylglyceride backbones. In total, four fatty acids chains are bound to CL, which differ in length and saturation. In most mammalian tissues, a multitude of different fatty acids bound to CL form a highly diversified CL pool (Maguire et al., 2017). In some tissues, like the mammalian heart, the CL pool consists of a defined species composition with linoleic acid (18:2) being the predominant form for all four acyl chains (Hoch, 1992). It seems that tissue specific metabolism and energy requirements, determine specific fatty acid composition of CL (Paradies et al., 2014). A significant adaption of the CL pool to external stress signaling has been reported in several studies. In yeast, increased total CL levels were found under temperature stress (Luévano-Martínez et al., 2015). A recent study observed increased total levels of CL and increased linoleic acid (18:2) content in CL, in skeletal muscle in response to overload stimuli (Fajardo et al., 2017).

The IM is the location of the CL biosynthesis and remodeling pathway (**Figure 1A**). Phosphatidic acid (PA) is imported from the ER and transported across the IMS with the help of the protein complex PRELID-TRIAP1 (Connerth et al., 2012; Potting et al., 2013; Tatsuta and Langer, 2016). After activation of PA by the CDP-DAG synthase TAMM41 (Kutik et al., 2008), the phosphatidylglycerol phosphate synthase (PGS1) catalyzes the committed step by converting CDP-DAG to phosphatidylglycerol phosphate (PGP) (Shen and Dowhan, 1998; Zhong and Greenberg, 2003; He and Greenberg, 2004). Phosphatidylglycerol (PG) is formed by the phosphatase PTPMT1 (Protein-tyrosine phosphatase mitochondrial 1) (Xiao et al., 2011; Zhang et al., 2011). A second molecule of CDP-DAG is used by the Cardiolipin Synthase (CLS1) to form premature cardiolipin (pCL) (Chang et al., 1998; Chen et al., 2006; Lu et al., 2006). After its initial synthesis CL is remodeled by the exchange of fatty acid moieties (**Figure 1B**). Several phospholipases ($iPLA_2$, $iPLA_2\gamma$, $iPLA_2$-VIA) have been suggested to deacytylate pCL to form monolysocardiolipin (MLCL) (Mancuso et al., 2007; Malhotra et al., 2009; Yoda et al., 2010; Hsu et al., 2013). The coenzyme A independent acyltransferase Tafazzin then mediates the reacylation to form mature CL (Bissler et al., 2002; Houtkooper et al., 2009a). The gene encoding for Tafazzin is mutated in Barth Syndrome, and the resulting inactivation of this enzyme causes a block in the generation of mature forms of CL (Lu et al., 2016). Despite the remarkable tissue specific composition of CL, isolated Tafazzin was found to have little or no specificity toward fatty acids (Houtkooper et al., 2009b). The specific remodeling of CL arises from the physical properties of the lipid environment and is ensured by the tissue specific availability of substrate fatty acids and the packing conditions of lipids within the membrane (Schlame et al., 2017). Interestingly, Tafazzin is not the only enzyme, capable of CL remodeling. Under pathological conditions, ROS induced upregulation of Acyl-CoA:lysocardiolipin acyltransferase 1 (ALCAT1) causes an aberrant remodeling of CL. ALCAT1 generates CL species with long and polyunsaturated acyl chains such as docosahexaenoic acid (DHA), which are prone to ROS (reactive oxygen species) induced oxidation (Li et al., 2010). ALCAT1 upregulation is associated with hyperthyroid cardiomyopathy and diabetes. The four fatty acid moieties in close proximity make CL susceptible to peroxidation by ROS. As damaged CL is harmful to mitochondria it has been suggested that oxidized CL is rapidly degraded. The CL phospholipase HSD10 has been identified recently to mediate the degradation of oxidized CL (Boynton and Shimkets, 2015).

The Role of CL in Mitochondrial Protein Transport

Ninety-nine percent of mitochondrial proteins are encoded in the nucleus and transported into mitochondria after their translation in the cytosol. These proteins are transported by specialized

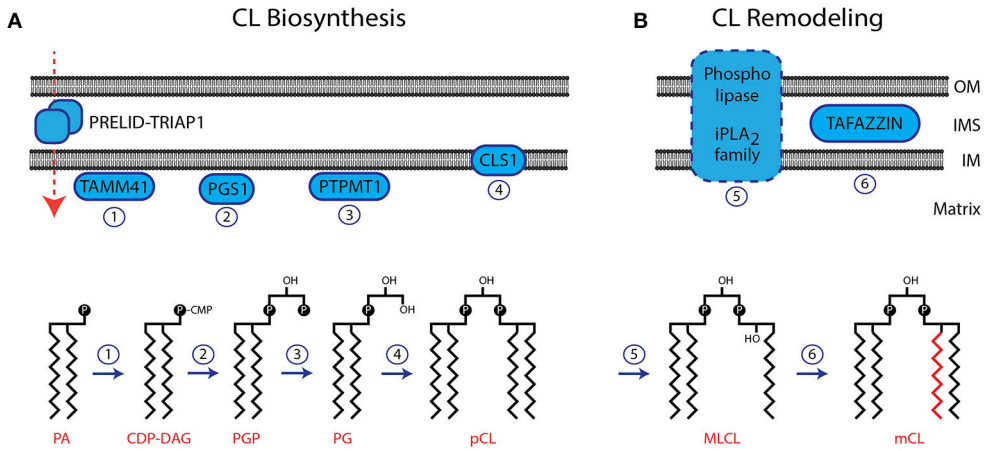

FIGURE 1 | Biosynthesis and remodeling of CL. **(A)** The biosynthesis pathway of CL is localized in the IM. The membrane topology of enzymes involved in CL biosynthesis is indicated. The structures of CL precursor molecules are shown in the lower panel. Numbers of enzymatic reactions correspond with the indicated enzymes in the upper panel. **(B)** CL undergoes remodeling after its initial biosynthesis. The phospholipase, involved in this process has not been identified. Tafazzin is localized in the IMS, associated to both, the IM and OM. OM, outer membrane; IM, inner membrane; IMS, intermembrane space; pCL, premature cardiolipin; mCL, mature cardiolipin.

translocases across mitochondrial membranes (**Figure 2A**). The Translocase of Outer Membrane (TOM) serves as a central entry gate for almost all mitochondrial proteins (Neupert and Herrmann, 2007; Dudek et al., 2013b). Protein import is highly regulated by posttranslational modification. Upon a metabolic switch to glycolysis in yeast, Protein Kinase A phosphorylates the TOM receptor subunit Tom70, exerting an inhibitory effect on protein import (Rao et al., 2011; Schmidt et al., 2011). PKA also reduces TOM assembly, whereas Casein kinase and Cyclin dependent kinase (Cdk1) promotes assembly of the TOM complex (Rao et al., 2012; Harbauer et al., 2014). After their import, β-barrel proteins are integrated into the outer membrane by the Sorting and Assembly Machinery (SAM) (Gebert et al., 2009). Inner membrane proteins are transported by the Translocase of inner membrane TIM23 and the associated Presequence Translocase-associated protein import motor (PAM) drives the import of proteins into the matrix (Dudek et al., 2013b). The TIM22 complex in the IM integrates metabolite carriers into the inner membrane. TOM, SAM, TIM22, TIM23 and the associated motor (PAM) require CL for their structural integrity indicating that CL is essential for mitochondrial protein import (Gallas et al., 2006; Tamura et al., 2006; Kutik et al., 2008). AGK has been recently identified a novel structural constituent of the TIM22 complex (Kang et al., 2017; Vukotic et al., 2017). Interestingly, AGK has a catalytic domain, which catalyzes the phosphorylation of DAG to PA. As PA is a precursor for CL, it has been suggested that AGK contributes to CL biosynthesis (Waggoner et al., 2004; Tatsuta and Langer, 2016). Defective mitochondrial import causes protein accumulation in the cytosol, and triggers the unfolded protein response activated by protein mistargeting (UPRam) in yeast (Wrobel et al., 2015). This signaling pathway reduces cytosolic translation and activates the proteasomal degradation pathway. An increase in proteasomal activity in response to a defect in the intermembrane space was also found in mammals suggesting that a similar pathway might also exist in mammals (Papa and Germain, 2011). After import and maturation, cofactors are integrated into mitochondrial proteins, which then acquire their native conformation. CL also plays a role in cofactor integration, as reduced enzymatic activity of Fe-S containing proteins has been reported in CL-deficient yeast (Patil et al., 2013).

The Role of CL in Mitochondrial Morphology

With four acyl chains bound to one head group, CL adopts a cone-shaped structure and segregates into regions of locally high membrane curvature or even induces membrane bends (Huang et al., 2006). CL is anticipated to localize in the highly bended regions within the cristae structures and preferentially resides in the monolayer facing the matrix side. In addition, the distribution of CL is determined by the lipid scaffolding proteins prohibitin-1 and prohibitin-2 (Merkwirth et al., 2008). Prohibitins belong to the stomatin/prohibitin/flotillin/HflK/C (SPFH) family, which also includes lipid raft-associated proteins. Prohibitin-1 and prohibitin-2 share more than 50% identical amino acids and form large hetero-oligomeric ring-like structures. The somatin like protein 2 (SLP-2) binds to prohibitins and was found to directly interact with cardiolipin (Da Cruz et al., 2008; Osman et al., 2009). Prohibitin complexes are suggested to segregate CL into specialized membrane domains (Merkwirth et al., 2012). A protein with similarities to the family of Hsp40 co-chaperones, DNAJC19, was identified as a prohibitin interaction partner. As DNAJC19 plays a detrimental role for the CL acetylation pattern, it was suggested that the prohibitin/DNAJC19 complex may segregate a specific membrane domain which facilitates Tafazzin mediated CL remodeling (Richter-Dennerlein et al., 2014).

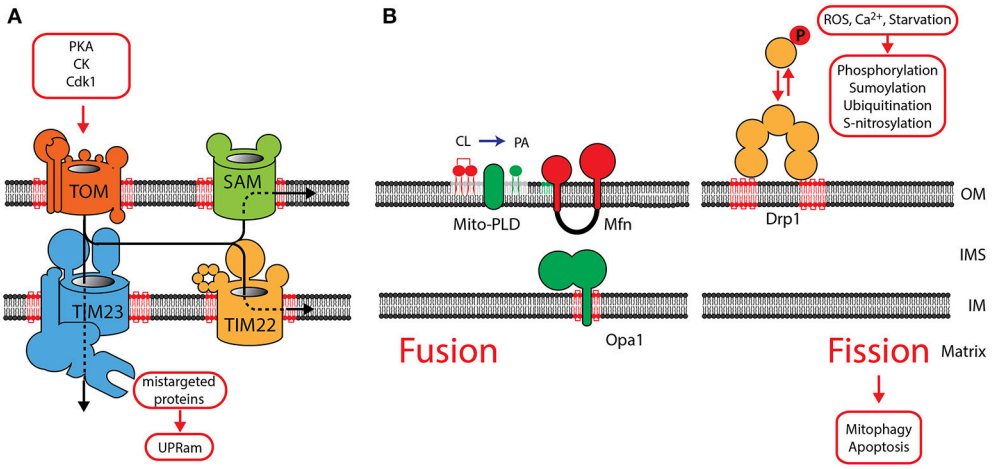

FIGURE 2 | The role of CL in mitochondrial protein translocation and morphology. **(A)** Protein translocases in the OM and IM are dependent on CL. Protein translocation is regulated by phosphorylation of components of the TOM complex. Mistargeted proteins activate the UPRam signaling pathway. **(B)** OM fusion is mediated by MFN1 and MFN2, fusion of the IM requires OPA1. See main text for a detailed description of the role of CL in the function of OPA1. The phospholipase MitoPLD converts CL into phosphatidic acid (PA), which facilitates MFN-mediated fusion. Fission is mediated by recruitment of DRP1 oligomers to the OM, where it interacts with CL. DRP1 oligomerization is intensively regulated by posttranslational modification. OM, outer membrane; IM, inner membrane; IMS, intermembrane space.

The role of CL in the mitochondrial morphology explains the morphological changes found in cardiolipin-deficient cells. CL deficiency results in an increase in mitochondrial size and absent or disorganized cristae structures (Xu et al., 2006; Acehan et al., 2007, 2009). Mitochondrial morphology is essential for normal mitochondrial function and plays a role in development, aging and apoptosis (Chan, 2006). A large amount of studies implicate changes of mitochondrial morphology in cardiovascular diseases (Brady et al., 2006; Cribbs and Strack, 2007; Williamson et al., 2010).

The complex mitochondrial morphology is determined by several protein structures, in which CL plays a role. The structural organization of the IM and the cristae structures are formed by the mitochondrial contact site and cristae organizing system (MICOS), located at the cristae junctions (Darshi et al., 2011; Gómez and Hagen, 2012). MICOS-deficient mitochondria show a reduction of the number of cristae junctions, resulting in stacks of lamellar cristae. The MICOS complex is formed by nine subunits and interaction partners in the inner membrane, exposing their functional domains into the IMS (Alkhaja et al., 2011; Darshi et al., 2011; Harner et al., 2011; von der Malsburg et al., 2011; Ding et al., 2015; Guarani et al., 2015; Ott et al., 2015; van der Laan et al., 2016). Two proteins MIC26 (APOO) and MIC27 (APOOL) are structurally related to the family of apolipoproteins. MIC27 directly interacts with CL and this interaction was found to be essential for MIC27 assembly into the MICOS complex (Weber et al., 2013; Friedman et al., 2015). The MICOS complex is a central component of a large interaction network which includes several complexes in the IM and OM (Rampelt et al., 2016). MICOS interacts with the protein import and assembly machinery (TOM, SAM in the OM and the IMS resident MIA40), and fusion proteins (OPA1 (see below) and SLC25A46, a mammalian homolog of the yeast fusion adaptor Ugo1) (Abrams et al., 2015; Janer et al., 2016).

Two opposing processes maintain the reticular network of mitochondria: fusion and fission. Fusion (merging of two mitochondria) is mediated by a set of dynamin related GTPases: the mitofusins (MFN1, MFN2) in the OM and OPA1 in the IM (**Figure 2B**) (de Brito and Scorrano, 2008; Westermann, 2010; Shirihai et al., 2015; Schrepfer and Scorrano, 2016). Their activity is counteracted by fission (segregation of two mitochondria), mediated by DRP1, which also belongs to the family of dynamin related GTPases. Fission and fusion maintains the homogeneity of mitochondria within a cell (Sesaki and Jensen, 1999) and has been shown to be functionally dependent on CL. The fusion protein OPA1 is anchored to the IM by an N-terminal transmembrane domain, exposing the GTPase domain into the IMS. Alternative splicing of OPA1 give rise to two long forms L-OPA1, which are processed by proteases to three short forms S-OPA1 (Ishihara et al., 2006; Anand et al., 2014). A balanced formation of both forms is required for the formation of active dimers and maintaining a reticulated morphology of mitochondria. CL was found to be necessary for the dimerization and induction of the GTPase activity (DeVay et al., 2009; Meglei and McQuibban, 2009). A recent study suggests that the IMS domain of OPA1 contains a CL-binding site. It has been proposed, that after fusion of the OM, this binding domain allows OPA1 to interact with CL in the opposing IM. Therefore, CL- and OPA1-mediated membrane bridging will induce fusion of the IM (Ban et al., 2017). Mitochondrial fission is mediated by DRP1, which is recruited from the cytosol to oligomerize into helical structures. Activation of its GTPase mediates constriction of the outer membrane and induces mitochondrial fission. DRP1 also shows a strong affinity to CL and recent studies

indicate that CL binding enhances oligomerization and GTP hydrolysis (Bustillo-Zabalbeitia et al., 2014; Stepanyants et al., 2015). A balanced equilibrium of fusion and fission is essential for maintaining mitochondrial morphology and necessary for mitochondrial inheritance, as recent data in yeast indicate (Böckler et al., 2017). The correct mitochondrial morphology is also a prerequisite for the many interactions of mitochondria with other organelles. Mitofusins act as tethering molecules, which mediate the contact to the ER (Schrepfer and Scorrano, 2016). In cardiac tissue, mitofusins are important for the SR-mitochondrial Ca^{2+} transmission and required for regulating mitochondrial metabolism (Chen et al., 2012).

Based on the functional dependence of the fission and fusion machinery on specific lipids, regulated synthesis and degradation of specific lipids may part of a signaling pathway on mitochondrial membranes. MitoPLD is an OM resident phospholipase, converting cardiolipin (CL) to phosphatidic acid (PA). The generation of PA was found to facilitate mitofusin-dependent fusion of the outer membrane (Choi et al., 2006). PA also recruits the PA phosphatase Lipin 1b, which converts PA to diacylglycerol (DAG). By an unknown mechanism DAG then mediates reduced fusion and increased fission (Huang et al., 2011). Also the phospholipase PA-PLA_1 was shown to play a role in hydrolyzing PA and counteracting MitoPLD (Baba et al., 2014). The generation of lipid signals might be important to locally define regions of membrane fusion and fission.

The Implication of Mitochondrial Morphology for Cellular Signaling

CL plays a fundamental role in forming the shape of mitochondria and mitochondrial morphology is strongly affected in CL-deficient cells. Interestingly, correct mitochondrial morphology is a prerequisite for many cellular signaling pathways, as these pathways are strongly affected in cells with disturbed mitochondrial morphology. Fragmentation of mitochondria in mouse skeletal muscle, induced by the mutation of genes involved in mitochondrial morphology (see above) was reported to cause severe changes insulin signaling, without interference with the contractile functions (Sebastián et al., 2012; Touvier et al., 2015; Wai et al., 2015). Other studies describe skeletal muscle atrophy induced by enforced expression of the fission machinery, which causes AMPK activation and FOXO3 signaling resulting in autophagy activation (Romanello et al., 2010).

Increased fission in apoptotic cells coincides with the release of cytochrome *c* and the execution of apoptosis. Inactivating DRP1 causes a delay in cytochrome *c* release, caspase activation and a block in apoptosis (Frank et al., 2001; Breckenridge et al., 2003; Barsoum et al., 2006). Mitochondrial fission is also necessary for mitophagy in many cell types, as mitochondria are too large for autophagic removal (Arnoult et al., 2005; Twig et al., 2008). Dedicated signaling pathways control mitochondrial morphology during mitophagy. Starvation activates PKA and mediates phosphorylation of DRP1. Phospho-DRP1 is retained in the cytosol resulting in mitochondrial elongation which excludes elongated mitochondria from mitophagy (Cribbs and Strack, 2007; Gomes et al., 2011). DRP1 acts as a central hub in the regulation of mitochondrial morphology and is modified by phosphorylation ubiquitination, sumylation, and S-nitrosylation (Karbowski et al., 2007; Zunino et al., 2007; Cereghetti et al., 2008; Han et al., 2008; Cho et al., 2009; Wu et al., 2011). The stress-induced mitochondrial hyperfusion pathway (SIMH) triggers mitochondrial hyperfusion in response to cellular stress. MFN1 and long isoforms of OPA1 have been found to be required for the SIMH response, which establishes a compensating increase in OXPHOS and ATP levels (Tondera et al., 2009). Interestingly, the CL binding protein Stomatin-like protein 2 (SLP-2) was found crucial for regulating OPA1 processing upon SIMH activation (Christie et al., 2011). SLP-2 was also found to be involved in affect supercomplex stabilization and in segregating cardiolipin into lipid domains the inner membrane (Mitsopoulos et al., 2015).

Role of CL in the Respiratory Chain and Metabolism

The oxidative metabolism in the cell generates NADH and $FADH_2$, which are oxidized by the respiratory chain in the mitochondrial inner membrane. The respiratory chain consists of five complexes (complex I–V) involved in the electron transport to molecular oxygen. The electron transport is coupled with proton export across the IM, generating the membrane potential, which serves as an energy source for the production of ATP by the F_1F_o-ATPase. Evidence for CL playing a role in the structural integrity and enzymatic activity exists for all complexes. Structural analysis found specific binding sites for CL in complex I (Fiedorczuk et al., 2016), complex III (Lange et al., 2001; Palsdottir et al., 2003), and complex IV (Itoh et al., 2007). The succinate dehydrogenase (complex II) is strongly reduced in BTHS, indicating a role of CL in the structural integrity of the complex (Dudek et al., 2015). For complex III and complex IV an active role of CL in the proton translocation has been suggested (Eble et al., 1990; Morelli et al., 2013; Duncan et al., 2016). Respiratory chain complexes assemble into large supercomplexes, in which complex I binds a dimer of complex III and several copies of complex IV (**Figure 3**; Letts et al., 2016; Wu et al., 2016). Supercomplex formation increases the efficiency of the electron translocation and minimizes the risk for the generation of ROS (Schagger and Pfeiffer, 2000). Structural analysis found about 200 CL molecules to be associated with the bovine mitochondrial supercomplex (Pfeiffer et al., 2003; Zhang et al., 2005; Mileykovskaya et al., 2012; Bazan et al., 2013). Analysis of CL-deficient mitochondria indicates a remodeling of supercomplexes with a shift from higher- to lower molecular weight complexes. Therefore, it has been concluded, that CL is required for the structural integrity of supercomplexes (Dudek et al., 2013a, 2015; Huang et al., 2015). CL is also essential for the structure of the family of mitochondrial carrier proteins, which includes the phosphate carrier (PiC), the pyruvate carrier, the tricarboxylate carrier and the carnitine/acylcarnitine translocase (Claypool et al., 2006, 2008; Claypool, 2009).

A large number of phosphorylation sites has been reported for respiratory chain subunits indicating that reversible

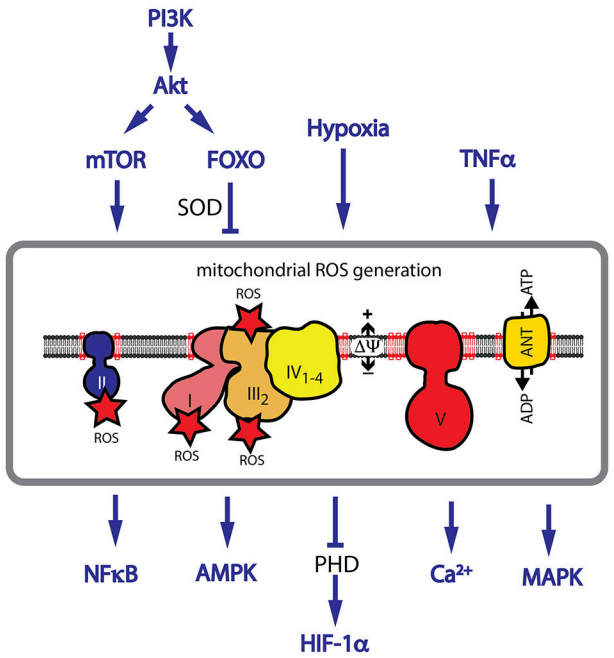

FIGURE 3 | CL deficiency causes an increase in mitochondrial ROS. CL is required for the structural assembly of the mitochondrial respiratory chain. CL deficiency causes a decrease in respiratory performance and an increase in ROS generation. Major pathways implicated in ROS signaling are shown. Hypoxia, TNFα signaling and activation of the PI3K/Akt pathway are associated with an increase in ROS levels. HIF-1α stabilization, and activation of AMPK, NF-κB and many MAPKs are dependent on ROS signaling.

phosphorylation plays a prominent role in regulation of respiration (Covian and Balaban, 2012). Other covalent modifications of respiratory chain subunits include the modification by lysine acylation and succinylation, which is reversed by the family of mitochondria-localized NAD^+-dependent deacetylases SIRT3, SIRT4, and SIRT5. SIRT3 was shown to regulate subunits in all five respiratory chain complexes upon dietary challenges such as calorie restriction (Bao et al., 2010; Cimen et al., 2010; Kim et al., 2010). SIRT5 regulates the respiratory chain by desuccinylation (Zhang et al., 2017). A specific binding of an N-terminal amphipathic helix in SIRT5 to CL was recently found to be essential for its activity (Zhang et al., 2017).

SIGNALING PATHWAYS AFFECTED BY CL

Mitochondria Play a Central Role in ROS Signaling

The structural remodeling of the respiratory chain in BTHS is associated with the aberrant transfer of electrons onto O_2, forming superoxide ($\cdot O_2^-$) and other forms of ROS (Dudek et al., 2015). When ROS defense mechanisms are overwhelmed, ROS directly reacts with lipids, proteins and DNA (Davies, 1995). ROS also targets proteins of the respiratory chain, affecting its structure and initiates a vicious cycle by further generating more ROS (Nickel et al., 2014, 2015). ROS-induced cellular damage is associated with many forms of cardiac disease in aging (Gómez and Hagen, 2012), ischemia/reperfusion (Ostrander et al., 2001) and heart failure (Saini-Chohan et al., 2009).

A role for ROS as a second messenger in cellular signaling pathways has also been described. ROS activates signaling proteins by changing their redox status. The most susceptible molecular targets of ROS are cysteine and methionine residues. Oxidation of cysteine causes intra- or intermolecular disulfide formation or nitrosylation and glutathiolation. Other posttranslational redox modifications include the hydroxylation of proline and arginine and the nitration of aromatic amino acids. ROS have been integrated as second messengers in many fundamental signaling pathways, modulating the cellular response. Activation of many pathways including the PI3K/Akt pathway and TNFα signaling are associated with an increase in ROS levels (Nogueira et al., 2008). The balance between ROS generating and ROS detoxifying mechanisms are crucial for the activation of downstream pathways. Hypoxia is associated with an increase in ROS levels in many cell types and ROS was identified as an essential contributor to the stabilization of the transcription factor HIF-1α (Diebold and Chandel, 2016). Other transcription factors, which are reported to be activated by ROS include NF-κB, FOXO3A, p53, and PGC1α (Gloire and Piette, 2009; Chae et al., 2013; Marinho et al., 2014). Upstream signaling components are also regulated by ROS, like the Protein kinase C (PKC), which is directly activated by oxidation of cysteine residues (Gopalakrishna et al., 1997). In cardiac hypertrophy AMPK and many MAPKs including p38, JNK, apoptosis-signaling kinase 1 (ASK-1) and ERK1/2 (Giordano, 2005) are activated by ROS. Mitochondria have the ability to significantly amplify a local increase in ROS levels. In a process called ROS-induced ROS release the opening of the mitochondrial permeability transition pore (MPT) and the inner membrane anion channel (IMAC) induces a ROS burst, which not only induces redox-sensitive signaling pathways but also triggers a similar ROS burst in neighboring mitochondria (Zorov et al., 2014).

Role of CL in MCU and Ca^{2+} Signaling

Mitochondrial morphology is critical for many signaling pathways. During excitation-contraction coupling, Ca^{2+} emitted from the sarcoplasmic reticulum is transmitted into the mitochondrion. Ryanodine receptors (RyR) in the sarcoplasmic reticulum are in close proximity to mitochondria and the mitochondrial calcium uniporter (MCU) in the inner membrane. The formation of this functional microdomain has been considered to be essential for the efficient transmission of Ca^{2+} into mitochondria. Transmission of Ca^{2+} into mitochondria was absent in fragmented mitochondria (Szabadkai et al., 2004).

Ca^{2+} plays a prominent role in the regulation of mitochondrial metabolism allowing adaptation to increased energy demands during increased cardiac workload (Dorn and Maack, 2013). Ca^{2+}-mediated activation of the pyruvate dehydrogenase, the isocitrate dehydrogenase and the α-ketogrutarate dehydrogenase triggers a significant increase in the metabolic flux in the Krebs cycle. At the same time respiration

is increased by activation of complex III, IV and V. The Ca^{2+} conducting pore of the mitochondrial Ca^{2+} uniporter (MCU) is formed by MCU and EMRE (Baughman et al., 2011; De Stefani et al., 2011; Sancak et al., 2013; Oxenoid et al., 2016). A novel form of regulation has been discovered recently with the finding that oxidative stress causes S-glutathionylation and persistent activity of MCU (Dong et al., 2017). MCUb was described as a dominant negative component of the Ca^{2+} channel (Raffaello et al., 2013). Regulation of MCU as a calcium induced calcium channel is mediated the Ca^{2+} sensitive subunits MICU1 and MICU2 (Petrungaro et al., 2015). This regulatory unit is bound to MCU complex via its interaction with EMRE (Tsai et al., 2016). Recently, MICU1 and MICU2 was found to interact with CL, indicating that the binding to CL also contributes to the membrane association (Kamer et al., 2017). Studies in cells, deficient of the regulatory subunits MICU1 and MICU2 revealed a deregulated calcium uptake into mitochondria (Patron et al., 2014). If deregulated Calcium uptake plays a role in BTHS has not been investigated.

Mitochondrial Protein Kinase C Signaling Depends on CL

Human cells have fifteen isoforms of the Protein kinase C (PKC). Overstimulation of some isoforms are associated with hypertrophy in cardiomyocytes, other forms are associated with a cardioprotective function (Mellor and Parker, 1998). One of the first data of a direct role of CL in PKC signaling was generated in yeast. CL-deficient yeast was found to have a defect in the integrity of the cell wall. Cell wall biogenesis and intracellular turgor pressure are regulated by the PKC-Slt2 and HOG pathway, respectively. CL deficiency caused a defect in the phosphorylation and activation of the MAPK Slt2, the downstream effector of PKC (Zhong et al., 2005, 2007; Zhou et al., 2009). Overexpression of individual genes involved in the PKC-Slt2 pathway rescued the mutant phenotype. As deletion of components of the HOG pathway also rescued the phenotype, it was concluded, that the balanced homeostasis of both pathways is affected in CL-deficient yeast (Patil and Greenberg, 2013). The PKC-Slt2 and the HOG pathway in yeast are also involved in the induction of mitophagy. Recent data indicates that defective activation of both pathways contribute to the described mitophagy defect in CL-deficient yeast (Shen et al., 2017). It is presently unclear, if a direct binding of CL may regulate PKC and Slt2 in yeast or loss of CL has an indirect effect on the components of the Slt2 pathway.

In the heart, several targets for PKC have been identified including the myofilament proteins troponin, cMyBP-C, titin, and the cytoskeletal protein desmin (Hidalgo et al., 2009; Kooij et al., 2010). Although most members of the PKC family are cytosolic proteins, the two diacyglycerol (DAG) sensitive but Ca^{2+} insensitive PKCε and PKCδ localize to the inner mitochondrial membrane (Budas et al., 2010; Yang et al., 2012). These proteins were found to interact with the cytosolic chaperone HSP90 and the mitochondrial import receptors TOM20 and TOM70. This led to the model of a regulated import into mitochondria upon mitochondrial stress, such as ischemia and reperfusion. Activation of mammalian PKCε was found to be dependent on CL (Konno et al., 1989; Shen et al., 2017). In mitochondria PKC was suggested to regulate mitochondrial proteins, involved in glycolysis, TCA cycle, β-oxidation, and ion transport and it physically interacts with regulatory subunits of the respiratory chain (Ping et al., 2001; Baines et al., 2003). Interestingly, PKCδ was shown to phosphorylate and activate the phospholipid scramblase 3 (PLS3), which is an important mediator of CL externalization (see below), revealing an interesting interdependence of CL and PKC (He et al., 2007). By supporting complex IV activity and preventing pathological ROS release, PKCε was suggested to have a cardioprotective effect (Guo et al., 2007; Yu et al., 2008).

CL is Precursor for Lipid Mediators Released under Stress Conditions

Lipid mediators such as prostaglandins, thromboxanes, leukotrienes play a role in many pathological processes such as inflammation, fewer, allergy and other immune responses. These signaling molecules are derived from phosphatidylserine (PS) by PLA_2-mediated (Ca^{2+} dependent phospholipases A_2) release of polyunsaturated fatty acids, which are subsequently oxidized by cyclooxygenases (COX) and lipoxygenases (LOX). Mitochondrial CL was recently found to be the origin of a new class of mitochondrial mediators. In a very similar process, cytochrome *c*-mediated oxidation of CL has been proposed to play a role in the production of specific lipid mediators under stress conditions. Oxidized CL was suggested to be a precursor for iPLA2γ-mediated release of oxidized polyunsaturated fatty acids and the accumulation was inhibited by specific inhibitors of iPLA2γ (Tyurina et al., 2014; Liu et al., 2017). Typical mediators, released by this process are 9-Hydroxyoctadecadienoic acid (9-HODE) and 13-HODE. A functional role for lipid mediators in mitochondria is not known, yet. Targets for 9-HODE and 13-HODE have been found predominantly in the cytosol where they were proposed to activate G2A, a G protein-coupled receptor and TRPV1 (Transient receptor potential vanilloid 1), which is expressed in the peripheral and central nervous systems (Patwardhan et al., 2009; Ogawa et al., 2010).

EXTERNALIZED CL FORMS BINDING SITES FOR SIGNALING MOLECULES

Mechanisms of CL Translocation into the OM

The specific exposure of phosphatidylserine (PS) on the plasma membrane serves as a well-studied "death signal" in apoptosis. In healthy cells, PS is maintained in an asymmetrical distribution on the cytosolic site of the plasma membrane. The activation of a scramblase upon apoptosis externalizes PS on the outer surface forming a recognition signal for macrophages. The unique biochemical properties of CL have fostered the hypothesis that CL enriched membrane sites might also serve as a binding site for specific protein complexes. Two negative charges allow the specific recruitment of protein interaction partners. CL, which is translocated from the IM and exposed on the OM under stress

conditions, will serve as a binding site for signaling molecules and establishes a signaling hub for mitophagy and apoptosis.

In the inner mitochondrial membrane CL shows an asymmetrical distribution with a majority of CL facing the matrix. Phospholipid scramblases (PLS) are a small family of phospholipid translocators, which mediate the translocation of phospholipids between the two monolayers of a lipid bilayer (**Figure 4A**). Mitochondrial phospholipid scramblase-3 (PLS-3) is involved in the redistribution of CL within the membrane, which is considered a necessary step for translocation of CL to the outer membrane. Remarkably, inactivation of PLS-3 causes increased resistance to apoptosis, highlighting the importance of CL translocation for cellular signaling (Liu et al., 2003; Van et al., 2007).

Two IMS proteins, with an established role in energy metabolism, were recently found to have a novel function in the transport of CL into the outer membrane. The Mitochondrial creatine kinase (MtCK) provides the cellular energy buffer phosphocreatine by ATP dependent phosphorylation of creatine. The nucleoside diphosphate kinase (NDPK-D/NM23-H4) converts nucleoside diphosphates to triphosphates by transferring the terminal phosphate group. Both enzymes were found to form large oligomeric complexes in the IMS, exposing specific binding sites for CL (**Figure 4B**). By binding to CL, these proteins were shown to mediate CL transfer between the inner and outer membrane (Lacombe et al., 2009; Schlattner et al., 2013).

CL Externalization in Mitophagy

Mitophagy is the selective degradation of dysfunctional mitochondria by autophagy. Mitophagy plays an essential role during cardiac development and maintains cardiac function in the adult heart (Billia et al., 2011; Hoshino et al., 2013; Kubli et al., 2013; Gong et al., 2015). During mitophagy, damaged mitochondria are enclosed by a double membrane structure, the phagophore, which forms the autophagosome and fuses with the lysosome to target its content for degradation (Youle and van der Bliek, 2012). Due to their size, mitochondria have to be separated from their reticular network in order to be incorporated into the autophagosomes. Mitochondrial fission has been shown to be a prerequisite for mitophagy in many cell types. Inhibition of mitochondrial fission decreases the level of mitophagy (Thomas and Jacobson, 2012). The two CL dependent proteins DRP1 and OPA1 activity mediate the balanced fusion and fission of mitochondria (see above). Interestingly, another CL binding protein is involved in mitochondrial fission during mitophagy. The mitochondrial human immunity-related GTPase, IRGM, was shown to play an essential role in mitophagy induced fission and translocates from the cytosol to mitochondria, upon mitophagy induction, where it binds to CL (Singh et al., 2010).

The concept of CL externalization forming an essential platform for mitophagy was emerging from the observation that CL translocates to the OM upon CCCP treatment, a well-studied inducer of mitophagy in mammalian cells. Both the content and species composition of CL in the OM were increased upon mitophagy induction (Chu et al., 2013). Externalized CL plays a central role in mitophagy. Phagophore formation is promoted by activation the class III phosphatidylinositol 3-kinase Vps34, which generates phosphatidylinositol-3-phosphate PtdIns(3)P, required for the recruitment of further effectors (Kaur and Debnath, 2015). Vps34 forms a complex with Beclin 1, Vps15, and Ambra1. Beclin 1 is a central regulator of this complex and multiple proteins interact with Beclin 1 to induce or inhibit autophagy (Kroemer et al., 2010). Beclin 1 has been shown to directly interact with CL on the OM, indicating a direct involvement of CL in mitophagy (Huang et al., 2012).

Elongation of the isolation membrane is mediated by two ubiquitin-like protein-conjugation systems. Conjugation of Atg12 to Atg5 allows the formation of the Atg12-Atg5-Atg16 complex, which itself functions as an E3 ligase for LC3. LC3 is the mammalian ortholog of Atg8 and is conjugated to phosphatidylethanolamine (PE) in a reaction involving Atg7 (E1-like) and Atg3 (E2-like) to form membrane-bound LC3-II. Lipidated LC3-II is recruited to both the outer and inner surfaces of the autophagosomal membrane and remains on the autophagosomes until fusion with lysosomes. LC3-II contains basic surface patches which bind to CL (**Figure 5A**; Chu et al., 2013). Consequently, reducing CL levels by knockdown of genes involved in CL biosynthesis causes reduced mitophagy in primary neurons, SH-SY5Y, and HeLa cells (Chu et al., 2013). Recent data suggest that also LC3B, a second member of the Atg8 family, is also specifically recruited to the OM by its interaction with externalized CL (Antón et al., 2016).

CL Peroxidation Triggers Cytochrome *c* Release during Apoptosis

Cytochrome *c*, mediates electron transfer from complex III to complex IV in the respiratory chain. A second function has been discovered during apoptosis, where cytochrome *c* acts as a catalyst for peroxidation of cardiolipin (CL). Externalization of CL on the IMS side of the IM increases availability of CL for cytochrome *c*. It has been proposed that conformational changes alter the interaction of CL with cytochrome *c* causing a change in the coordination of heme (Rajagopal et al., 2012; Vincelli et al., 2013). The resulting increase in peroxidase activity targets the closely bound unsaturated fatty acids in CL and results in a substantial increase in oxidized CL (Abe et al., 2011). Cytochrome *c* loses its interaction with oxidized CL, allowing cytochrome *c* release during apoptosis (**Figure 5B**; He, 2010). CL peroxidation precedes the internal/mitochondrial apoptotic pathway (see below) and peroxidized CL was also found to be a major contributor to the opening of the Mitochondrial Permeability Transition Pore (MPTP). The MPTP consists of the OM protein VDAC, the IM protein adenine nucleotide transporter (ANT) and the peripherally associated IMS protein peptidyl-prolyl cis-trans isomerase cyclophilin D. Direct interactions of CL with ANT explain the role of peroxidized CL in triggering the opening of the MPTP (Schlame et al., 2012; Powers et al., 2013).

A major contributor of cytochrome *c* peroxidase activity has been identified in the protein $p66^{Shc}$. $p66^{Shc}$ is a splice variant of the growth factor adapter Shc (Francia et al., 2009). Activation of the $p66^{Shc}$ pathway was found in hypergylcemia, which significantly increases the severity of cardiovascular

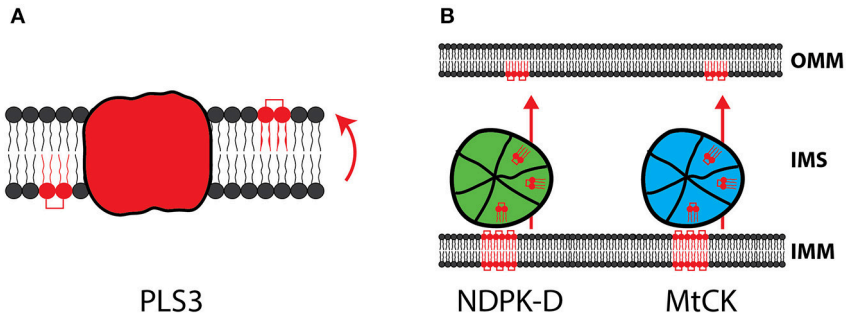

FIGURE 4 | CL trafficking from IM to OM. **(A)** The mitochondrial scramblase PLS3 allows the translocation of CL from the inner to the outer leaflet of the membrane. **(B)** NDPK-D and MtCK form large oligomeric complexes in the IMS and are capable to transport CL from the IM to the OM.

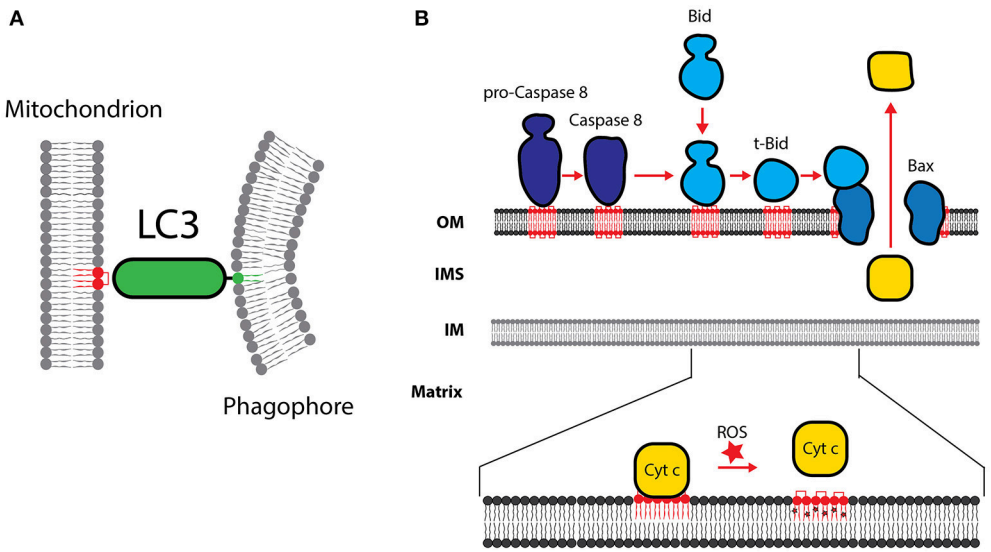

FIGURE 5 | Cardiolipin in apoptosis and mitophagy. **(A)** CL is necessary for the recruitment of LC3, which mediates binding of the phagophore membrane. **(B)** The role of CL in the processing of procaspase-8 is shown. Caspase-8-mediated processing of Bid to form t-Bid is stimulated by CL. Formation of t-Bid is required for the CL dependent oligomerization of Bax and Bak. Cytochrome *c* is detached from oxidized CL and released into the cytosol. OM, outer membrane; IM, inner membrane; IMS, intermembrane space.

diseases. Activated PKCβ induces phosphorylation of $p66^{Shc}$ (Pinton et al., 2007). Phosphorylated $p66^{Shc}$ translocates from the cytosol into the mitochondria where it binds to components of the TOM and TIM protein translocases. Proapoptotic stimuli destabilize this complex and induce the release of $p66^{Shc}$. During apoptosis, $p66^{Shc}$ plays an active role in supporting the peroxidase activity of cytochrome *c* and the generation of reactive oxygen species. Therefore, $p66^{Shc}$ might have an essential role in the oxidation of CL.

Role of CL during Execution of Apoptosis

Apoptosis is a controlled process of cell death and maintains the homeostasis of cell populations in tissues and occurs as a defense mechanism in immune reactions or in damaged cells. A role for apoptosis in the heart is described during cardiac development, in ischemia, infarction and in the end stage of heart failure (Bennett, 2002). Apoptosis is executed on two main apoptotic pathways: the extrinsic and the intrinsic pathway. The extrinsic pathway is triggered by the activation of the death receptor such as CD95/FAS/APO-1 in the plasma membrane, followed by the formation of the death-inducing signaling complex (DISC) and the subsequent activation of caspase-8. Caspase-8-mediated processing of Bid to form tBid is necessary to trigger the intrinsic pathway. The intrinsic apoptotic pathway is triggered, when diverse stimuli converge at the mitochondria and induce Bcl-2 family proteins. Bcl-2 proteins are involved in the permeabilization of the outer membrane and the release of pro-apoptotic factors, including cytochrome *c* and SMAC/DIABLO. It has been speculated that the externalization of CL into the outer membrane forms a binding platform for the recruitment of multi-protein complexes, which are required for the execution of apoptosis.

Proteins of the Bcl-2 family play a key role in the regulation of apoptosis and can be divided into the pro-apoptotic proteins (Bax

and Bak), the anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-W, and Mcl1) and the pro-apoptotic BH3-only proteins (Bid, Bim, Bad, Puma and Noxa). The balanced homeostasis of Bcl-2 family proteins is an important regulator of apoptosis. CL has been suggested to serve as a receptor to recruit tBid to the mitochondrial outer membrane (**Figure 5B**). tBid binding to the mitochondria was significantly decreased in mitochondria from cells deficient in CL (Lutter et al., 2000). Upon membrane binding, tBid promotes Bax membrane insertion. The recruitment and oligomerization of Bax and Bak in the outer mitochondrial membrane was also found to be a CL dependent process (Sorice et al., 2004; Lovell et al., 2008; Lucken-Ardjomande et al., 2008). CL-mediated changes in the structure and curvature of the membrane may be a prerequisite for Bax and Bak oligomerization and pore formation.

Execution of apoptosis depends on caspases, a family of cysteine proteases, which are synthesized in the cell as inactive procaspases. In an amplifying proteolytic cascade, active caspases cleave, and activate, other procaspases, resulting in the targeted degradation of key proteins in the cell. Procaspase-8 predominantly localizes in the cytosol. Apoptotic triggers induce the translocation of active caspase-8 to the mitochondria. Mitochondrial recruitment was shown to be dependent on the presence of CL on the outer mitochondrial membrane (**Figure 5B**). Lymphoblastoid cells from BTHS patients have been shown to be resistant to apoptotic triggers (Gonzalvez et al., 2008).

Role of CL in inflammasome Activation

Myocardial injury is commonly associated with inflammatory signaling. Upregulation of cytokines (TNF), interleukins (IL-1, IL-6), and chemokines (MCP-1) has been observed in heart failure. Inflammatory signaling was shown to increase fibrosis, is involved in myocardial remodeling, myocyte hypertrophy and decreased contractility and has been considered as a driving force for heart failure progression. The inflammasome is a cytosolic protein complex consisting of the receptor NLRP3, the procaspase-1 and ASC, and is essential for caspase-1 activation (Butts et al., 2015). Upon inflammasome activation, caspase-1 is activated and triggers the processing of pro-IL18 and pro-IL-1β into their active forms (Gurung et al., 2015). In cardiac myocytes, IL-1β induces calcium release from the sarcoplasmic reticulum, causing cardiac deficient contractility. The caspase-1-mediated inflammatory form of a programmed cell death (pyroptosis) is responsible for loss of cardiomyocytes and causes heart failure progression. Knockout of NLRP3 in mice results in smaller infarct sizes in an experimental model of acute myocardial infarction (Mezzaroma et al., 2011).

The inflammasome is activated by external molecular signals summarized as PAMPs (pathogen associated molecular patterns, LPS, proteoglycans, double stranded RNA) or endogenous signals, which are called DAMPS (danger associated molecular patterns, ATP, DNA, Chaperones). The finding of mitochondrial derived DAMPs (mito-DAMPs) including ROS, NAD^+ and mitochondrial Ca^{2+} release lead to the hypothesis that mitochondrial dysfunction is a contributor to inflammasome activation (Guo et al., 2015; Chakraborty et al., 2017). Mitochondria play a prominent role for inflammasome activity as they provide CL, which was found to bind to the leucin rich LRR domain in NLRP3. In an *in vitro* setting, CL was found sufficient for activation of NLRP3. In CLS1 knockdown cells with reduced CL levels, NLRP3 activity was significantly alleviated (Iyer et al., 2013).

CONCLUSIONS

The mitochondrial phospholipid CL is essential for a large array of functions. Therefore, CL forms multiple interactions with different proteins. As the interaction of CL with proteins serves multiple different functions, a uniform binding site has not been identified. In many of the described binding sites, CL's phosphate groups forms electrostatic interactions with positively charged residues, particularly with Arginine or Lysine (Rytömaa and Kinnunen, 1995). The acyl chains form hydrophobic interactions with Leucin, Isoleucine, and Valine (Kalanxhi and Wallace, 2007). A recent analysis of 62 CL interacting proteins also revealed a role for Glycine in the binding sites, indicating an increased requirement for structural flexibility in the CL binding site (Planas-Iglesias et al., 2015).

CL is a structural constituent of the respiratory chain and required for efficient respiration. A defect in the CL-biogenesis as found in BTHS patients, causes a structural remodeling of the respiratory supercomplexes and a reduction in respiratory performance. Reduced activity of the succinate dehydrogenase affects not only respiration but also the TCA cycle, which is a central hub for the entire cellular metabolism. Mitochondria are integrated in cellular signaling pathways, which monitor mitochondrial function and trigger an adequate nuclear response. Increased generation of ROS caused by the structural changes in the respiratory chain has been described in many models of BTHS. Persistent exposure to ROS is potentially harmful for the cell. Therefore, the cell closely monitors ROS levels in order to trigger defense mechanisms. ROS is an integrative part of a large number of cellular signaling pathways, and affects a wide variety of biological processes including response to hypoxia, apoptosis, autophagy, cell proliferation and differentiation. Several signaling pathways are dependent on CL directly. PKC family proteins are involved in maintaining cardiac structure and function. Some members of this family locate to the mitochondria and require CL for activation. If deficient PKC signaling contributes to BTHS pathology needs to be investigated. CL externalization is evident in mitophagy and apoptosis. Upon stress signals CL forms a binding platform for signaling molecules like LC3, which promotes the engulfment of defective mitochondria into autophagosomes and for pro-apoptotic molecules like caspse-8, Bax, Bak, and tBid. Defective mitophagy and apoptosis has been documented in cellular models for BTHS, but the physiological impact for BTHS patients remains obscure. Most interestingly, the CL molecule itself may even serve as a signaling molecule. A large number of different fatty acids can be bound to four positions within the CL molecule, giving rise to a highly diversified CL pool. The specific function of CL species is vastly unexplored. Interesting new data have elucidated that CL itself serves as a substrate for the

generation of lipid mediators. Correlating these new mediators with specific signaling function will be the start of an expanding research field.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Role of Mitochondrial Retrograde Pathway in Regulating Ethanol-Inducible Filamentous Growth in Yeast

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In yeast, ethanol is produced as a by-product of fermentation through glycolysis. Ethanol also stimulates a developmental foraging response called filamentous growth and is thought to act as a quorum-sensing molecule. Ethanol-inducible filamentous growth was examined in a small collection of wine/European strains, which validated ethanol as an inducer of filamentous growth. Wine strains also showed variability in their filamentation responses, which illustrates the striking phenotypic differences that can occur among individuals. Ethanol-inducible filamentous growth in Σ1278b strains was independent of several of the major filamentation regulatory pathways [including fMAPK, RAS-cAMP, Snf1, Rpd3(L), and Rim101] but required the mitochondrial retrograde (RTG) pathway, an inter-organellar signaling pathway that controls the nuclear response to defects in mitochondrial function. The RTG pathway regulated ethanol-dependent filamentous growth by maintaining flux through the TCA cycle. The ethanol-dependent invasive growth response required the polarisome and transcriptional induction of the cell adhesion molecule Flo11p. Our results validate established stimuli that trigger filamentous growth and show how stimuli can trigger highly specific responses among individuals. Our results also connect an inter-organellar pathway to a quorum sensing response in fungi.

Keywords: filamentous growth, pseudohyphal growth, quorum sensing, mitochondria-to-nucleus pathway, krebs cycle

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INTRODUCTION

Fungal species represent a diverse group of microorganisms. Most fungal species exist in the wild. Other species live in commensal or pathogenic relationships with host organisms, while others still have been domesticated for food and technological benefits. *Saccharomyces sensu stricto* represents a group of highly related yeasts (Borneman and Pretorius, 2015). *Saccharomyces cerevisiae* and its relatives are commonly used in research laboratories and a variety of industrial processes. The ability of *Saccharomyces* to produce ethanol from several sugar sources makes it an essential component of the brewing and wine-making industries. Yeast not only produces ethanol as the major by-product of the alcoholic fermentation of sugars but also produces minor compounds such as aromatic (or fusel) alcohols that impart flavor and bouquet to wines. These properties have been studied to improve ethanol production and to understand the molecular basis of nutrient

sensing and regulatory mechanisms in eukaryotes (Fleet and Heard, 1993; Ribéreau-Gayon et al., 2000; Beltran et al., 2004, 2008; Alper et al., 2006; Zaman et al., 2008).

Many fungal species, including yeasts, can undergo filamentous growth. Filamentous growth in yeast is a developmental foraging response, where cells become elongated and grow in connected chains (Gimeno et al., 1992; Kron et al., 1994). In some settings, cells can penetrate surfaces, which is known as invasive growth (Roberts and Fink, 1994). Some fungal species grow as multinucleate hyphae. Other species, like *S. cerevisiae*, produce pseudohyphae where cells undergo cytokinesis at each cell division. Filamentous growth has been extensively studied in yeast and other species, particularly pathogens, which require filamentous growth for virulence (Madhani and Fink, 1998; Lengeler et al., 2000; Polvi et al., 2015). Such studies have led to insights into the triggers, signaling pathways and transcriptional targets that control developmental responses in fungi and other eukaryotes.

One inducer of filamentous growth is nitrogen limitation (Gimeno et al., 1992). Another is the limitation of fermentable sugars like glucose (Cullen and Sprague, 2000). The morphogenetic response to limiting glucose is mediated by several pathways, including a mitogen-activated protein kinase pathway called the filamentous growth (fMAPK) pathway (Saito, 2010; Karunanithi and Cullen, 2012; Adhikari and Cullen, 2014; Adhikari et al., 2015), the AMP-dependent kinase AMPK Snf1p (Celenza and Carlson, 1989; Woods et al., 1994; Lesage et al., 1996; Cullen and Sprague, 2000; McCartney and Schmidt, 2001; Kuchin et al., 2002), and the RAS-cAMP-protein kinase A (PKA) pathway (Toda et al., 1985; Gimeno et al., 1992; Mosch et al., 1996, 1999; Colombo et al., 1998; Robertson and Fink, 1998a,b; Rupp et al., 1999b; Robertson et al., 2000; Pan and Heitman, 2002). Filamentous growth is also regulated by the Rim101 pathway, which regulates the response to pH (Lamb et al., 2001; Lamb and Mitchell, 2003; Barrales et al., 2008). Other regulators include the chromatin remodeling complex Rpd3(L) (Carrozza et al., 2005; Barrales et al., 2008; Ryan et al., 2012), the tRNA modification complex Elongator (Krogan and Greenblatt, 2001; Winkler et al., 2001; Petrakis et al., 2004; Li et al., 2007; Svejstrup, 2007), and the Pho80p-Pho85p cyclin and cyclin-dependent kinase (Measday et al., 1997; Huang et al., 2002, 2007; Shemer et al., 2002; Moffat and Andrews, 2004; Chavel et al., 2014). In addition to these pathways, genetic (Lorenz and Heitman, 1998; Palecek et al., 2000), genomic and proteomic screens (Jin et al., 2008; Xu et al., 2010; Ryan et al., 2012) have identified many other proteins and pathways that impact filamentous growth. Thus, filamentous growth resembles cell differentiation in metazoans, where global reorganization of cellular processes results in the construction of a new cell type.

Fungal species also utilize small molecules to interpret information about their environment. Like many other microbial species (Miller and Bassler, 2001; Parsek and Greenberg, 2005; Rumbaugh et al., 2009), *S. cerevisiae* exhibits quorum-sensing responses (Hlavacek et al., 2009; Prunuske et al., 2012). Yeast can sense and respond to ammonia (Palkova et al., 1997), aromatic (fusel) alcohols (Chen and Fink, 2006), and ethanol (Dickinson, 1994, 1996; Lorenz et al., 2000). By products of the Ehrlich reactions (Hazelwood et al., 2008), fusel alcohols are formed by conversion of several amino acids into glutamate as a nitrogen source under nitrogen-limiting conditions (Ljungdahl and Daignan-Fornier, 2012). Fusel alcohols are produced at higher levels in nitrogen-limiting medium and sensed in a density-dependent manner by a PKA-dependent mechanism to regulate filamentous growth (Chen and Fink, 2006). Multiple fungal species produce and sense a variety of aromatic alcohols, which may impart selectivity in this type of cellular communication (Chen et al., 2004; Chen and Fink, 2006; Sprague and Winans, 2006; Kruppa, 2008; Langford et al., 2013). Recent efforts have expanded the diversity alcohols that can be sensed and measured their impact on fungal behavioral responses (Ghosh et al., 2008; Wuster and Babu, 2010; Sharma and Prasad, 2011; Albuquerque and Casadevall, 2012; Bojsen et al., 2012; Avbelj et al., 2015; Williams et al., 2015). An open question has been to identify the regulatory pathways that control alcohol-mediated morphogenesis and understand how cells detect and respond to these stimuli. Addressing this problem has a practical benefit, as industrial manipulation of yeast may be accelerated by understanding density-dependent growth and behavioral responses (Westman and Franzen, 2015).

To better understand common and unique elements of the filamentous growth response, a diverse collection of strains was examined from the "wine/European" group (Goffeau et al., 1996; Wei et al., 2007; Borneman et al., 2008, 2011; Argueso et al., 2009; Liti et al., 2009; Novo et al., 2009). Most strains tested underwent filamentous growth in response to limiting glucose, limiting nitrogen, or the presence of ethanol or fusel alcohols. A specific role for the mitochondrial retrograde (RTG) pathway, which controls the response to compromised mitochondrial function (Liu and Butow, 2006) and is known to regulate filamentous growth (Jin et al., 2008; Chavel et al., 2010, 2014; Aun et al., 2013; Starovoytova et al., 2013), was identified as a specific regulator of ethanol-inducible invasive growth. RTG regulated TCA cycle flux in response to ethanol to modulate filamentous growth. Thus, the study connects an inter-organellar signaling pathway to a quorum-sensing morphogenetic response in fungi.

Abbreviations: AMPK, AMP-dependent protein kinase; DIC, differential-interference-contrast; fMAPK, filamentous growth mitogen activated protein kinase; HOG, high osmolarity glycerol pathway; MM, minimal medium; OD, optical density; PWA, plate-washing assay; PKA, protein kinase A; qPCR, quantitative polymerase chain reaction; SAD, synthetic medium with ammonium and dextrose; SALG, synthetic medium with ammonium and low glucose; SLAD, synthetic medium with dextrose and low-ammonium; TOR, target of rapamycin; TCA, tricarboxylic acid; Trp-OH, tryptophol; v/v, volume-to-volume percent; YNB, yeast nitrogen base; YPD, yeast peptone dextrose.

MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions

Yeast strains are described in **Table 1**. Standard media was used (Rose et al., 1990). Yeast strains were generated by polymerase chain reaction (PCR)-based homologous recombination

TABLE 1 | Yeast strains used in the study.

Strain	Genotype	References
S288c	*MAT***α** *SUC2 gal2 mal2 mel flo1 flo8-1 hap1 ho bio1 bio6*	Mortimer and Johnston, 1986
Nsa[a]	*MAT***a**/*MAT*α	Wang et al., 2015
S1[b]	*MAT***a**/*MAT*α	Padilla et al., 2016
QA23[c]	*MAT***a**/*MAT*α	Borneman et al., 2011
T73[d]	*MAT***a**/*MAT*α	Querol et al., 1992
SB	*HO/HO, asp1-H142/asp1-H142*	Marullo et al., 2007
P5[e]	*MAT***a**/*MAT*α	García-Ríos et al., 2014
P24[c]	*MAT***a**/*MAT*α	García-Ríos et al., 2014
VIN7[f]	*Triploid allohybrid S. cerevisiae* × *S. kudriavzevii*	Borneman et al., 2012
W27[c]	*Hybrid S.cerevisiae* × *S. kudriavzevii*	Schütz and Gafner, 1994
PC312[g]	*MAT*α *ura3-52*	Liu et al., 1993
PC313	*MAT***a** *ura3-52*	Liu et al., 1993
PC318	*MAT*α *ura3-52 rho0*	Chavel et al., 2010
PC344	*MAT***a**/*MAT*α *ura3-52/ura3-52*	Cullen and Sprague, 2000
PC443	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 snf1::URA3*	Cullen and Sprague, 2000
PC471	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud6::KlURA3*[h]	Cullen and Sprague, 2002
PC538	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52*	Cullen et al., 2004
PC539	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KLURA3*	Cullen et al., 2004
PC549	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3*	Cullen and Sprague, 2000
PC563	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 bud8::KlURA3*	Cullen and Sprague, 2002
PC611	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::URA3*	Cullen et al., 2004
PC999	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA*	Cullen et al., 2004
PC2549	*MAT***a** *ura3-52 ras2::KlURA3*	Chavel et al., 2010
PC2584	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 tpk1::NAT*	Chavel et al., 2010
PC2763	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 elp2::KlURA3*	Abdullah and Cullen, 2009
PC2953	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rim101::ura3*	Chavel et al., 2010
PC3030	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA sin3::NAT*	Chavel et al., 2010
PC3035	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA mks1::NAT*	Chavel et al., 2010
PC3097	*MAT***α** *ura3-52 leu2 pex3::HYG*	This study
PC3363	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA nrg1::KLURA3*	Chavel et al., 2010
PC3642	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg3::NAT*	Chavel et al., 2010
PC3643	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tco89::NAT*	Chavel et al., 2014
PC3652	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT*	Chavel et al., 2010
PC3654	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA tor1::NAT*	Chavel et al., 2010
PC3695	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg1::NAT*	Chavel et al., 2014
PC3909	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste12::KLURA3 mks1::NAT*	This study
PC3910	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste20::URA3 mks1::NAT*	This study
PC3911	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 ste11::URA3 mks1::NAT*	This study
PC4041	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 MSB2-HA rtg2::NAT ssk1::KlURA3*	This study
PC4141	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-5 tpk2::URA3*	Chavel et al., 2014
PC5059	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 mig2::HYG*	This study
PC5084	*MATa ste4 FUS1-lacZ FUS1-HIS3 ura3-52 Msb2-HA tpk3::NAT*	Chavel et al., 2014
PC5582	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 pbs2::KanMX6*	This study
PC5594	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 flo11::KlURA3*	This study
PC5864	*MAT***a** *ste4 FUS1-lacZ FUS1-HIS3 ura3-52 sch9::KlURA3*	This study
PC6017[i]	*MAT***α** *can1*Δ*::Ste2pr-spHIS5 lyp1*Δ*::Ste3pr-LEU2 his3::hisG leu2*Δ*0 ura3*Δ*0*	Ryan et al., 2012
PC6018	*MAT***a**/*MAT*α *can1*Δ*::Ste2pr-spHIS5/can1*Δ*::Ste2pr-spHIS5 lyp1*Δ*::Ste3pr-LEU2/lyp1*Δ*::Ste3pr-LEU2 his3::hisG/his3::hisG leu2*Δ*0/leu2*Δ*0 ura3*Δ*0/ura3*Δ*0*	Ryan et al., 2012

[a] *Natural isolate from wine.*
[b] *Natural isolate from wine (CECT 13132).*
[c] *Commercial wine yeast Lalvin® Lallemand.*
[d] *Commercial wine yeast Lalvin® Lallemand (CECT1894).*
[e] *Commercial wine yeast Lalvin® ICVGRE Lallemand.*
[f] *Commercial wine yeast AWRI1539®.*
[g] *All PC strains are in the Σ1278b strain background.*
[h] *KlURA3 refers to the Kluyveromyces lactis URA3 gene cassette.*
[i] *Mutants derived from this strain were constructed in a genomic collection and were also tested in the study.*

techniques using auxotrophic or antibiotic resistant markers (Goldstein and McCusker, 1999). Yeast were grown on YPD (2% peptone, 1% yeast extract, 2% glucose, and 2% agar), minimal medium [(MM) 1X Yeast Nitrogen Base (YNB) without amino acids or ammonium, 2% glucose, and 10 mM $(NH_4)_2SO_4$], synthetic media [(SD) 1X YNB, 2% glucose, and 37 mM $(NH_4)_2SO_4$] with ammonium and dextrose [(SAD) 1X YNB, 1% glucose, and 37 mM $(NH_4)_2SO_4$], or with ammonium and low glucose [(SALG) 1X YNB, 0.5% glucose, and 37 mM $(NH_4)_2SO_4$]. To evaluate pseudohyphal growth, yeast were grown on synthetic medium with dextrose and low-ammonium [(SLAD) 1X YNB, 2% glucose, 50 μM $(NH_4)_2SO_4$, and 2% agar (Gimeno et al., 1992)]. Media was supplemented with uracil for auxotrophic mutants. For some experiments, SD and SLAD media were supplemented with 500 μM tryptophol, tyrosol, or phenylethanol and 2%(v/v) ethanol. The *CIT2-lacZ* plasmid has been described (Liu and Butow, 1999) and was provided by Dr. Zhengchang Liu (Louisiana State University, New Orleans). Beta-galactosidase assays were performed as described (Chavel et al., 2014).

Pseudohyphal Growth Assays

Examination of pseudohyphae was determined as described (Gimeno et al., 1992). Strains were grown for 16 h at 28°C in MM and harvested by centrifugation (1,000 rpm for 3 min). To obtain single colonies, cells were diluted by a factor of 10^6 in sterile water, and 100 μL of cells were spread onto media (SAD, SALG, and SLAD). Plates were incubated at 28°C and observed daily for 10 d by microscopy for colony morphology.

Invasive Growth Assays

Strains were grown for 16 h at 30°C in MM, harvested by centrifugation (10,000 rpm for 3 min) at an optical density (O.D. A_{600}) of 2.0, washed once in sterile water and resuspended in sterile water. Ten microliters of cells were spotted on semisolid agar media. Plates were incubated at 28°C. Invasive growth was determined by the plate-washing assay (Roberts and Fink, 1994). Colonies were photographed before and after washing over a 10 days period. Plates were washed in a stream of water (soft wash) and colonies were rubbed from the surface with a gloved finger (hard wash). ImageJ (http://rsb.info.nih.gov/ij/) was used to quantitate invasive growth (Zupan and Raspor, 2008). Background intensity was determined for each spot and subtracted from the densitometry of the area of invaded cells. Densitometric analysis was performed on invasive patches over multiple days. Tukey's *t*-test was used to determine statistical significance and generate *p*-values. The Shapiro-Wilk and Jarque-Bera normality tests showed that the data fit a normal distribution. A non-parametric statistics test (Wilcoxon test) showed the same results as the Tukey's *t*-test.

Quantitative Polymerase Chain Reaction (qPCR) Analysis

Quantitative PCR was performed as described (Beltran et al., 2004). Ethanol addition stimulated the expression of *FLO11* at all-time points except 24 h. Strains were grown in MM for 24 h at 28°C, washed with MiliQ sterile water (Millipore Q-PODTM Advantage A10) and resuspended in the indicated media at an O.D. A_{600} of 2.0. Cells were inoculated in SLAD media and in SAD media, and samples were taken at 2 h. To study the effect of nitrogen concentration in *FLO11* expression, strains were grown in MM for 24 h at 28°C, washed with MiliQ sterile water (Millipore Q-PODTM Advantage A10) and resuspended in SAD and SLAD media at an O.D. A_{600} of 2.0. Samples were taken at 2 h to analyze the *FLO11* expression. To study the effect of ethanol in *FLO11* expression, cells were inoculated at an O.D. A_{600} of 2.0 in SLAD medium with or without ethanol (2% v/v) Samples were taken at 45 min, 2, 8, and 24 h. RNA extraction was performed using an RNeasy Mini Kit (Qiagen). RNA concentration was adjusted to 320 ng/μL. Reverse transcription was performed using SuperScript® III Reverse Transcriptase (Invitrogen) and Oligo (dt) 20 Primer (Invitrogen).

qPCR was performed using an Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems, USA). SyberGreen master mix was used according to the manufacturer's instructions (Applied Biosystems, USA). Reactions contained 25 μL sample (5 μL cDNA, 1 μM each primer, 10 μL SyberGreen master mix, H_20 q.s.p. 25 μL). The starting quantity of genes was normalized with *ACT1* (Chavel et al., 2010). Relative gene expression was calculated using the $2^{-\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence was determined to be statistically significant above background; ΔCt is the difference in Ct of the *FLO11* gene and housekeeping gene (*ACT1*). The primers used were *FLO11* forward (5′-CACTTTTGAAGTTTATGCCACACAAG-3′) and *FLO11* reverse (5′-CTTGCATATTGAGCGGCACTAC-3′) based on Chen and Fink (2006), and *ACT1* forward (5′-TGGATTCCGGTGATGGTGTT-3′) and *ACT1* reverse (5′-CGGCCAAATCGATTCTCAA-3′).

Microscopy

Differential-interference-contrast (DIC) and bright-field microscopy was performed using an Axioplan 2 fluorescent microscope (Zeiss) with a PLAN-APOCHROMAT 100X/1.4 (oil) objective (N.A. 0.17). Digital images were obtained with the Axiocam MRm camera (Zeiss). Axiovision 4.4 software (Zeiss) was used for image acquisition and analysis and for rendering 3D Z-stack images. Images were further analyzed in Adobe Photoshop, where adjustments of brightness and contrast were made.

RESULTS

Exploring Filamentous Growth in a Collection of Wild and Industrial Yeast Strains

To understand the common and unique features of filamentous growth in yeast, a collection of wild and industrial yeast strains used in wine making was examined (**Table 1**). Strains were compared to ∑1278b, a well-characterized strain background that undergoes filamentous growth (Gimeno et al., 1992), and S288c, which is commonly used in research laboratories (Mortimer and Johnston, 1986) but has acquired mutations

due to genetic manipulation that render it unable to undergo filamentous growth (Liu et al., 1996; Dowell et al., 2010; Chin et al., 2012).

One aspect of filamentous growth is invasive growth, which can be assessed by the plate-washing assay (PWA), and which measures penetration of filamentous cells into surfaces (Roberts and Fink, 1994). Invasive growth in nutrient-rich (SAG) conditions was compared to conditions that induce filamentous growth, nitrogen limitation (SLAD; Gimeno et al., 1992) and glucose limitation (SALG; Cullen and Sprague, 2000) as shown in **Figure 1A**. The results were quantitated by densitometric analysis (**Figure 1B**). As expected, S288c did not undergo invasive growth, and ∑1278b underwent invasive growth that was higher in media lacking glucose or nitrogen (**Figure 1A**, washed and **Figure 1B**). Most wine strains underwent invasive growth, which was stimulated in nitrogen- and glucose-limited medium (including VIN7, W27, QA23, T73, SB, and S1; **Figures 1A,B**). Three strains showed a different trend: P5 invaded equally well in glucose-rich and glucose-limiting media, P24 did not invade nitrogen-limiting medium, and Nsa showed constitutive invasion. Moreover, the pattern of invasive growth varied widely among strains (**Figure 1A**).

Another aspect of filamentous growth is pseudohyphal growth, which can be measured by microscopic examination of colony peripheries (Gimeno et al., 1992). As expected, S288c did not form pseudohyphae, and ∑1278b formed pseudohyphae in nitrogen-limiting medium (**Figure 1C**, SLAD). Most strains formed pseudohyhae in nitrogen-limiting media (**Figure 1C**, including VIN7, W27, QA23, T73, SB, P5, S1, and Nsa), except SB, which did not form pseudohyphae until day 16 (for **Figure 1C**, day 5 is shown) and P24, which did not form pseudohyphae by day 20 when the experiment was terminated. The pattern of pseudohyphae varied among strains. With the exception of Nsa, which formed pseudohyphae in glucose- (Figure S1A, Nsa SALG, arrow) and nitrogen-limiting media, all other strains formed pseudohyphae exclusively under nitrogen-limitng conditions. Invasive and pseudohyphal growth require cell adhesion mediated by the flocculin Flo11p (Lambrechts et al., 1996; Lo and Dranginis, 1996; Guo et al., 2000). *FLO11* expression is induced during filamentous growth (Rupp et al., 1999a). A subset of wine strains that were tested all showed induction of *FLO11* expression under nitrogen-limiting conditions (Figure S1B). Therefore, above results agree with the widely accepted notion that glucose and nitrogen limitation are general inducers of filamentous growth.

Ethanol also stimulates filamentous growth (Dickinson, 1994, 1996; Lorenz et al., 2000). Ethanol induced filamentous growth specifically in nitrogen-limiting medium (Figure S2A) and showed a maximal effect at a concentration of 2% (Figure S2B). At this concentration, ethanol did not impact growth (Figure S2C; yeast can survive in 12% ethanol; Lleixà et al., 2016). Thus, tests were performed at 2% ethanol in nitrogen-limiting media. As expected, S288c did not show invasive growth by the addition of ethanol (**Figures 2A–C**), and ∑1278b showed ethanol-inducible invasive growth (**Figures 2A,B**). In particular, cells invaded the agar more robustly (**Figures 2A,B**), and pseudohyphae formed at earlier time points (**Figure 2C**, colonies were grown for 2 days compared to 5 days in **Figure 1C**). With the exception of P24 and Nsa, most strains showed increased invasive growth in response to ethanol (**Figures 2A–C** including VIN7, W27, QA23, T73, SB, S1, and P5). By these criteria, ethanol can also be viewed as a general inducer of filamentous growth. The fusel alcohol tryptophol stimulates filamentous growth in ∑1278b strains (Figure S3; Chen and Fink, 2006). Tryptophol stimulated invasive growth of most wine strains in nitrogen-rich (SAD) but not nitrogen-limiting (SLAD) medium (Figure S3, including VIN7, W27, QA23, T73, and S1). Thus, in line with previous studies, fusel alcohols like tryptophol are general inducers of filamentous growth.

Major Filamentation Regulatory Pathways are not Required for Ethanol-Inducible Filamentous Growth

We focused on ethanol-inducible filamentous growth because ethanol was a stronger inducer of filamentous growth than fusel alcohols. How ethanol is sensed and triggers filamentous growth has not been extensively studied. The ethanol response occurred in diploid (**Figure 2**) and haploid (**Figure 3**) strains of the ∑1278b background, which facilitated genetic analysis of the response.

Signaling pathways known to regulate filamentous growth were tested for a role in regulating ethanol-inducible filamentous growth. Specifically, mutants were tested that lack key regulators of fMAPK (*ste11*Δ; Ste11p is the MAPKKK), Ras2p-cAMP-PKA (*ras2*Δ) and PKA (Tpk in yeast) subunits Tpk1p, Tpk2p, and Tpk3p (*tpk1*Δ, *tpk2*Δ, and *tpk3*Δ), Snf1p (*snf1*Δ), Rim101p (*rim101*Δ), Rpd3p(L) (*sin3*Δ), Elongator (*elp2*Δ), and Pho85p (*pho85*Δ). Surprisingly, all of the mutants showed enhanced invasive growth in media containing ethanol (**Figures 3A,B**). The examination of colony perimeters generally bore this out, either showing enhanced filament formation or clumpiness (**Figure 3C**, arrows), which is indicative of elevated cell-cell adhesion. Colony perimeters did not show a change for the *ste11*Δ and *rim101*Δ mutants. Thus, fMAPK and Rim101 pathways may play some role in mediating ethanol-dependent filamentous growth. In summary these results show that ethanol exerts its effect on filamentous growth independent of several of the major regulatory pathways that control filamentous growth.

Unexpectedly, several mutants did not show an invasive growth defect in SLAD media. Specifically, the *rim101*Δ, *sin3*Δ, *snf1*Δ, *elp2*Δ, and *pho85*Δ mutants invaded the agar as well as or better then wild-type cells [**Figures 3A,B**; *tpk3*Δ is not defective for invasive growth (Robertson and Fink, 1998a; Robertson et al., 2000; Chavel et al., 2010)]. We have previously shown that the *rim101*Δ (Chavel et al., 2014), *sin3*Δ (Chavel et al., 2010), *snf1*Δ (Cullen and Sprague, 2000), *elp2*Δ (Abdullah and Cullen, 2009), and *pho85*Δ (Chavel et al., 2014) mutants have an invasive growth defect on rich media, and we verified that phenotype here (Figure S4A; YPD). Thus, there may be differences in the roles these pathways play in regulating

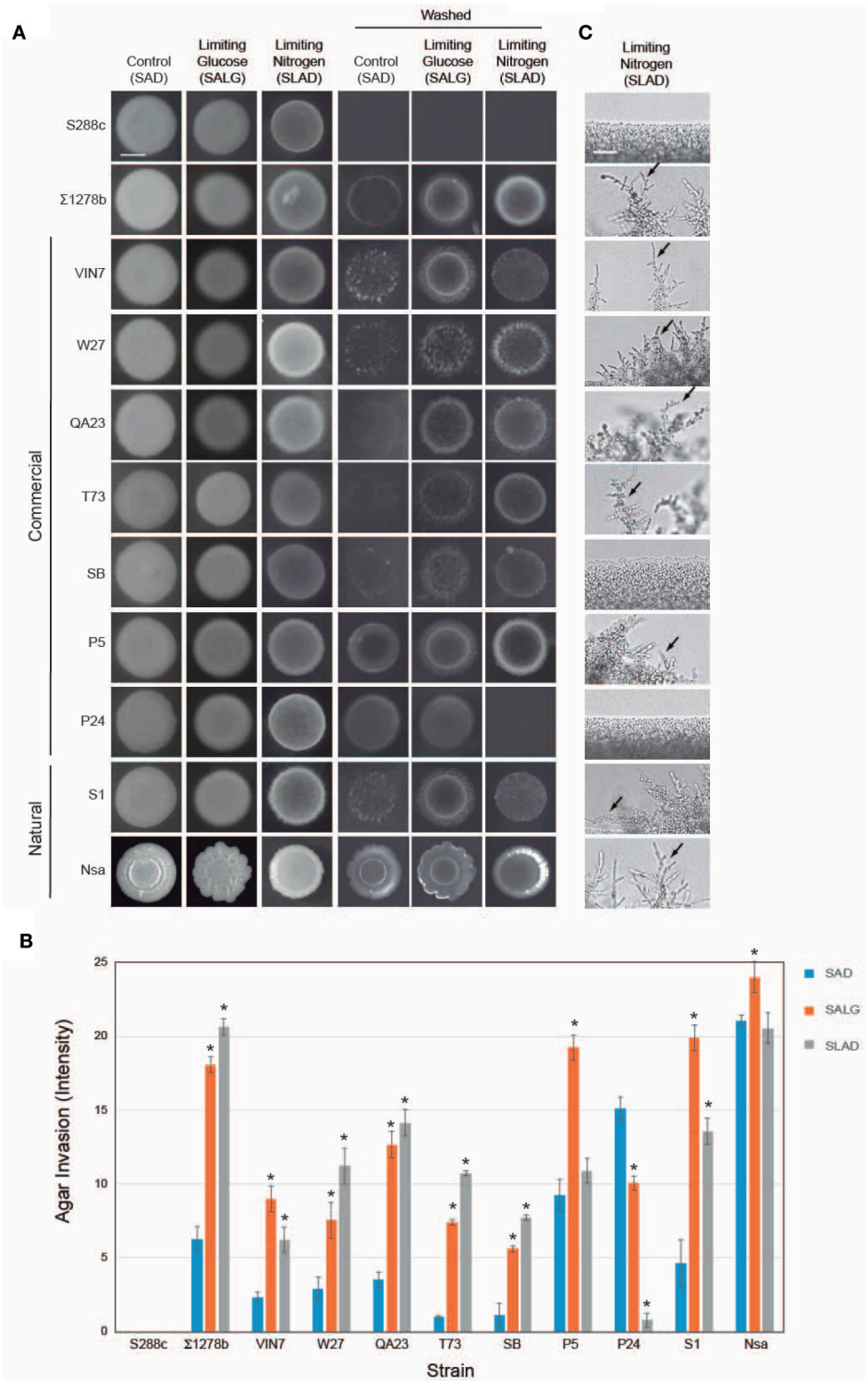

FIGURE 1 | Filamentous growth phenotypes of wine strains. (A) Plate-washing assay (PWA). Equal concentrations of cells ($OD_{600\ nm} = 2$) were spotted in 10 μL aliquots onto the indicated media. Plates were incubated for 5 days at 30°C and washed in a stream of water. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SAD. **(C)** Pseudohyphal growth of micro-colonies. Cells were grown for 3 days in minimal medium (MM) at 30°C, diluted by a factor of 10^6 and spotted onto SLAD media. Plates were incubated for 5 days. Colonies were examined by microscopy at 40X magnification. A representative image is shown. Bar, 25 μm. Arrows mark examples of pseudohyphae.

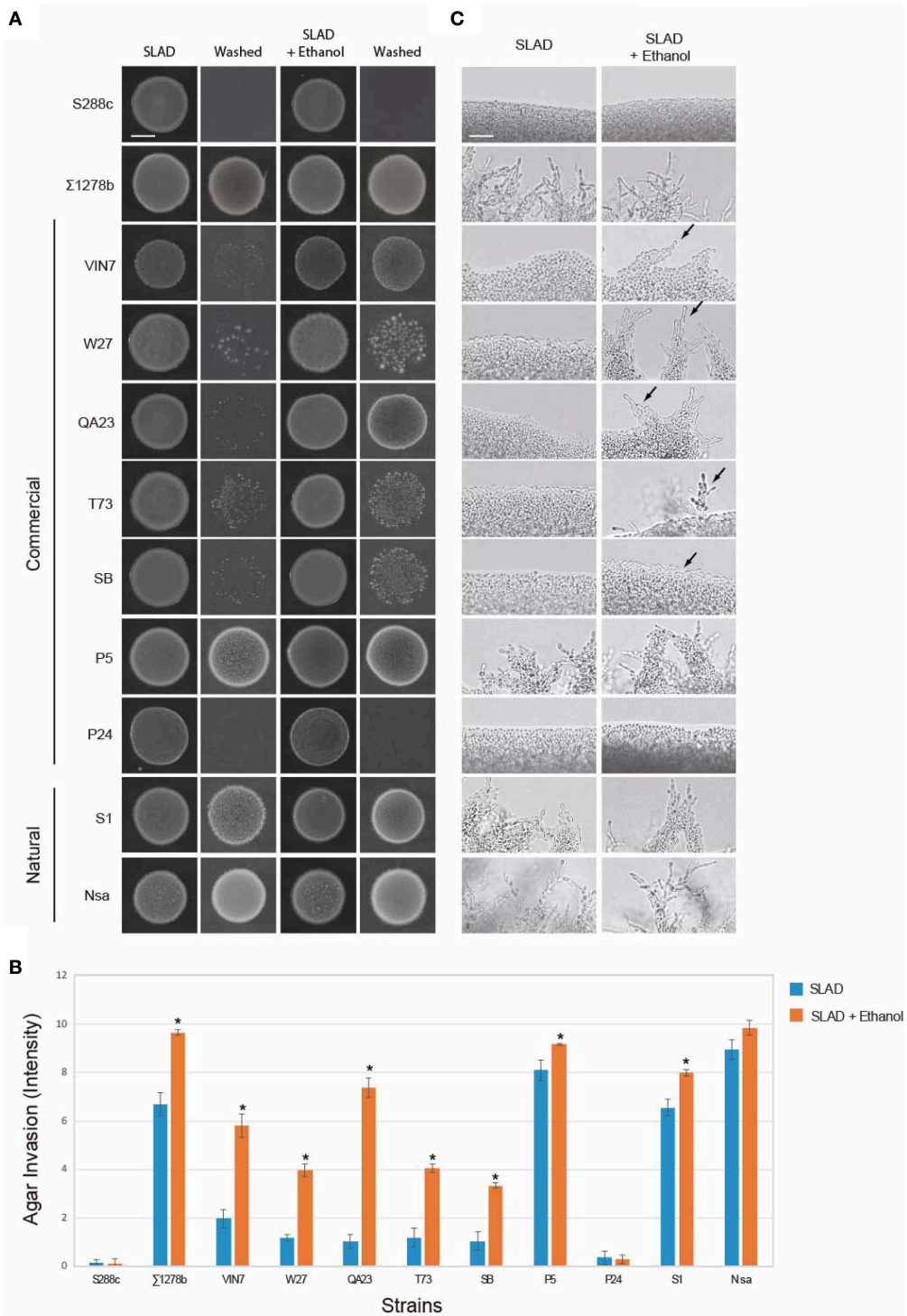

FIGURE 2 | Response of wine strains to ethanol. (A) PWA of cells spotted onto nitrogen-limited medium (SLAD) with or without ethanol (2% v/v). Plates were incubated for 2 days at 30°C and washed in a stream of water. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry, performed as described in **Figure 1B**. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. **(C)** Microscopy of colony perimeters with or without ethanol at 40X magnification. Bar, 25 μm. Arrows mark examples of pseudohyphae.

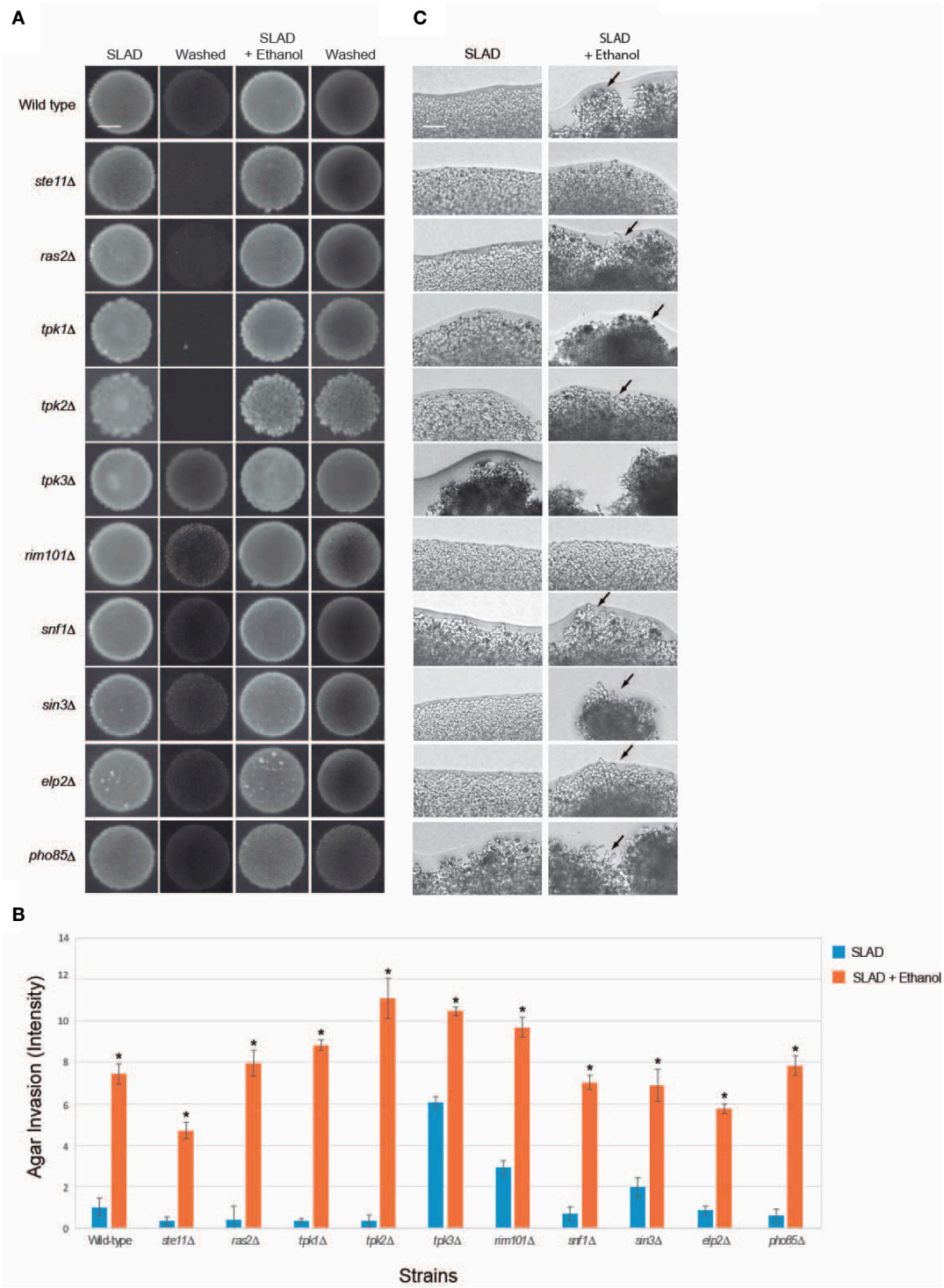

FIGURE 3 | Evaluating mutants lacking established filamentation regulatory pathways for ethanol-inducible invasion. (A) Wild-type cells (PC538, Σ1278b *MAT***a** haploid) and the indicated isogenic mutants were spotted onto nitrogen-limited medium (SLAD) with or without 2% ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry, performed as described in the legend for **Figure 1B**. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. **(C)** Colony peripheries from the plates in panel **(A)** were examined at 20X magnification. Bar, 50 μm. Arrows mark examples of pseudohyphae.

invasive growth depending on growth on YPD or SLAD. This hypothesis is consistent with the fact that mutants scored for pseudohyphal and invasive growth do not completely overlap in a genome-wide screen (Ryan et al., 2012) and with the fact that several pathways, like Snf1p, play different roles in response to carbon and nitrogen limitation (Orlova et al., 2010).

Mitochondrial Retrograde Pathway is Required for Ethanol-Inducible Invasive Growth

Other proteins and pathways regulate filamentous growth than those tested above (Ryan et al., 2012). A broader collection of genes implicated in filamentous growth regulation was examined. One of these is the mitochondrial retrograde pathway (or RTG pathway; Sekito et al., 2002; Liu et al., 2003; Liu and Butow, 2006; Kleine and Leister, 2016), which senses changes in metabolic respiration (Aun et al., 2013) to regulate filamentous growth. The RTG pathway has recently been shown to regulate the filamentation response to the alcohol butanol (Starovoytova et al., 2013). Rtg2p is a positive regulator of the retrograde pathway (Ferreira Junior et al., 2005). The *rtg2*Δ mutant was defective for ethanol-dependent invasive growth (**Figures 4A–C**). The RTG pathway is composed of two other regulators, the basic helix-loop-helix leucine zipper transcription factors Rtg1p and Rtg3p, which hetero-dimerize to regulate transcription (Jia et al., 1997). The *rtg1*Δ and *rtg3*Δ mutants were also defective for ethanol-dependent invasive growth (**Figures 4A–C**).

The RTG pathway controls expression of genes that function to ameliorate defects in mitochondrial function (Epstein et al., 2001). The activity of the RTG pathway can be assessed by examining the expression of the *CIT2* gene, which is a target of the retrograde pathway (Liao and Butow, 1993; Chelstowska and Butow, 1995; Kos et al., 1995; Jia et al., 1997; Liu and Butow, 1999) that encodes peroxisome citrate synthase (Kim et al., 1986). Ethanol stimulated the activity of a *CIT2-lacZ* transcriptional reporter (**Figure 4D**) in a manner that was dependent on Rtg2p (**Figure 4D**). Interestingly, the data indicates that ethanol induces the RTG pathway. One possibility is that nitrogen and ethanol both activate the RTG pathway. The addition of ethanol to cells grown in nitrogen-limiting media showed an additional stimulation (**Figure 4D**). Thus, nitrogen limitation and ethanol both contribute to RTG pathway activity. Therefore, the mitochondrial retrograde pathway regulates ethanol-inducible filamentous growth.

Mitochondrial Retrograde Pathway Regulates Ethanol-Inducible Filamentous Growth Independent of fMAPK, TOR, and HOG Pathways

To define how the RTG pathway connects to the ethanol response, known regulators of that pathway were examined. Mks1p is a negative regulator of multiple pathways, including Rtg2p in the mitochondrial retrograde pathway (Dilova et al., 2004; Ferreira Junior et al., 2005). Mks1p was not required for invasive growth in response to ethanol (**Figures 5A–C**), which indicates that another negative regulator of the pathway might function in this context. The RTG pathway can regulate the fMAPK pathway (Chavel et al., 2010), as part of a highly coordinated transcriptional sensing and signaling circuit among the pathways that regulate filamentous growth (Borneman et al., 2006; Bharucha et al., 2008; Chavel et al., 2014). We tested whether cells with an up-regulated RTG pathway functioned through fMAPK. An *mks1*Δ *ste11*Δ double mutant, which has an up-regulated retrograde pathway and lacks the MAPKKK for the fMAPK pathway (Ste11p), showed ethanol-inducible invasive growth. This result aligns with the abovementioned results that fMAPK does not regulate ethanol-dependent filamentous growth and indicates that the mitochondrial retrograde pathway does not control filamentation through fMAPK (**Figures 5A,B**). As shown above, the *mks1*Δ *ste11*Δ double mutant did not show an increase in filamentation at colony peripheries (**Figure 5C**).

Another major regulator of the mitochondrial retrograde pathway is the TOR pathway, which is a ubiquitous nutrient-regulatory pathway in eukaryotes (Bar-Peled and Sabatini, 2014). TOR plays an important role in nutrient-regulated responses in yeast (Heitman et al., 1991) and is a master regulator of nitrogen control (Beck and Hall, 1999; Cardenas et al., 1999; Bruckner et al., 2011; Kingsbury et al., 2015). TOR signaling also links nitrogen quality to the activity of the Rtg1p and Rtg3p transcription factors (Komeili et al., 2000). TOR specifically regulates the expression of genes encoding RTG pathway components (Crespo et al., 2002; Dilova et al., 2004). We found that the TOR pathway was not required for ethanol-inducible filamentous growth (**Figures 5A–C**; *tor1*Δ, *tco89*Δ). In addition, the AGC-type kinase Sch9p, which is phosphorylated by and is a major target of TORC1, and which contributes to TORC1-mediated regulation of ribosome biogenesis (Urban et al., 2007; Wei and Zheng, 2009), was not required for ethanol-dependent invasion (*sch9*Δ **Figures 5A,B**, although it was required for filamentation at colony perimeters **Figure 5C**). These results may not be entirely surprising, because although TOR and the mitochondrial retrograde pathway are functionally connected, the retrograde response to mitochondrial dysfunction is not dependent on TOR1-dependent regulation of retrograde gene expression (Giannattasio et al., 2005). Therefore, the mitochondrial retrograde pathway controls ethanol-inducible filamentous growth independent of TOR and at least partly independently of Sch9p.

In addition to TOR, the SAP- or p38-type high osmolarity glycerol response (HOG) MAP kinase pathway, which controls the response to osmotic and other stresses (Westfall et al., 2004; Saito, 2010), also regulates the RTG pathway (Ruiz-Roig et al., 2012). The HOG pathway was not required for ethanol-inducible filamentous growth (**Figures 5A–C**, *pbs2*Δ). Another function of the RTG pathway is to stimulate peroxisome biogenesis in periods of mitochondrial stress (Liao and Butow, 1993; Chelstowska and Butow, 1995; Kos et al., 1995; Epstein et al., 2001). Peroxisomes, which control elements of metabolism and can be regulated by the RTG pathway (Chelstowska and Butow, 1995), may impact ethanol-dependent filamentous

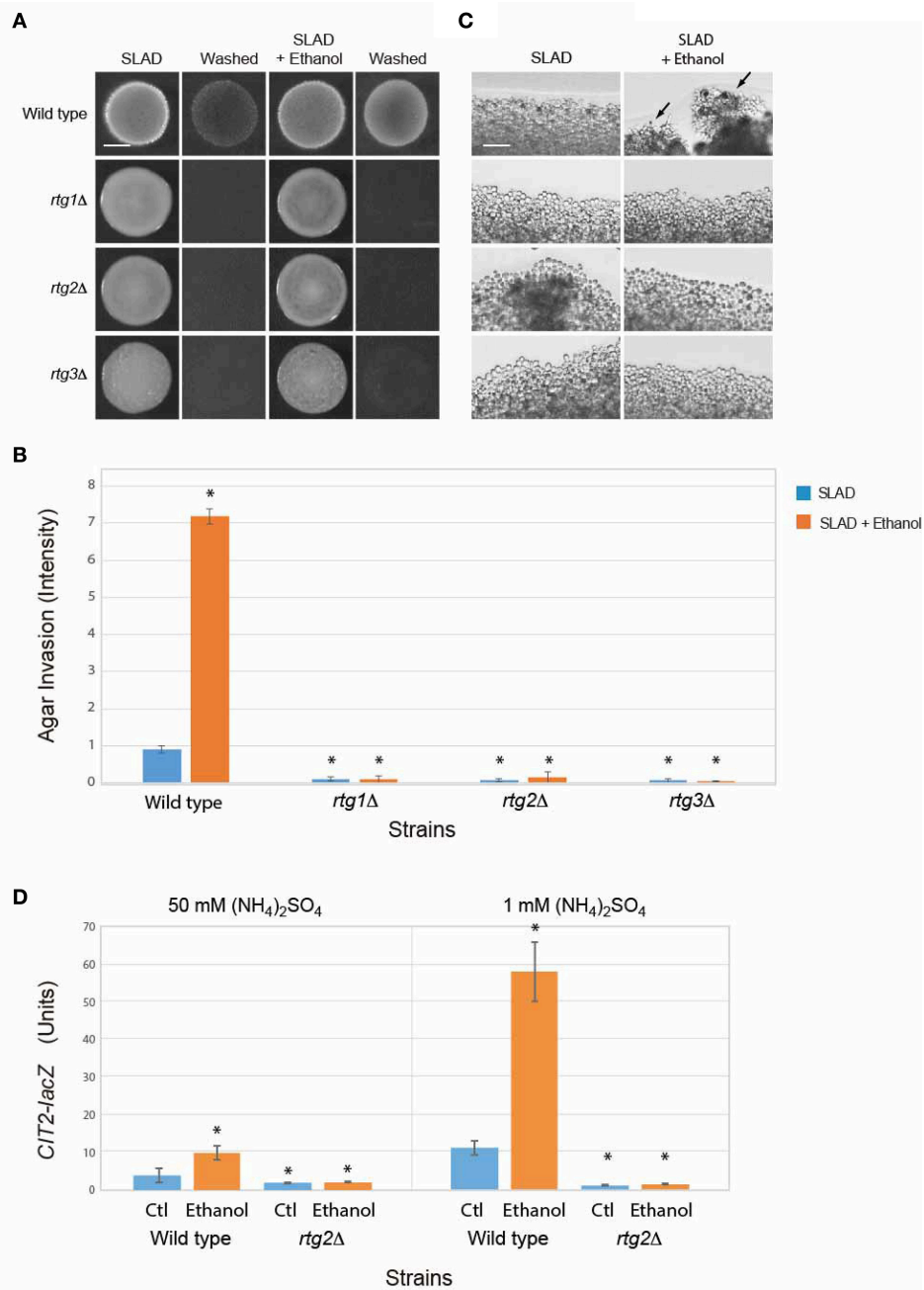

FIGURE 4 | Role of the RTG pathway in regulating ethanol-inducible invasive growth. (A) Wild-type cells (PC538, Σ1278b *MAT***a** haploid) and the indicated isogenic mutants were spotted onto nitrogen limiting medium (SLAD) with or without 2% ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry, performed as described in the legend for **Figure 1B**. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to wild type in SLAD. **(C)** Colony peripheries from the plates in panel **(A)** were examined by microscopy at 20X magnification. Bar, 50 μm. Arrows mark examples of pseudohyphae. **(D)** Beta-galactosidase activity of the *CIT2-lacZ* reporter in wild-type cells and the *rtg2*Δ mutant grown in 1 or 50 mM $(NH_4)_2SO_4$ with or without 2% ethanol (v/v). Experiments were performed in triplicate from independent inductions. Error bars represent the standard deviation between experiments. Asterisk denotes a $p < 0.01$ for samples relative to wild type in media lacking ethanol (Ctl).

growth. A mutant lacking peroxisomes was not required for ethanol-dependent filamentous growth, indicating that this is not the case (**Figures 5A–C**, *pex3*Δ). However, the *pex3*Δ mutant did show some defect (**Figure 5B**), and Cit2p, which is a target of RTG, was induced by ethanol (**Figure 4D**). These proteins regulate the glyoxylate cycle (Jazwinski, 2013) and

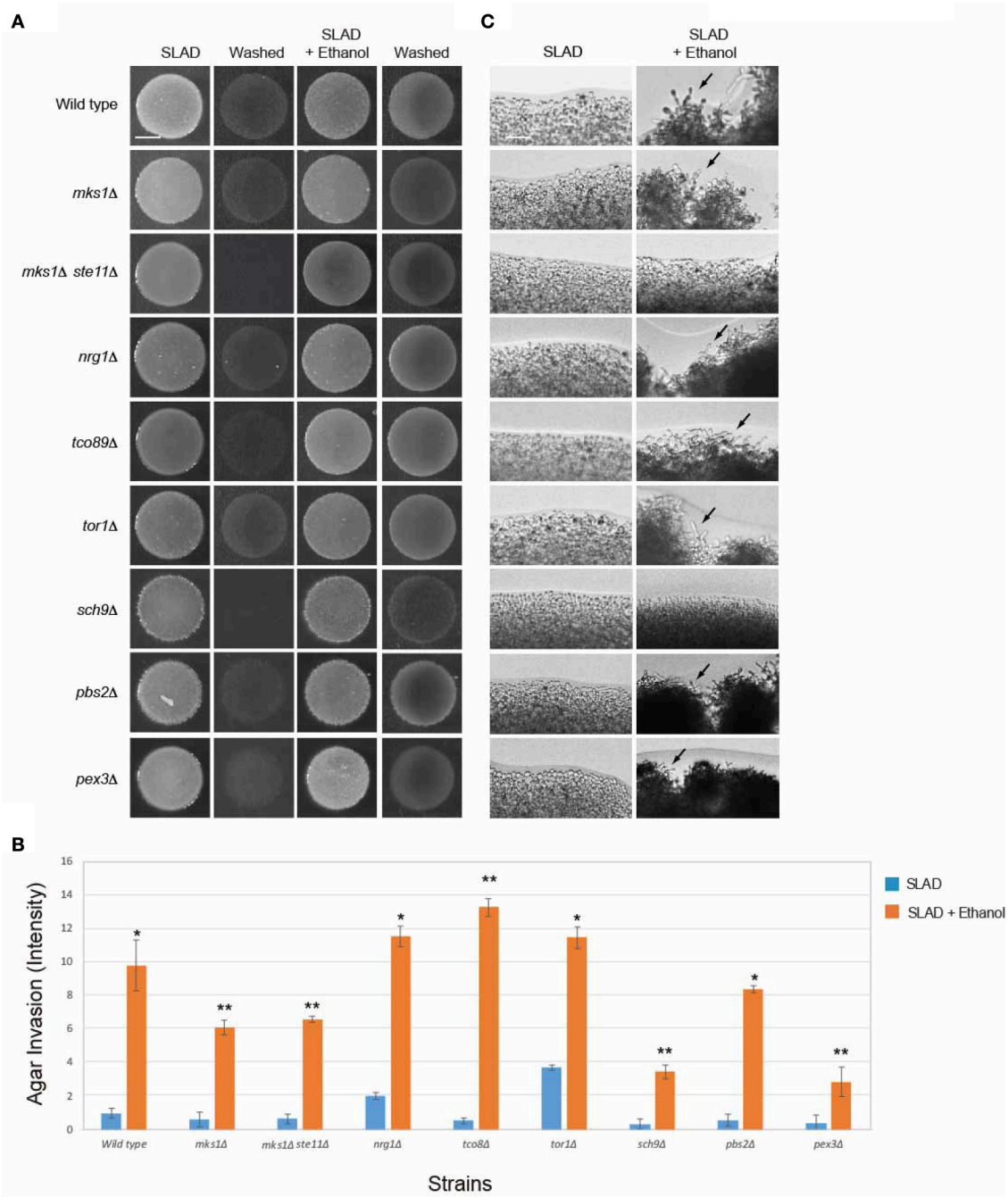

FIGURE 5 | Role of RTG pathway regulators in controlling ethanol-inducible invasive growth. (A) Wild-type cells (PC538, Σ1278b *MAT***a** haploid) and the indicated isogenic mutants were spotted onto nitrogen-limiting medium (SLAD) with or without 2% ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry, performed as described in the legend for **Figure 1B**. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.01$ for samples relative to each strain's invasion in SLAD. Double asterisk refers to a $p < 0.01$ for samples relative to each strain's invasion in SLAD compared to wild-type in SLAD with ethanol. **(C)** Colony peripheries from the plates in panel **(A)** were examined by microscopy at 20X magnification. Bar, 50 μm. Arrows mark examples of pseudohyphae.

it is possible that that metabolic pathway plays a role in regulating ethanol-inducible filamentous growth. Therefore, the mitochondrial retrograde pathway regulates ethanol-inducible filamentous growth in a manner that is separate from TOR, fMAPK, and HOG, and partly independent of peroxisome function.

Regulation of the TCA Cycle Underlies the Role of the Mitochondrial Retrograde Pathway in Controlling Ethanol-Inducible Filamentous Growth

The tricarboxylic acid (TCA or citric acid/Krebs) cycle functions through a series of reactions to generate ATP and produce reducing agents necessary for mitochondrial electron transport and energy generation. The TCA cycle is compromised in cells experiencing mitochondrial defects, but flux through the pathway can be maintained by the action of the RTG pathway (Liu and Butow, 1999; Lin et al., 2011), which is a major function of the RTG pathway (Butow and Avadhani, 2004). Glutamate can suppress the requirement for the retrograde pathway in the TCA cycle by increasing metabolic flux (Liu and Butow, 1999). Glutamate suppressed the defect in ethanol-inducible filamentous growth of the *rtg1*Δ, *rtg2*Δ, and *rtg3*Δ mutants (**Figures 6A–C**). The role of the RTG pathway in regulating ethanol-inducible invasion suggests that mitochondrial respiration is important for ethanol-dependent invasive growth. Consistent with this possibility, rho^0 cells, which lack a functional mitochondria, were defective for ethanol-inducible invasive growth (**Figures 6A–C**). Thus, one function of the RTG pathway in ethanol-dependent filamentous growth is to stimulate flux through the TCA cycle. Glutamate did not suppress the invasive growth defect of the *flo11*Δ mutant (**Figures 6A–C**). Given that Flo11p is the main cell adhesion molecule that regulates filamentous growth, these results suggest that glutamate-dependent invasive growth in *rtg* mutants is mediated (in some manner) through Flo11p.

The mitochondrial retrograde pathway has also been shown to regulate deoxyribonucleotide pools by impacting the rate of threonine metabolism (Hartman, 2007). Hydroxyurea induces a cell-cycle delay (Adams and Lindsay, 1967) and reduces the rate of DNA synthesis (Niu et al., 2008), and accordingly triggers a filamentation-like response (Jiang and Kang, 2003). Ethanol may impact threonine levels and DNA synthesis rates and induce retrograde-dependent filamentation. However, hydroxyurea, unlike ethanol, did not cause invasive growth in SLAD medium (Figure S4B). Moreover, the elongated cell morphology induced by hydroxyurea was retrograde-independent (Figure S4C). Therefore, the mitochondrial retrograde pathway probably does not regulate ethanol-dependent filamentous growth by influencing the rate of threonine metabolism.

Several other mutants that are defective in pathways surrounding the TCA cycle, ethanol uptake and metabolism, signaling, and the cell cycle were examined for a role in ethanol-inducible filamentous growth (Figure S5). Most of the mutants examined showed a detectable reduction in ethanol-inducible invasive growth (Figure S5). Two mutants stood out. One lacked Adh2p, which might be expected as that protein catalyzes the conversion of ethanol to acetaldehyde (Bennetzen and Hall, 1982; Young and Pilgrim, 1985; Dickinson et al., 2003). The other lacked Csf1p (Figure S5), a protein that is required for fermentation at low temperatures (Tokai et al., 2000). Notably, the wine yeast P24, which does not invade the agar in SLAD medium, is defective for growth at low temperatures (García-Ríos et al., 2014). Thus, the regulators of ethanol-inducible filamentous growth may encompass a more diverse collection of proteins than has been defined here.

Ethanol-Inducible Filamentous Growth Requires the Polarisome and Occurs through Induction of FLO11 Expression

Filamentous growth involves at least three major regulatory changes. One is an increase in cell length, which is mediated by a delay in the cell cycle (Kron et al., 1994) and by an increase in polarized growth by a Cdc42p-dependent mechanism that involves the polarisome (Cullen and Sprague, 2002). The formin Bni1p (Evangelista et al., 1997) and accessory proteins Bud6p, Pea2p, and Spa2p comprise the polarisome (Amberg et al., 1997; Sagot et al., 2002; Graziano et al., 2011; Tu et al., 2012). Another change is a switch in polarity to distal-unipolar budding that requires the distal-pole landmark Bud8p (Gimeno et al., 1992; Cullen and Sprague, 2002). Bud8p is a distal-pole marker that localizes to the distal pole of the cell (Harkins et al., 2001). The third change, as discussed above, is an increase in adhesion mediated by the cell adhesion molecule Flo11p (Lambrechts et al., 1996; Lo and Dranginis, 1996; Guo et al., 2000). The different aspects of filamentous growth are genetically separable and can be examined by mutants that specifically compromise each aspect of the response (Cullen and Sprague, 2002). Mutants were examined that were specifically defective for polarized growth (*bud6*Δ), polarity reorganization (*bud8*Δ), or cell adhesion (*flo11*Δ). Ethanol-inducible filamentous growth occurred in cells lacking Bud8 (**Figures 7A–C**, *bud8*Δ), which indicates that ethanol does not function mainly through the switch in polarity. Ethanol-inducible filamentous growth was reduced in cells lacking the polarisome component Bud6p (**Figures 7A–C**, *bud6*Δ). Thus, ethanol induces filamentous growth by a mechanism that is partly dependent on the increase in polarized growth driven by the polarisome. This is consistent with studies of fusel alcohols, which induce dramatic changes in cell length (Dickinson, 1996; Lorenz et al., 2000). Ethanol-inducible filamentous growth was also dependent on Flo11p (**Figures 7A–C**, *flo11*Δ). Consistent with this result, ethanol stimulated the expression of the *FLO11* gene (**Figure 7D**). Therefore, ethanol-inducible filamentous growth, which is controlled by the RTG pathway, requires polarisome function and occurs by a mechanism that involves Flo11p-dependent transcriptional induction.

DISCUSSION

Filamentous growth in yeast has been mainly studied in one strain background (∑1278b; Gimeno et al., 1992), in part because most laboratory strains have lost filamentation properties due to genetic manipulation in the laboratory (Liu et al., 1996; Dowell et al., 2010; Chin et al., 2012). Although filamentous growth is common among "wild" *S. cerevisiae* strains (Carstens et al., 1998; Sidari et al., 2014), the triggers of filamentous growth have not been extensively characterized in other backgrounds. By

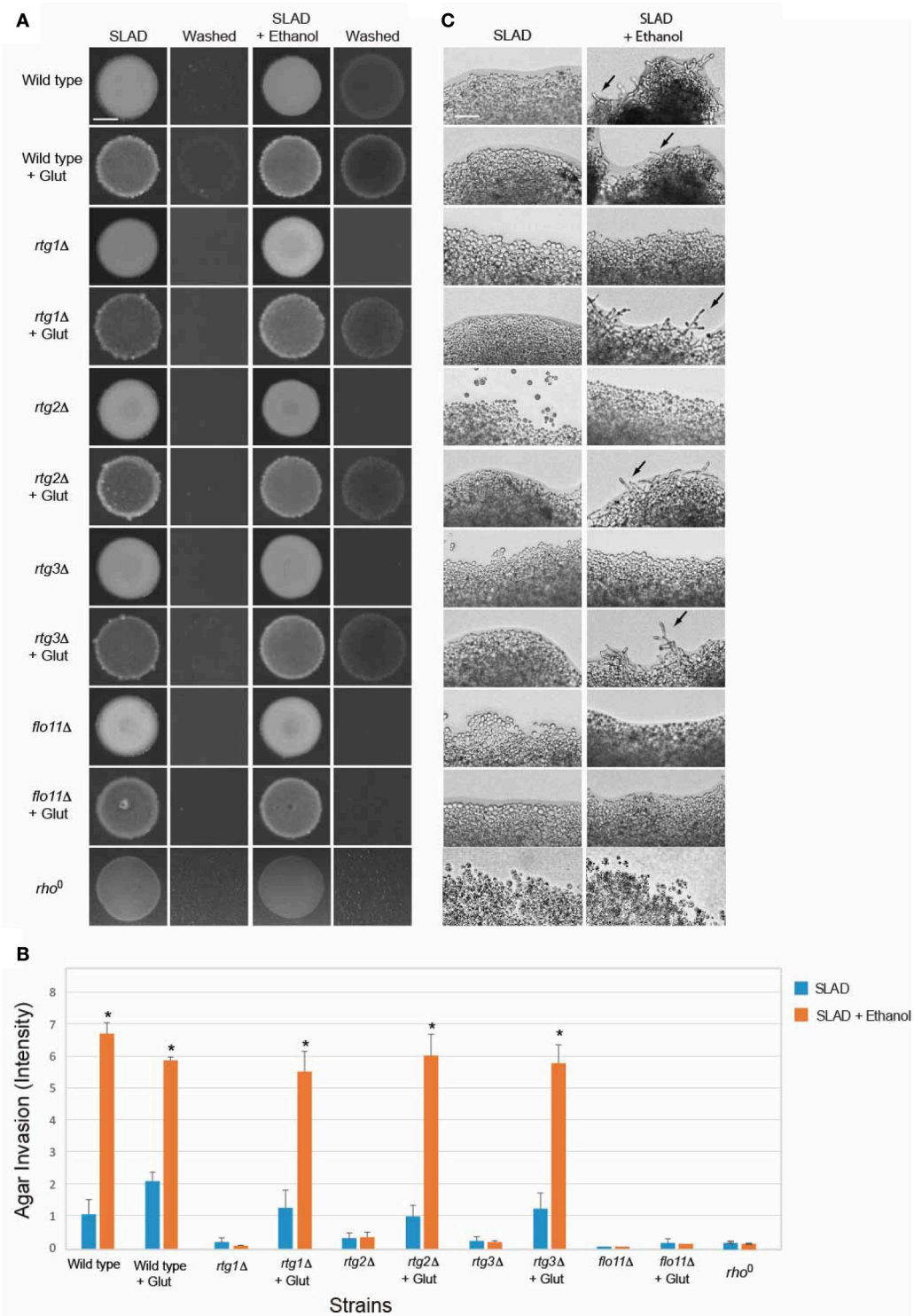

FIGURE 6 | Impact of glutamate on ethanol-inducible invasive growth defect of *rtg* mutants. (A) Wild-type cells (PC538, Σ1278b *MAT***a** haploid) and the indicated isogenic mutants were spotted onto nitrogen limiting medium (SLAD) with or without 2% ethanol (v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. Glutamate was added at a concentration of 200 μM. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry. Cells were spotted in triplicate, and the average values are shown. Error bars represent the standard difference between experiments. Asterisk denotes a $p < 0.05$ for samples relative to each strain's invasion in SLAD. **(C)** Colony peripheries from the plates in panel **(A)** were examined by microscopy at 20X magnification. Bar, 50 μm. Arrows mark examples of pseudohyphae.

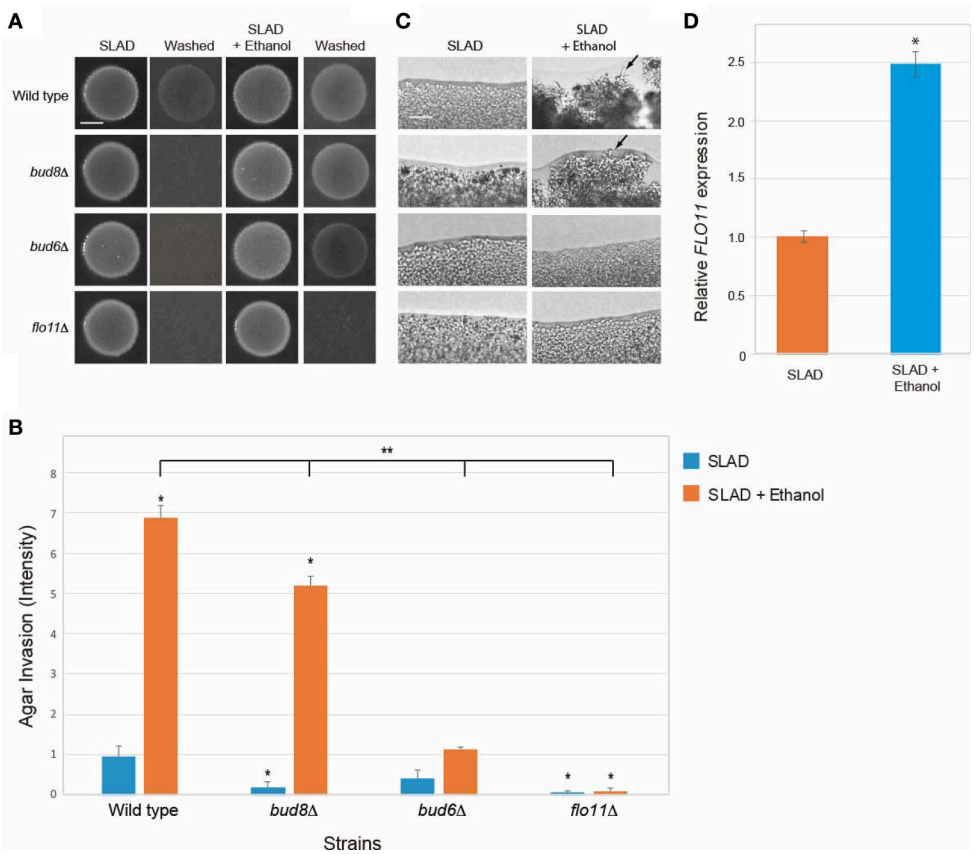

FIGURE 7 | Requirement for Bud8, Bud6, and Flo11 in mediating ethanol-inducible invasive growth. (A) Wild-type cells (PC538, Σ1278b *MAT***a** haploid) and the indicated isogenic mutants were spotted onto nitrogen-limiting medium (SLAD) with or without ethanol (2%v/v). Plates were incubated for 4 days at 30°C, photographed, washed in stream of water, and photographed again. Bar, 5 mm. **(B)** Quantitation of invasive growth in panel **(A)** by densitometry, performed as described in the legend for **Figure 1B**. Asterisk denotes a $p < 0.01$ for samples relative to wild type invasion in SLAD. Double asterisk denotes a $p < 0.01$ for samples relative to wild type invasion in SLAD with ethanol. **(C)** Colony peripheries from the plates in panel **(A)** were examined by microscopy 20X magnification. Bar, 50 μm. **(D)** Ethanol stimulates *FLO11* expression in SLAD medium. Cells were incubated in SLAD (orange bar) or SLAD with ethanol (blue bar). Gene expression was examined by qPCR at time 45 min and normalized to a control transcript (*ACT1*). Error bar represents standard difference between samples. Asterisk denotes a $p < 0.01$.

examining a collection of wine yeast, we show that most wine strains undergo filamentous growth. The strains also showed a high degree of phenotypic variation. Phenotypic variation is common among individual strains (Dowell et al., 2010) and may not be surprising given that these strains have undergone selection based on flavor, cold-sensitivity, alcohol tolerance, and flocculation (Suzzi et al., 1984; Fleet, 2003; Borneman et al., 2011).

We show here that nitrogen limitation and carbon limitation induce filamentous growth in most strains. This is consistent with previous claims that nitrogen limitation (Gimeno and Fink, 1994) and carbon limitation (Cullen and Sprague, 2000) trigger the filamentation response. We also show that ethanol and fusel alcohols induce filamentous growth. Ethanol (Dickinson, 1994; Lorenz et al., 2000) and fusel alcohols (Dickinson, 1996; Chen and Fink, 2006) are known to stimulate filamentous growth. Fusel alcohols induced filamentous growth under nutrient-replete conditions, and ethanol stimulated filamentous growth under nitrogen-limiting conditions. Ethanol is a by-product of glycolysis, whereas fusel alcohols are by-products of Ehrlich reactions. Thus, the two types of alcohols may provide information about different nutritional states. During alcoholic fermentation, *S. cerevisiae* produces ethanol when it has reached a maximum population density that corresponds with consumption of nitrogen (Beltran et al., 2005). Because nitrogen limitation is itself a trigger for filamentous growth, ethanol may be a coincidence detector of nitrogen levels and TCA compromise. Alternatively, glucose uptake correlates with the rate of the TCA cycle (Heyland et al., 2009). We also identify a potential role for the glyoxylate cycle in regulating ethanol-dependent filamentous growth. Thus, ethanol production may be a readout of nitrogen or glucose availability.

The cellular response to mitochondrial stress is important for biological responses in many systems. Generally speaking, cellular responses to mitochondrial disfunction have been implicated in cancer (Guha and Avadhani, 2013), aging (Friis et al., 2014; da Cunha et al., 2015; Jazwinski, 2015), development (Berkowitz et al., 2016), and inter-organellar homeostasis (Liu

and Butow, 2006). Here, we show that the fungal-specific RTG pathway controls ethanol-inducible invasive growth in yeast. Lorenz and Heitman argued that the fMAPK pathway mediates the response to alcohols (Lorenz et al., 2000), and we show that it may play a minor role. Here we establish the RTG pathway as a key pathway in the response. How does the RTG pathway control ethanol-dependent filamentous growth without involving other major filamentation regulatory pathways? One possibility is that the RTG pathway is part of the sensing/signaling mechanism that controls the rate of flux through the TCA cycle (Liu and Butow, 1999; Lin et al., 2011). TCA cycle rate is dependent on carbon and nitrogen levels, which are key inducers of filamentous growth in yeast and other fungal species. Canonical metabolic regulatory pathways that control filamentous growth also control TCA cycle flux including Snf1 (Hedbacker and Carlson, 2008) and TOR (Komeili et al., 2000); thus, TCA cycle activity may be a nexus for monitoring nutritional health.

The connection between TCA cycle flux and filamentous growth may be relevant from the perspective of pathogenecity. TCA cycle flux has been connected to the evolution of pathogenicity in filamentous fungi (Hogan et al., 2015) and apicomplexan parasites (Oppenheim et al., 2014). TCA cycle reprogramming is becoming increasingly tied to developmental transitions in pathogens ranging from *C. albicans* (Askew et al., 2009; Guedouari et al., 2014; Grahl et al., 2015), to *Plasmodium falciparum* (Ke et al., 2015) to *Yersinia pseudotuberculosis* (Bucker et al., 2014). The boost in TCA cycle flux is critical for phagososomal escape of the bacterial pathogen *Francisella* (Ramond et al., 2014). Moreover, the fungal RTG pathway is responsible for evasion of programmed cell death in yeast cells growing on non-repressing carbon sources (Guaragnella et al., 2013). Both the RTG pathway and relief of carbon catabolite repression are required for programmed cell death resistance. Evasion of programmed cell death and filamentous growth may be two hallmarks that fungi must acquire to become pathogenic. Our study therefore connects TCA cycle flux, as regulated by the RTG pathway, to an aspect of filamentous growth. Perhaps TCA flux controls developmental and morphogenetic responses in other eukaryotic systems.

AUTHOR CONTRIBUTIONS

BG designed and performed experiments. GB designed experiments. AM designed experiments. MJT designed experiments. PC helped with experimental design and writing the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2017.00148/full#supplementary-material

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