Review

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A new pathway for mitochondrial quality control: mitochondrial-derived vesicles

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Abstract

The last decade has been marked by tremendous progress in our understanding of the cell biology of mitochondria, with the identification of molecules and mechanisms that regulate their fusion, fission, motility, and the architectural transitions within the inner membrane. More importantly, the manipulation of these machineries in tissues has provided links between mitochondrial dynamics and physiology. Indeed, just as the proteins required for fusion and fission were identified, they were quickly linked to both rare and common human diseases. This highlighted the critical importance of this emerging field to medicine, with new hopes of finding drugable targets for numerous pathologies, from neurodegenerative diseases to inflammation and cancer. In the midst of these exciting new discoveries, an unexpected new aspect of mitochondrial cell biology has been uncovered; the generation of small vesicular carriers that transport mitochondrial proteins and lipids to other intracellular organelles. These mitochondrial-derived vesicles (MDVs) were first found to transport a mitochondrial outer membrane protein MAPL to a subpopulation of peroxisomes. However, other MDVs did not target peroxisomes and instead fused with the late endosome, or multivesicular body. The Parkinson's disease-associated proteins Vps35, Parkin, and PINK1 are involved in the biogenesis of a subset of these MDVs, linking this novel trafficking pathway to human disease. In this review, we outline what has been learned about the mechanisms and functional importance of MDV transport and speculate on the greater impact of these pathways in cellular physiology.

Keywords mitochondria; Parkin; PINK1; quality control; vesicle transport
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Introduction

Mitochondria are very complex organelles, housing hundreds of biochemical reactions from energy production to amino acid and lipid synthesis, to hormone production. These biochemical reactions involve substrates and products that flow between the many organelles within the cell. Rather than metabolite shuttling through free diffusion mechanisms, there is increasing evidence that direct interorganellar contacts are required. For example, elemental iron uptake into the mitochondria has been shown to require "kiss-and-run" contacts between the endosome and mitochondria (Zhang *et al*, 2005; Sheftel *et al*, 2007). Direct contacts between mitochondria and lipid droplets and peroxisomes are thought to facilitate fatty acid transport. The most advanced understanding of these contacts is between the ER and the mitochondria. It has long been known that ER is the source of lipids for mitochondrial biogenesis (Shiao *et al*, 1995) and that these contacts are important for cellular calcium homeostasis (Rizzuto *et al*, 1998). More recently, it was discovered that ER wrapping around the mitochondria marks the sites for mitochondrial division. A molecular understanding of these contacts has been advanced through studies in yeast and mammalian models (Csordas *et al*, 1999; de Brito & Scorrano, 2008; Kornmann *et al*, 2009) and is reviewed elsewhere. It is now clear that there is extensive biochemical cross talk between organelles, but the mechanisms are only beginning to emerge (Sheftel *et al*, 2007; Zehmer *et al*, 2009; Rowland & Voeltz, 2012; Mesmin *et al*, 2013).

This review will outline the emerging role of vesicular transport as another means of interorganellar communication. Mitochondrial-derived vesicles (MDVs) are generated through the selective incorporation of protein cargoes, which can be limited to the outer membrane, or can include outer, inner membrane, and matrix content, as illustrated in Fig 1 (Neuspiel *et al*, 2008; Soubannier *et al*, 2012a,b). Ultrastructural analysis revealed their size to be relatively uniform, between 70 and 150 nm, and their scission does not require the established mitochondrial fission GTPase Drp1 (Neuspiel *et al*, 2008; Soubannier *et al*, 2012a,b). Two distinct fates were identified for MDVs, with their targeting either to the late endosome/multivesicular body for degradation (Soubannier *et al*, 2012a), or to a subpopulation of peroxisomes (Neuspiel *et al*, 2008). Although this area of research is just emerging, this review will outline the molecular details of cargo selection, vesicle formation, and delivery, as well as the established and predicted impact of these pathways in cellular physiology.

The selection of cargo for transport

Mitochondrial-vesicle transport carries cargo to peroxisomes and lysosomes. Cargo destined for the lysosomes is ultimately degraded (Soubannier *et al*, 2012a), and *in vitro* studies have shown

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enrichment of oxidized proteins within MDVs (Soubannier *et al*, 2012b). However, the purpose of vesicle delivery to the peroxisomes is unclear (Mohanty & McBride, 2013). Currently, only one protein is known to traffic to the peroxisomes, a membrane anchored protein ligase called MAPL (also called MULAN, MUL1, GIDE and HADES) (Neuspiel *et al*, 2008; Braschi *et al*, 2009). With just one known cargo, it is difficult to predict the mechanisms and principles that govern cargo selection. However, we can look to evolution to help generate testable, working hypotheses. We will first consider the mechanisms of cargo selection based on each target destination separately.

Cargo selection for transport to lysosomes

There are two primary pieces of evidence that contribute to our understanding of the nature of the cargo en route to the late endosome/lysosome. Firstly, MDVs generated *in vitro* from isolated mitochondria were shown to be enriched in oxidized protein, in a process that was stimulated by mitochondrial stress (Soubannier *et al*, 2012b). This reconstitution of MDV formation further revealed a selective incorporation of protein cargo based on the nature of the mitochondrial stress induced. For example, the generation of ROS in the reaction with xanthine oxidase/xanthine led to a stimulation of MDVs carrying the outer membrane pore protein VDAC, but generation of ROS within the mitochondria, upon addition of a complex III inhibitor antimycin A, led to MDVs carrying the complex III subunit core2, without any enrichment in VDAC (Soubannier *et al*, 2012b). These data suggest that potentially any cargo could be included within MDVs; assuming that they are first oxidized, which would "damage" the complex. This also suggests that oxidation may trigger aggregation or oligomerization, acting as a seed to initiate membrane curvature from the inside.

The process of MDV formation has likely been conserved from archaebacteria, the mitochondrion's ancestors (Deatherage & Cookson, 2012). So, are there clues as to the cargo selection mechanisms within these ancient systems? All gram-negative bacteria, including *Archae* strains, shed vesicles, from those living within the soil to infectious strains like *Helicobacter pylori*, causing ulcers, or *Treponema pallidum,* the cause of syphilis (Kulp & Kuehn, 2010; Bonnington & Kuehn, 2014). These bacterial strains bud a variety of vesicles carrying specific cargoes, with unique tasks from the transport of virulence factors, or peptides that arrest the host cell cycle, to the generation of a biofilm. In addition, the quantity of bacterial protein incorporation into vesicles ranges from 0.1% to 8–12%, showing a remarkable > 100-fold variation in cargo incorporation (Soubannier *et al*, 2012b; Bonnington & Kuehn, 2014). Environmental changes in pH or nutrients are often a signal for bacterial vesicle secretion; however, the molecular mechanisms responsible for such varied cargo selection are not well understood (Deatherage & Cookson, 2012). Given the diversity and complexity of bacterial vesicle formation, we consider it unlikely that a single evolutionary set of machinery could be mapped to MDVs from the ancestral mechanisms of shedding. However, the utility of vesicles in all membrane systems has been demonstrated and mitochondria are no exception. Indeed, the lessons from bacteria help to frame our understanding of the use of vesicles as a highly selective way to sort mitochondrial proteins.

The second important finding was that the generation of MDVs destined for lysosomes required the protein kinase PINK1 and the cytosolic ubiquitin E3 ligase Parkin (McLelland *et al*, 2014). PINK1 and Parkin are both mutated in familial cases of Parkinson's disease (PD) (Trinh & Farrer, 2013) and were initially shown to act in a common pathway in mitochondrial quality control in *Drosophila* models of PD (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006). More recent work has shown that PINK1 is targeted to the mitochondria but is normally degraded very rapidly. During import, PINK1 is first cleaved by the matrix processing peptidases and PARL (Jin *et al*, 2010; Greene *et al*, 2012); however, almost all of the cleaved PINK1 is then released from the import channel and degraded in the cytosol through the N-end rule proteolytic pathway (Kondapalli *et al*, 2012; Lazarou *et al*, 2012; Yamano & Youle, 2013). Upon mitochondrial depolarization, the import machinery is inactivated and PINK1 becomes trapped either within the import channel or becomes anchored to the mitochondrial outer membrane near the import channel (Greene *et al*, 2012; Lazarou *et al*, 2012). This exposes the kinase domain to the cytosol where it phosphorylates ubiquitin and Parkin, leading to stable Parkin recruitment and activation at the mitochondrial surface (Kim *et al*, 2008; Shiba-Fukushima *et al*, 2012; Iguchi *et al*, 2013; Kane *et al*, 2014; Kazlauskaite *et al*, 2014). Parkin ubiquitinates a series of proteins on the mitochondrial surface, which are then recognized by autophagic adaptor proteins and delivered to the autophagosome (Narendra *et al*, 2008; Gegg *et al*, 2010; Lee *et al*, 2010; Matsuda *et al*, 2010; Tanaka *et al*, 2010; Chan *et al*, 2011; Chen & Dorn, 2013; Sarraf *et al*, 2013).

Given that PINK1 and Parkin are also required for MDV transport, we predict that the same mechanisms apply, but at a much more localized level (see model, Fig 2). The import channels are spatially restricted upon the mitochondrial surface, as shown with super-resolution microscopy or immunoelectron microscopy (Wurm *et al*, 2011). Protein misfolding in the matrix was recently shown to trigger mitophagy after long incubations (Jin & Youle, 2013), without any loss of electrochemical potential. Therefore, we consider that local protein aggregation at the import site, perhaps due to local oxidative damage, or complex assembly defects, would block the import process. Should the matrix chaperones become saturated, or cardiolipin become oxidized locally, then the inner membrane import channel may fail. Cardiolipin oxidizes to phosphatidic acid, a lipid known to alter membrane curvature (Yurkova *et al*, 2008; Donaldson, 2009; Horvath & Daum, 2013), and may help initiate the outward bending of the membrane. Upon complete depolarization or organelle dysfunction, the mechanism may switch from a local removal of a "patch" of mitochondrial content to the global arrest of PINK1 in all import channels, activation of the autophagic machinery, and entire engulfment of the organelle. This "patch" may not be strictly cargo selective, since whatever aggregated or oxidized proteins and lipids reside in proximity to an arrested import channel would be ejected. Supporting this concept, the kinetic analysis of events following treatment with antimycin A revealed the generation of MDVs at an early stage of ROS production, while global mitochondrial depolarization led to the kinetically slower process of mitophagy (McLelland *et al*, 2014). This indicates that MDVs are likely a first round of defense for the mitochondria to eject damaged proteins in order to avoid the complete failure of the organelle. This first response does not require the activation of autophagy machinery, as it occurs in the absence of Atg5, Rab9, or beclin (Soubannier *et al*, 2012a; McLelland *et al*, 2014).

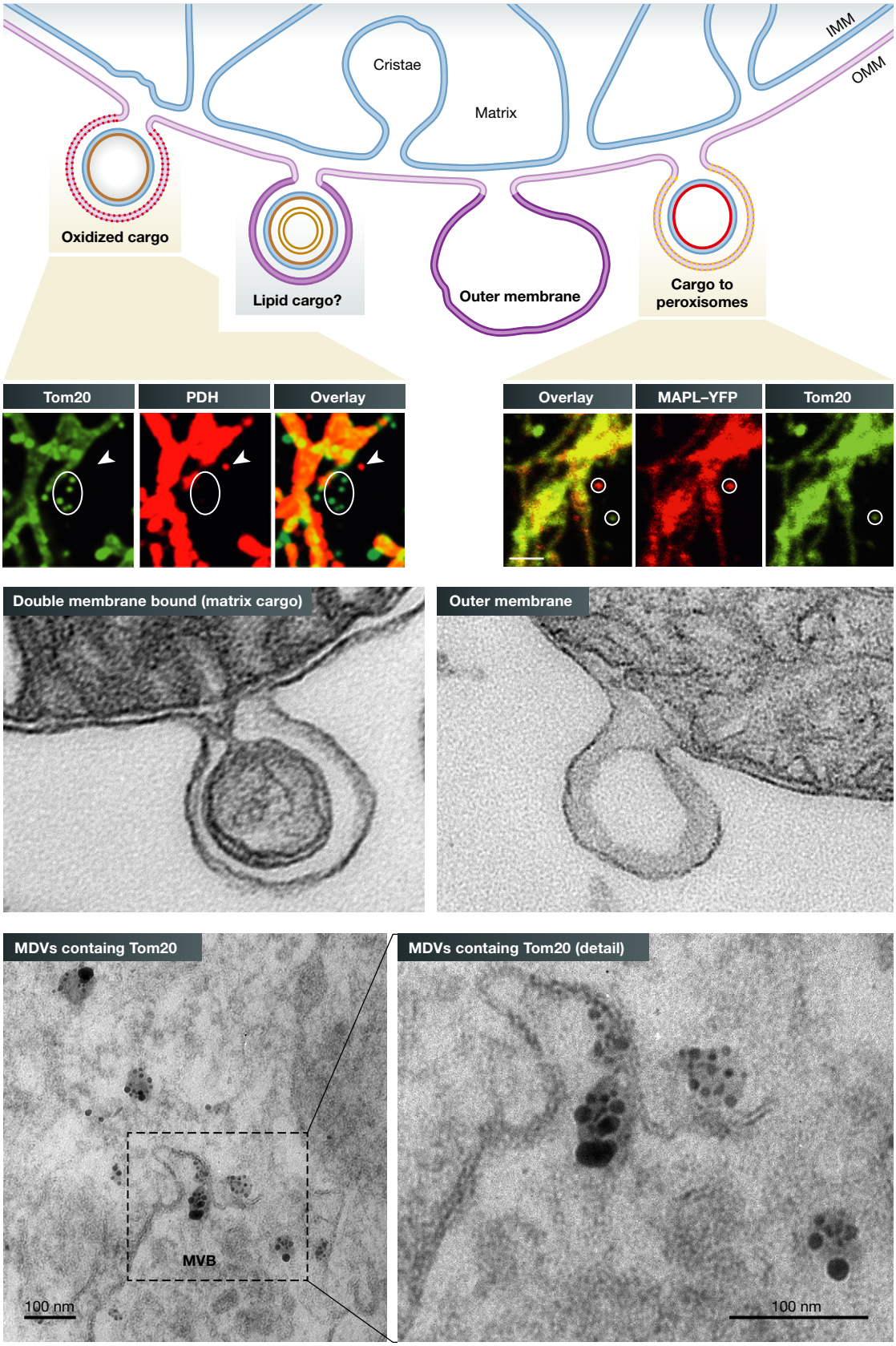

Figure 1. Summary of MDVs cargo variability.
Immunofluorescent and EM images illustrate the diversity of cargo-selected MDVs. Immunofluorescent staining of Tom20 (an outer membrane protein) and pyruvate dehydrogenase (PDH, matrix protein) reveals a number of cargo-selected vesicular structures lying outside of the mitochondria (top left panels, circles versus arrowheads). Although Tom20 is absent from PDH-positive structures (arrowheads), EM and biochemical experiments confirm that these vesicles are double membrane bound. An example is shown to the left where both membranes are seen within the vesicle emerging from the intact mitochondria [with permission from Soubannier *et al* (2012b)]. Similar cargo selectivity is seen for MDVs carrying MAPL that target the peroxisomes [top right panel of immunofluorescent images, taken with permission from Neuspiel *et al* (2008)]. We also observe single membrane MDVs derived from just the outer mitochondrial membrane (EM panel on right side). Bottom electron microscopic pictures show MDVs containing Tom20 labeled by immunogold particles enter the multivesicular body [taken with permission from Soubannier *et al* (2012a)]. Scale bars in EM pictures represent 100 nm.

A second argument in favor of the import channel acting as a sentinel for MDV formation is that import channels are restricted to the boundary membranes, where the inner and outer membrane are in close apposition to thread precursor proteins into the matrix and inner membrane. This explains how the two membranes may bud out together, since they would be locked in place by the arrested PINK1 precursor. It also lends insight into why PINK1 and Parkin are not required for the generation of MDVs that carry only outer

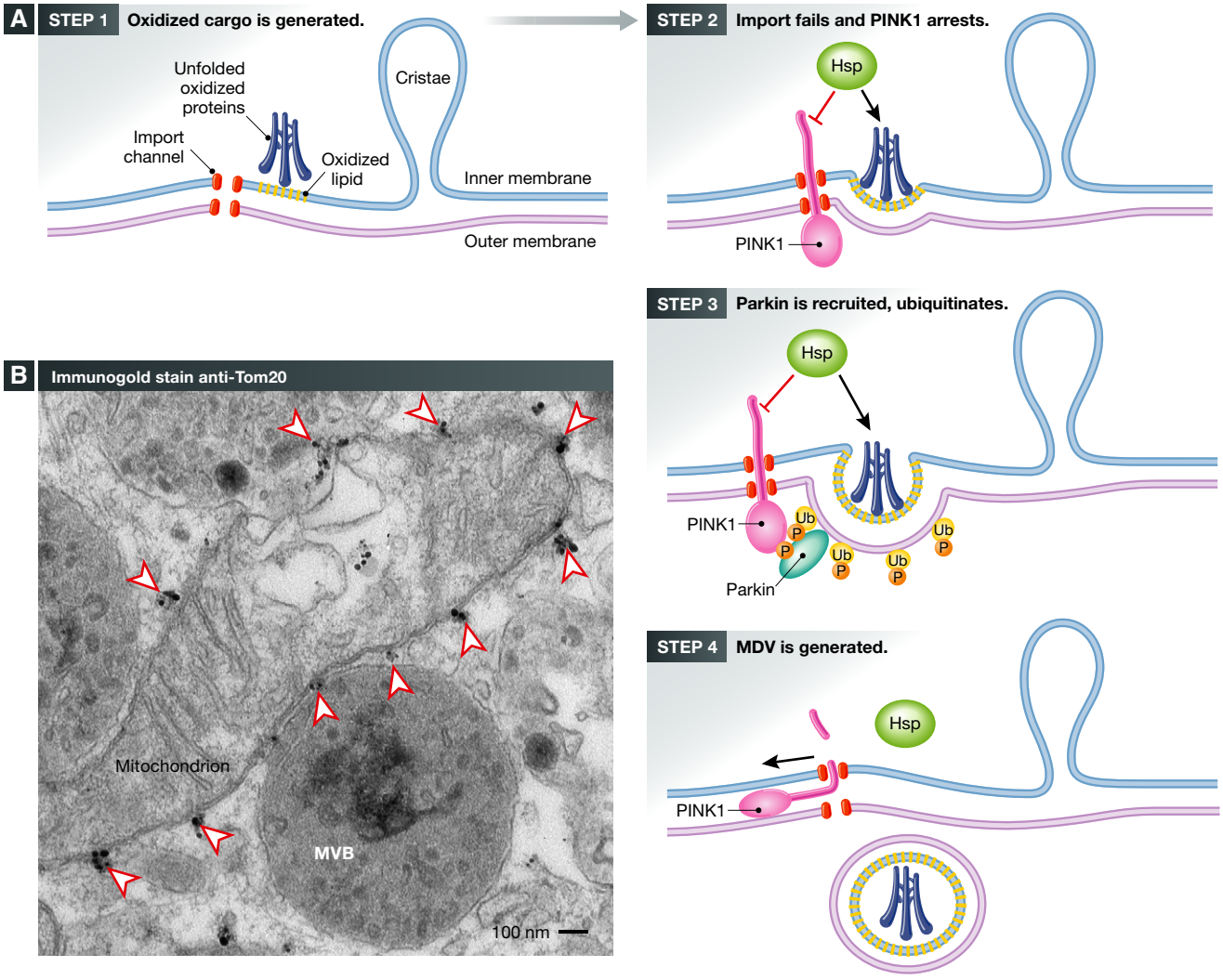

Figure 2. Working hypothesis for vesicle initiation by PINK1 and Parkin.
(A) Immunogold staining of endogenous Tom20 within COS7 cells reveals the regular spacing of the import channels indicated by arrowheads. Note the close tethering of three multivesicular bodies to the mitochondria. (B) An illustration of our working hypothesis of PINK1/Parkin-mediated MDV formation. In Step 1, unfolded, oxidized proteins within matrix, triggered by ROS or failure to assemble, leads to protein aggregation (blue). Oxidation of cardiolipin will generate PA, contributing to altered membrane curvature. In Step 2, protein aggregates may saturate chaperones, leading to a very localized failure to import at an individual channel. In addition, local oxidation of cardiolipin would further interfere with import channels. PINK1, which is rapidly imported, would then accumulate at these failed import channels. In Step 3, PINK1 phosphorylates both ubiquitin and the ubiquitin-like domain of Parkin, stabilizing the recruitment of activated Parkin. The ubiquitination activity of Parkin is required to generate MDVs, suggesting that domains on the surface may be cleared. In Step 4, a vesicle is formed and released in a process that will certainly involve a number of unidentified proteins. Future studies are needed to test this hypothesis and uncover the details governing the generation of MDVs.

membrane proteins like the import receptor Tom20. Ultrastructural analysis of mitochondrial single membrane vesicles reveal a more pleotropic appearance, rather like "blebs" than true, well-constructed vesicles (Fig 1, Soubannier *et al*, 2012b). The trigger for these vesicles may more closely mirror the bacterial mechanisms of outer membrane vesicle release, the mechanisms of which remain unclear. Indeed, despite the relatively greater abundance of these Tom20 outer membrane vesicles compared to, for example, MDVs carrying matrix pyruvate dehydrogenase that lack Tom20, to date no protein machineries required for their biogenesis have been identified.

A role for Parkin in vesicle trafficking has been shown previously. In receptor-mediated endocytosis, a ubiquitin-interacting motif (UIM) within the adaptor protein Eps15 was shown to bind the ubiquitin-like domain (UBL) of Parkin (Fallon *et al*, 2006). This led to the monoubiquitination of Eps15 and inhibition of its capacity to recruit the endocytic machinery, thereby regulating its function as an adaptor for endocytosis of the EGF receptor (EGFR). In this way, by delaying EGFR endocytosis and degradation, Parkin can enhance signaling downstream of the receptor. Parkin was also shown to bind and monoubiquitinate the endocytic BAR domain protein endophilin A via a Ubl-SH3 interaction (Trempe *et al*, 2009). As BAR domains are involved in membrane remodeling and curvature, this finding further links Parkin to vesicle budding and trafficking machinery. It is unclear what the signal is to recruit and activate Parkin at the cell surface as it is unlikely to be PINK1, which is constitutively targeted to mitochondria. However, very recent data have shown that PINK1, upon stabilization at the mitochondrial outer membrane, phosphorylates ubiquitin at position S65 (Kane *et al*, 2014; Kazlauskaite *et al*, 2014; Koyano *et al*, 2014). Three independent studies demonstrated that phosphorylated ubiquitin efficiently activated Parkin ubiquitin ligase activity at the mitochondrial surface in acute settings of mitochondrial uncoupling. In this situation, there was a nearly stoichiometric phosphorylation of cellular ubiquitin, which is likely to reach other cellular ubiquitin targets. In this way, the generation of phosphor-ubiquitin at the mitochondrial surface could act as a signaling mechanism for a global cellular response to mitochondrial stress. Regardless of the mechanisms by which Parkin is activated in endocytosis, the data indicate that Parkin may have a multifaceted role in vesicle transport, at the plasma membrane, mitochondrial surface, and perhaps elsewhere. It will be particularly interesting to determine whether these or other adapters are involved in the membrane budding and trafficking involved in the biogenesis of the subset of MDVs involving PINK1 and Parkin at mitochondria. Thus, whereas Parkin clearly plays a role in mitophagy, it also has a steady-state role in the removal of selected, oxidized cargo in a pathway parallel to mitophagy.

Cargo selection for transport to peroxisomes

The fate of mitochondrial cargo transiting to the lysosome is to be degraded. However, it is much less obvious why there may be a need for vesicle transport to the peroxisomes. The only cargo identified to date transits to a subpopulation of peroxisomes, about 10–20% of the total peroxisomes in the cell (Neuspiel *et al*, 2008; Braschi *et al*, 2010). Immunogold analysis of MAPL-positive MDVs revealed the presence of two membranes (Neuspiel *et al*, 2008), leading us to consider that the cargo is not limited to outer membrane content. There is some information on the mechanisms of MAPL enrichment within peroxisome-bound MDVs. The retromer complex containing Vps35, Vps26, and Vps29 was identified as a MAPL binding partner in an affinity chromatography approach (Braschi *et al*, 2010). The retromer complex was first established as a coat-like complex that binds and enriches cargo into vesicles from the endosome for their return to the Golgi apparatus (Seaman *et al*, 1998; Arighi *et al*, 2004; Seaman, 2012). The retromer complex also binds to the sorting nexin family of proteins that contain a PX-BAR domain that facilitates membrane curvature required for vesicle formation. More recent experiments reveal a much broader role for the retromer complex in many transport pathways, where specificity is granted through the combinatorial use of different sorting nexin members, and variants of the retromer subunits (Rojas *et al*, 2007; Collins *et al*, 2008; Cullen & Korswagen, 2012). In each case, the Vps35 subunit of the retromer binds to cargo tails, hinting that transport to the peroxisome will be more signal specific compared with the mechanisms of transport to the lysosome. Silencing Vps35 blocked the delivery of MAPL to peroxisomes, confirming the functional requirement for this complex in MDV transport (Seaman, 2012). MAPL contains ubiquitin and SUMO E3 ligase activities within the cytosolic domain (Braschi *et al*, 2009), however mutations in the RING finger domain did not alter the delivery to the peroxisome (Neuspiel *et al*, 2008; Braschi *et al*, 2010), indicating that the SUMOylation/ubiquitination activity of MAPL are not mechanistically required for MDV formation. At this time, we consider that MAPL constitutes vesicle cargo that does not function in the generation of peroxisome-bound vesicles. Clearly, there is a great deal of work remaining to elucidate the extent of cargo incorporation and the role of the retromer complex in this pathway.

Vps35 participates in a variety of transport pathways throughout the cell. However, with mutations in Vps35 being recently linked to PD and Alzheimer's disease (Vilarino-Guell *et al*, 2011; Zimprich *et al*, 2011), its role in MDV transport has emerged as an intriguing functional arc that may link defects in Vps35 with mitochondrial dysfunction. Future work will determine whether an alteration in cargo delivery to peroxisomes may contribute to PD.

Mechanisms of MDV transport and delivery

As described above, we have identified three factors required for the generation of at least a subset of MDVs; those carrying matrix content for delivery to the lysosome (PINK1 and Parkin), and MDVs destined for the peroxisome (retromer complex). However, if we look to other vesicle transport paradigms, it is apparent that this is likely the tip of the iceberg. In addition to cargo selection mechanisms, MDV formation will require machineries that facilitate membrane curvature, potential coat complexes, incorporation of fusion machinery, and motility factors. MDVs are formed in the absence of the mitochondrial dynamin GTPase Drp1, indicating additional mechanisms are also required for the final scission event (Neuspiel *et al*, 2008; Soubannier *et al*, 2012a; McLelland *et al*, 2014). The independence of Drp1 is consistent with the diameter of the yeast mitochondrial dynamin (Dnm1) ring limited to 100 nm, which would be too large to constrict an MDV neck (Ingerman *et al*, 2005).

We may find clues as to the identity of MDV factors within the MitoCarta, an annotated map of the mitochondrial proteome (http://www.broadinstitute.org/pubs/MitoCarta/). For example, there are a number of vesicle-related proteins whose roles have not yet been characterized on the mitochondrial outer membrane. Two enzymes are predicted to modulate phosphatidylinositol phosphates (PIPs) on the surface; PI(4)-kinase IIIβ and splice variant of the PI(5)-phosphatase synaptojanin-2A (Nemoto *et al*, 2001). Although the presence of PIP-based microdomains on the mitochondria has not been studied intensively, PI(3)P domains were observed to form during mitophagy (Yang & Yang, 2013). PIP-related microdomains are known to recruit adaptor proteins that could favor membrane bending and vesicle generation (Mayinger, 2012). Consistent with adaptors that facilitate alterations in membrane curvature, another endophilin family member, endophilin B1 (Karbowski *et al*, 2004; Takahashi *et al*, 2005, 2007), and a mitochondrial phospholipase D, MitoPLD (Choi *et al*, 2006; Huang *et al*, 2011), may also modulate membrane dynamics at the outer membrane. Endophilin B1 has been implicated in the binding of Bax (Pierrat *et al*, 2001), beclin (Takahashi *et al*, 2007) and in the process mitochondrial fission (Karbowski *et al*, 2004). These lipid binding and modifying enzymes are all candidates for MDV transport machinery given the established roles for their activities in vesicle transport within the biosynthetic and endocytic pathways. Finally, several Rab GTPases have been shown to impact mitochondrial morphology, biogenesis or turnover, including Rab32, Rab11, Rab4, and Rab7 (Alto *et al*, 2002; Bui *et al*, 2010; Caza *et al*, 2013; Landry *et al*, 2014; Talaber *et al*, 2014; Yamano *et al*, 2014). Therefore, it would not be surprising if some of these small GTPases were involved in MDV transport as well.

It is also critical to learn how MDVs may fuse with their target organelle. A splice variant of VAMP1A, called VAMP1B, was identified in 1998 and contains a mitochondrial targeting sequence in place of the C-terminal tail anchor (Isenmann *et al*, 1998). VAMP1B is ubiquitously expressed, whereas VAMP1A variants are exclusive to neurons. The function of VAMP1B is currently unknown, but it is a prime candidate to mediate fusion events of MDVs with target organelles. Mitochondrial proteomic studies have not identified a t-SNARE or SNAP25 homologue, suggesting that the mitochondria may be unable to receive incoming vesicles.

Interestingly, a high-resolution proteome of a very divergent mitochondrion-related organelle, called a mitosome, from the parasite *Giardia intestinalis* was recently published (Jedelsky *et al*, 2011). Mitosomes have lost their mtDNA, as well as their capacity to respire, and have almost-unrecognizable import machinery. Their major role is in fact to generate iron sulfur clusters for distribution throughout the cell. Despite its divergence from a typical mitochondrion, the highly purified mitosome proteome included potential orthologues of the retromer component Vps35, an R-SNARE 3 (a v-SNARE) and VAP, a VAMP (vesicle associated membrane protein)-interacting protein (Jedelsky *et al*, 2011). Like mammalian mitochondria, *Giardia* mitosomes are also limited to v-SNAREs in the absence of t-SNAREs or SNAP orthologues. This provides a clue that even the simplest mitosome may sort cargo within vesicles for delivery within the cell, perhaps to distribute iron sulfur clusters to other organelles, or for degradation as we see in mammalian cells.

The physiological contribution of MDV transport to mitochondrial quality control

MDV transport to lysosomes adds a fourth mechanism to the paradigms of mitochondrial quality control. MDVs function alongside the actions of mitochondrial proteases, ubiquitin-mediated proteasomal degradation, and mitophagy. The unanswered question is to define the relative contributions and potential hierarchy of these 4 mechanisms (Fig 3). Mitochondrial proteases degrade unfolded and oxidized proteins within the matrix and intermembrane space (Tatsuta & Langer, 2009). In yeast, an *in vitro* peptide export assay indicated that mitochondrial proteases degrade between 6–12% of proteins per hour, consistent with proteases as a front line of mitochondrial quality control (Augustin *et al*, 2005). It is also possible that proteases may trim down complexes and cargoes, leaving more hydrophobic regions to be subsequently removed via MDVs, a possible example of the overlap among these pathways. Loss of mitochondrial proteases leads to various forms of neurodegeneration, including spastic paraplegia (Casari *et al*, 1998; Atorino *et al*, 2003; Nolden *et al*, 2005). For example, mutations in AFG3L2, an m-AAA protease within the inner membrane, are responsible for spinocerebellar ataxia 28 (SCA28) (Di Bella *et al*, 2010). In addition, mitochondrial proteases are required for the processing of PINK1 (Greene *et al*, 2012), further supporting the interdependence between multiple quality control pathways.

Some outer membrane proteins are ubiquitinated and degraded by p97-dependent retrotranslocation and delivery to the cytosolic proteasome (Heo *et al*, 2010; Tanaka *et al*, 2010; Chan *et al*, 2011; Xu *et al*, 2011). This process efficiently removes surface proteins, which may be linked to quality control or to the selective removal of mitochondrial proteins in response to cellular signals (Neutzner *et al*, 2007). For example, the anti-apoptotic Bcl-2 family protein Mcl-1 is ubiquitinated by MULE during apoptosis, and its removal facilitates Bax activation and cell death (Warr *et al*, 2005; Zhong *et al*, 2005).

Mitophagy is an important mechanism to remove entirely dysfunctional mitochondria, whether linked to global protein misfolding or depolarization (Youle & Narendra, 2011). But what is the contribution of MDVs to steady-state quality control, and how does this compare with mitophagy? Unfortunately, since PINK1 and Parkin are required for both pathways, gene editing or siRNA approaches will not allow us to easily answer this. Loss of these genes in flies leads to a loss of dopaminergic neurons and flight muscle defects, consistent with their importance in mitochondrial quality control (Clark *et al*, 2006; Park *et al*, 2006; Yang *et al*, 2006). However, the loss of PINK1 or Parkin in mice leads to very mild phenotypes and a notable absence of neurodegeneration (Goldberg *et al*, 2003; Kitada *et al*, 2009). This hints at the existence of redundancies in both mitophagy and MDV formation in higher eukaryotes. Until we identify MDV-specific factors essential for their formation, we cannot determine the relative contributions *in vivo*. We do know that MDVs are released within 2–6 h following a mild stress like antimycin A, where mitophagy occurs between 12–24 h (McLelland *et al*, 2014). The prediction is that MDV formation protects the mitochondria from mitophagy by removing PINK1 and Parkin from each failed import channel. Any loss of MDV-specific machinery should therefore trigger premature mitophagy. Alternatively, the loss of MDVs may lead to increased global cellular

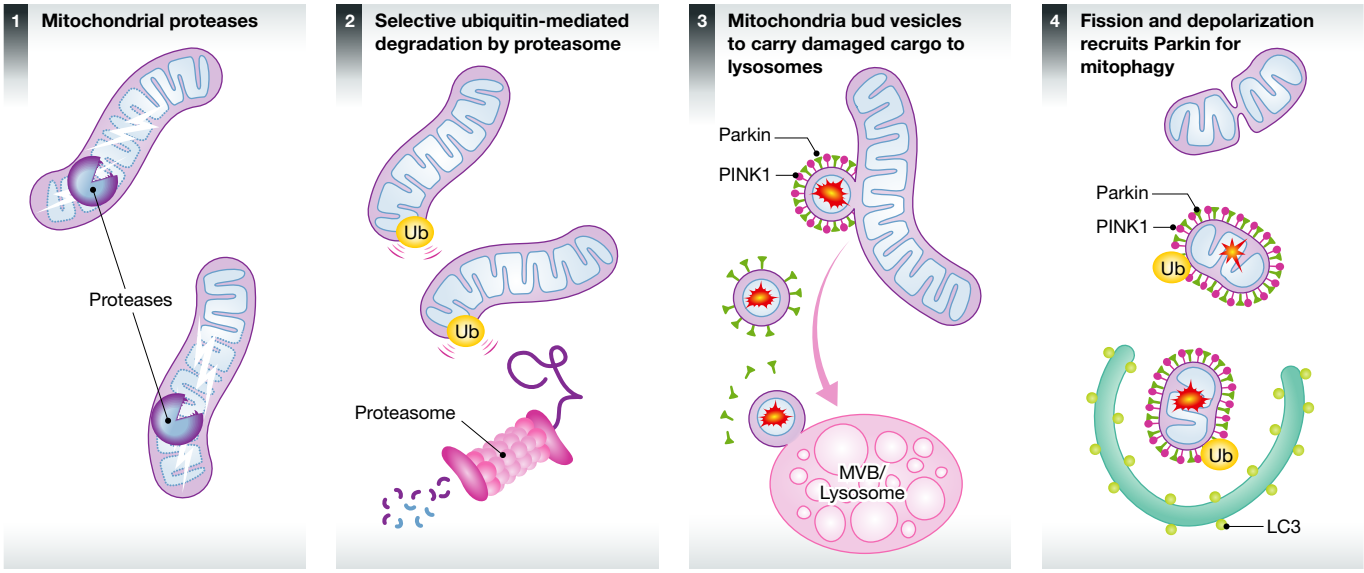

Figure 3. Outline of the 4 pathways of mitochondrial quality control.
A schematic diagram depicting the presence of mitochondrial proteases within the mitochondrial matrix and intermembrane space, which likely acts as a first line of defense against unfolded and oxidized soluble proteins. Outer membrane proteins are instead removed from the mitochondria through a retrotranslocation pathway following ubiquitination. Degradation of these proteins is completed within the cytosolic proteasome, similar to the ER-associated degradation pathway. We propose that the third line of defense is the removal of mitochondrial patches through the generation of MDVs, which transit to the late endosome. Only upon complete mitochondrial dysfunction, or upon a failure of most import channels would the entire organelle be targeted to the autophagosome. Different tissues and cellular states may rely on each of these mechanisms to a variable degree, making it important to understand the levels of redundancy and overlap among these pathways.

damage since the mitochondria may need to deteriorate to an appropriate level in order to engage the mitophagy pathway. Deteriorating mitochondria would release increased levels of ROS and likely begin to consume cellular ATP in efforts to maintain their electrochemical potential.

Beyond a global consideration of MDVs acting upstream of mitophagy in quality control, there are a few clues that shed some light on the functional importance of MDV formation in mitochondrial homeostasis. Treating cultured cells with the lysosomal inhibitors bafilomycin A or pepstatin A/E-64D led to a significant accumulation of MDVs in the cytosol or within the multivesicular bodies (Soubannier *et al*, 2012a; McLelland *et al*, 2014). This demonstrated that MDV transport occurs in steady state, even in the absence of mitochondrial or redox-based stress. In addition, the amount of cargo released into MDVs using the *in vitro* budding assay was quantified, indicating that up to 4% of some proteins are ejected per hour (Soubannier *et al*, 2012b). While there are certainly limitations to these kinds of quantifications, it suggests that MDVs are transporting significant amounts of cargo in steady state.

In other approaches, a number of recent studies have quantified the half-lives of mitochondrial proteins under various conditions, from yeast to flies and mammalian tissues. The most striking observation from all of these studies is the extreme variety of half-lives of mitochondrial proteins—from minutes to weeks. For example, a recent study in mice demonstrated a threefold range in the turnover rates of different complex I subunits, indicating that mitochondrial proteins within the same macromolecular complex have differing half-lives (Kim *et al*, 2012). This is consistent with the existence of multiple, overlapping pathways for mitochondrial protein degradation. In an elegant study in *Drosophila* by the Pallanck lab, the accumulation of cellular proteins in flies lacking the core autophagy protein Atg5, compared with those lacking Parkin or PINK1, was quantified by mass spectrometry (Vincow *et al*, 2013). In the absence of Atg5, many cellular proteins—including ones targeted to mitochondria—accumulated, indicating that Atg5 was required for their turnover. There was a great deal of overlap between the Atg5 and Parkin accumulated proteomes, consistent with them functioning along the same pathway in mitophagy. However, there was a distinct subset of highly hydrophobic proteins of the mitochondrial inner membrane that accumulated specifically in the absence of Parkin (Vincow *et al*, 2013). This indicates that Parkin plays an additional role the selective removal of these proteins, most likely through vesicular carriers.

In yeast, the Abeliovich lab recently confirmed the selective degradation of mitochondrial content using a similar approach (Abeliovich *et al*, 2013). In this case, they observed a selective accumulation of proteins in stationary phase yeast upon the loss of Dnm1, the functional homologue of the fission GTPase Drp1. The authors concluded that mitophagy results in the selective removal of damaged content at very different rates and that this process requires ongoing fusion and fission events. Exactly how the cargo would be sorted into two halves of a dividing mitochondrion remains unclear. The authors proposed a type of "percolation" of specific proteins into large domains, perhaps based on aggregation. These large domains would eventually be separated from the functional organelle by dynamin-mediated constriction (Abeliovich *et al*, 2013). On the other hand, it is formally possible that yeast mitochondria bud vesicles in a Dnm1-dependent manner, where the lateral segregation of cargo may occur in an analogous manner to MDV transport in mammalian systems. There is a great deal of

evolutionary distance between these two models, so these possibilities should not be ruled out without formally testing them with higher resolution imaging and biochemical analysis. Overall, quantitative mass spectrometry approaches are helping define the turnover of critically important mitochondrial proteins and lipids under a myriad of conditions.

The field of mitochondrial quality control has exploded with the causal links to many degenerative diseases. So do any of the recent studies support a role for MDVs in quality control? The most common approach is to use the protonophore CCCP to globally depolarize mitochondria and induce Parkin-dependent mitophagy. However, recent studies are expanding the analysis to more physiological systems, including the unfolded protein response (Jin & Youle, 2013), laser-induced damage (Yang & Yang, 2013), and the manipulation of mitochondrial proteases that interfere with PINK1 processing, such as the matrix processing protease (MPP) (Greene *et al*, 2012). Indeed, modest knockdown of the catalytic subunit MPPβ leads to PINK1 accumulation, Parkin recruitment, and mitophagy, without the widespread effects seen with CCCP on protein import and mitochondrial function (Greene *et al*, 2012). Using a different approach, the Youle lab employed a non-cleavable mutant of ornithine carbamyl transferase (Jin & Youle, 2013), first used to induce the mitochondrial unfolded protein response by Nick Hoogenraad (Zhao *et al*, 2002). Cells expressing this construct accumulated PINK1-YFP at specific foci along the mitochondrial tubules (Jin & Youle, 2013), presumably reflecting sites of failed import. Parkin was recruited to these sites as well, and after extended accumulation of unfolded proteins, respiratory competent mitochondria were cleared by mitophagy. Similarly, foci of Parkin and PI(3)P were observed along mitochondria following laser ablation (Yang & Yang, 2013), and these small fragments were targeted for mitophagy. In all these studies, the authors did not explore whether they were actually imaging MDVs as an early, targeted response to the stress. Certainly, if the magnitude of the damage is great enough, mitophagy will ensue, as is commonly observed. Interestingly, two tail-anchored mitochondrial membrane proteins, FKBP38 and Bcl-2, were shown to translocate to the endoplasmic reticulum to escape CCCP-induced mitophagy (Saita *et al*, 2013). The mechanism for this relocalization is not known, but the authors suggested the possibility of vesicle-based transport prior to mitochondrial removal by mitophagy. As the size of MDVs is smaller than the wavelength of light, routine confocal microscope approaches cannot distinguish between a mitochondrial fragment (~400 nm diameter) and an MDV (~100 nm) (Neuspiel *et al*, 2008; Soubannier *et al*, 2012a). Antibody staining can reveal the selection of specific mitochondrial cargo in these structures, and silencing Drp1 can help address the question of fission vs. budding. In the end, ultrastructural analysis is critical to directly visualize these events, something lacking from current studies. Some experimental considerations relevant to the analysis of cellular MDVs are listed in Box 1.

In order to ultimately define the functional contribution of MDV transport to mitochondrial quality control *in vivo*, we will continue to identify the core machinery required to generate and transport MDVs. We are currently utilizing the cell-free mitochondrial budding assay to generate vesicles in the presence of non-hydrolyzable GTP, for example, to stabilize potential coat proteins and other machinery. Mass spectrometry of isolated vesicles will help identify both the cargo and machinery. Lipidomics should lend insights into any lipid oxidation or enrichments that are likely to be very important cargoes as well. As the mechanisms gain in resolution, we will be able to directly assess the consequences on mitochondrial function and cell survival in different cell types and tissues.

Box 1. Experimental considerations for MDV analysis

- MDVs are visualized as small vesicular structures that show evidence of cargo selectivity. This is done using highly specific antibodies against endogenous mitochondrial proteins, or with a combination of transfected mitochondrial GFP-tagged constructs with antibodies to label a second or third mitochondrial protein. The limitation of antibody usage is the absolute dependence on the specificity and low or zero background signal. GFP-tagged and overexpressed proteins have not been efficient cargoes in general, perhaps because they are first targeted by proteases. We continue to evaluate mitochondrial content in MDVs and look to proteomics approaches for future analysis.
- To increase the ability to visualize MDVs, it is useful to minimize mitochondrial fragmentation by silencing the fission machinery, or expressing a dominant-negative Drp1. This results in hyperfused mitochondria, enabling the visualization of small, Drp-independent MDVs within the cell.
- To capture MDVs within the cytosol, prior to delivery to the lysosome, cells can be pre-treated with bafilomycin A. Alternatively, inhibition of lysosomal proteases with E64D/pepstatin A allows delivery to lysosomes, but blocks protein degradation. For transport to peroxisomes, the cargo is not degraded, so MAPL-positive MDVs en route to peroxisomes (or already within peroxisomes) is monitored by triple-labeling mitochondria, MAPL and peroxisomes. We do not yet have the tools to block the fusion of MAPL-positive MDVs with peroxisomes and accumulate them within the cytosol.
- Our experience with immunofluorescence approaches indicates a requirement for high percentages of PFA (5–6%), added directly to cells at 37°C without a PBS wash. This hints that the lipid/protein ratio in MDVs is not easily crosslinked and may be lost with low concentrations of fixative.
- The signal intensity of cargo within MDVs varies; Tom20-positive MDVs are brighter in Tom20 signal compared with Tom20 intensity within mitochondria, but PDH is less enriched in MDVs compared with the mitochondria. Given this variability, it is important to use an appropriate objective lens, preferably 100×, with the highest NA possible, at least a 1.4. Cameras for spinning confocal continue to improve, increasing the ability to visualize MDVs, and laser scanning confocal at high resolution also works well.
- Ultrastructural analysis is important to directly visualize MDVs within the cell using immunogold labeling. This is to confirm their size and differentiate them from Drp1-dependent fragments. The probabilities of capturing an unbudded MDV from the mitochondria is low unless cells are treated with stress agents like xanthine oxidaze/xanthine. Overexpressing MAPL in cells led to a dramatic stabilization of unbudded structures, suggesting that the GFP tag interfered with efficient removal. With the ongoing identification of new machinery required to generate MDVs, their silencing should help capture MDVs in various stages of formation. Tomography of tissues from disease animal models should also help visualize MDVs emerging in situ at high resolution.

The meaning of MDV transport to the peroxisome

Next to the ER, it can be argued that the peroxisome is the most closely linked organelle to the mitochondria (Mohanty & McBride, 2013). In mammalian cells, both organelles are responsible for the

beta-oxidation of fatty acids, where peroxisomes selectively oxidize very long-chain fatty acids and perform alpha-oxidation reactions. Both organelles neutralize damaging oxidative by-products—peroxides (peroxisomes) or ROS (mitochondria)—and the biogenesis of both organelles is signaled by the peroxisome proliferator-activated receptor coactivator 1 alpha (PGC1α) pathway (Mohanty & McBride, 2013). The biogenesis and growth of both organelles is also coupled to the ER, which provides lipids to facilitate their growth. Finally, the organelle division machinery is shared, including the core fission GTPase Drp1 and its receptor Mff (Koch *et al*, 2003; Gandre-Babbe & van der Bliek, 2008). Indeed, membrane-anchored proteins like Mff are commonly localized to both peroxisomes and mitochondria, (Koch *et al*, 2005; Gandre-Babbe & van der Bliek, 2008). Most of these tail-anchored proteins appear to be imported into peroxisomes using the canonical, peroxisomal import machinery (Delille & Schrader, 2008).

We identified MAPL as a SUMO E3 ligase containing two transmembrane domains, with a C-terminal RING domain exposed to the cytosol and a approximately 40 kDa intermembrane space domain of unknown function (Li *et al*, 2008; Neuspiel *et al*, 2008; Zhang *et al*, 2008; Braschi *et al*, 2009; Jung *et al*, 2011). This protein has a unique evolutionary phylogeny, with the intermembrane space and transmembrane domains being conserved within distant bacteria, as well as plants (Andrade-Navarro *et al*, 2009). MAPL promotes the sumoylation of Drp1, stabilizing its recruitment to the mitochondria and promoting fission (Neuspiel *et al*, 2008; Braschi *et al*, 2009).

If peroxisomes can import their own proteins, why would MAPL require such a complex vesicular transport pathway? We are actively pursuing an answer to this question, so will only speculate here. Peroxisomes grow and divide as autonomous organelles. However, unlike the mitochondria, which multiply exclusively as a result of the enlargement and fission of existing organelles, peroxisomes can also be generated *de novo* (Dimitrov *et al*, 2013). Current evidence, primarily in yeast, supports a role for the ER in the generation of new peroxisomal precursor vesicles that may mature (Hoepfner *et al*, 2005), or fuse to form a mature organelle (Lam *et al*, 2010). The population of "young" versus "mature" peroxisomes varies depending on the cell type and growth conditions. As a regulator of Drp1, MAPL is likely to participate in peroxisomal biogenesis and division. The silencing of MAPL does not phenocopy Drp1, so it is not essential for mitochondrial fission, rather it can promote and stabilize Drp1 recruitment. It may be that MAPL is targeted to "young" or newly formed peroxisomes, which may have a higher rate of fission, or different regulatory mechanisms than the "older", more mature peroxisomes.

A second reason to consider that MAPL-containing MDVs would target "young" peroxisomes is that old peroxisomes cannot fuse, only the ER-derived pre-peroxisomes are thought to be fusogenic (Dimitrov *et al*, 2013). Therefore, MDVs may deliver their cargo to the pool of nascent peroxisomes, and this cargo exchange may be necessary for peroxisomal maturation. As these peroxisomes grow and divide, MAPL would become diluted, or perhaps selectively degraded in some way. From an evolutionary perspective, it is interesting that most of the peroxisomal enzymes appear to derive from an archaebacterial origin (Gabaldon *et al*, 2006). This has led some to suggest that peroxisomes originated as specialized mitochondria responsible for the breakdown of very long-chain fatty acids (and a number of other biochemical processes) (Speijer, 2014). Perhaps, this ancient vesicular transport pathway allowed the selective segregation of potentially damaging (or highly ROS generating) mitochondrial pathways to a newly formed organelle within the primitive eukaryotic cell.

Supporting the idea that the mitochondria contribute to the generation of peroxisomes *de novo* is the fact that many peroxisomal membrane proteins default to the mitochondrial outer membrane in patient fibroblast cells lacking peroxisomes (Sacksteder *et al*, 2000; South *et al*, 2000; Kim *et al*, 2006; Toro *et al*, 2009). This is not an exclusive pathway, as other peroxins target the ER under these conditions (Kim *et al*, 2006; Toro *et al*, 2009; Yonekawa *et al*, 2011), as in yeast. To date, this mitochondrial default pathway in mammalian cells has not been considered physiologically meaningful. However, this observation is consistent with the mitochondria as a contributor to peroxisomal biogenesis. Indeed, a recent study from the Erdman laboratory showed that a yeast mutant strain lacking Pex3 was rescued by ectopically targeting Pex3 to the mitochondria, resulting in the biogenesis of functional peroxisomes in cells that previously lacked these organelles (Rucktaschel *et al*, 2010). This indicates that the machinery exists to generate peroxisomes from mitochondria, even in yeast. We are currently exploring whether the retromer subunit Vps35 may bind to mitochondrial-localized peroxins like Pex3. Upon transfection of Pex3-GFP, some cells show high levels of mitochondrial targeting even when peroxisomes are functioning normally (Kim *et al*, 2006). It is also possible to test whether the retromer is required to generate new peroxisomes in patient fibroblasts upon rescue with the missing peroxin. Approaches like this should help define the functional contribution of MDV transport to peroxisomes.

What mitochondrial cargo do peroxisomes require that cannot be simply imported directly from the cytosol? These organelles share many metabolites, including heme, which is generated from iron sulfur clusters in the mitochondria and required for peroxisomal enzymes like catalase (Lazarow & de Duve, 1973; Stehling *et al*, 2014). Phospholipids like phosphatidylethanolamine, which is used to generate plasmalogens (Braverman & Moser, 2012), and perhaps even very long-chain fatty acids that may be misdirected to the mitochondria could be carried more easily within MDVs rather than via a soluble, cytosolic intermediate. With the identification of the retromer complex as requisite for this vesicle transport pathway, we can dissect the functional consequences on peroxisomal function when Vps35 is lost (Braschi *et al*, 2010). For example, if heme is a cargo, then loss of Vps35 may lead to oxidative stress within peroxisomes through catalase dysfunction. This would be particularly dangerous for neurons, since it is established that patients suffering from peroxisomal disorders present with a primarily neurological phenotype (Powers, 2001). Patients suffering from Zellweger syndrome carry mutations in core peroxisomal import proteins and therefore lack peroxisomes. The primary manifestation of the disease is a loss of myelination and neuronal cell death through the accumulation of very long-chain fatty acids and ROS production (Barry & O'Keeffe, 2013). Although primarily a disease of infants, an analysis of peroxisomal dysfunction in age-related diseases like PD is currently gaining momentum (Fransen *et al*, 2013). In general terms, it is clear that the mitochondria and peroxisomes are intimately linked and that the dysfunction of one can lead to the dysfunction of the other (Baumgart *et al*, 2001).

An alternative fate for MAPL-containing MDVs may be to a specialized functional population of peroxisomes. For example, it is possible that peroxisomes may not all be identical; some may specialize in bile acid synthesis, some in long-chain beta-oxidation, and others in plasmalogen synthesis. It is difficult to envision how the import machinery would select for a subset of functional enzymes. On the other hand, the delivery of an MDV to a peroxisome would change the content by supplying metabolite substrates or enzymes, thereby actively generating a functionally distinct class of peroxisome. Although some evidence supports the existence of different populations of peroxisomes in isolated tissue, identified by altered buoyant densities (Schrader *et al*, 1994), the functional implications of these different pools have not been established. Again, once the fusion machinery and cargo are identified, we can directly test these hypotheses.

Other fates for MDVs?

The fate of mitochondrial cargo entering the late endosome/multivesicular body is primarily to be degraded, since the inhibition of lysosomal proteases led to an accumulation of mitochondrial content (Soubannier *et al*, 2012a; McLelland *et al*, 2014). However, there may be other fates of this cargo. The multivesicular body can be routed to the cell surface, where its limiting membrane can fuse with the plasma membrane (Raposo & Stoorvogel, 2013). This leads to the release of its internal contents—intralumenal vesicles (exosomes), which can contain protein and microRNAs, as well as vacuolar proteases—that can have broad-ranging effects on neighboring cells; from microRNA-based reprogramming to cancer cell migration (Raposo & Stoorvogel, 2013). Proteomic analysis of exosome content, compiled within the "Vesiclepedia" (http://www.microvesicles.org) indicates that up to 10% of secreted proteins are mitochondrial (Choi *et al*, 2013; Burke *et al*, 2014), although it is not known whether this is functionally significant. Autophagosomes can also be a source of unconventional secretion, which can include a subset of leaderless cytosolic proteins (Zhang & Schekman, 2013), with some evidence of transit through the recycling endosome (Duran *et al*, 2010; Manjithaya *et al*, 2010). The most robust example of secreted autophagosomes occurs during erythrocyte development when the cytosolic organelles are ejected to form the red cell (Ney, 2011). MDV transport to the late endosome in steady state would predict the presence of selected mitochondrial content in exosomes under most conditions (Fig 2). Therefore, both mitophagy and MDV transport to the late endosome provide a means for the secretion of mitochondrial content from the cell.

The steady-state presence of mitochondrial content in exosomes may have some important physiological consequences. Under the conditions we have examined so far, we have shown that MDVs can contain at least three of the five complexes of the electron transport chain. Complexes III and IV both include subunits encoded by the mitochondrial DNA, which are translated with a formylated initiating methionine, conserved from bacterial translation (Kozak, 1983). The presence of extracellular formylated proteins or peptides can launch an immune response, whether of bacterial or mitochondrial origin (Zhang *et al*, 2010). This is because non-methylated DNA (Pollack *et al*, 1984) and formylated peptides bind and activate Toll-like receptor 9 leading to cytokine release and lymphocyte infiltration (Zhang *et al*, 2010). This pathway was shown to become activated within the endosome of cells lacking the lysosomal DNAseII (Oka *et al*, 2012). The subsequent accumulation of mtDNA within the autophagosome eventually escaped into the endosomal compartments, activating the receptors in the lumen of the endosome. Therefore, whether secreted through exosomes, or accumulated within the endosome, mitochondrial content has the potential to launch an inflammatory response.

Another possible link between MDVs, exosomes and disease may involve α-synuclein. α-synuclein is an aggregation-prone cytosolic protein important in the pathogenesis of sporadic PD. Moreover, mutations in α-synuclein, like those in Parkin and PINK1, lead to a familial form of PD, albeit a dominant iteration of the disease (Klein & Westenberger, 2012). Secretion and cell-to-cell transfer of α-synuclein has been shown both in cells and *in vivo* (Lee *et al*, 2004, 2005, 2008a,b; Desplats *et al*, 2009; Luk *et al*, 2012a,b), and exosomes constitute a proposed method of propagation (Emmanouilidou *et al*, 2010; Kong *et al*, 2014; Lee *et al*, 2014). While a functional role for α-synuclein has been proposed in the assembly of presynaptic SNARE complexes (Chandra *et al*, 2005; Burre *et al*, 2010; Diao *et al*, 2013), α-synuclein has also been shown to function at mitochondria. A number of studies have identified α-synuclein as present either within mitochondria (Cole *et al*, 2008; Devi *et al*, 2008), or localized to mitochondrial-associated membranes of the ER (Guardia-Laguarta *et al*, 2014) and have implicated it in binding to anionic and cardiolipin-containing membranes (Nakamura *et al*, 2011; Zigoneanu *et al*, 2012; Diao *et al*, 2013) and in promoting Drp1-independent fission (a hallmark of MDV formation) in cells (Kamp *et al*, 2010; Nakamura *et al*, 2011). Thus, the incorporation of α-synuclein into multivesicular bodies via MDVs represents an intriguing candidate mechanism for its exosomal secretion, with additional implications for PD.

Final thoughts

With advances in the mechanistic characterization of MDV transport, we hope the field will be encouraged to carefully examine these structures within various physiological paradigms. There are many lines of evidence supporting the importance of MDV transport in mitochondrial cell biology; from an unbiased evolutionary consideration of the process (Kulp & Kuehn, 2010; Jedelsky *et al*, 2011), to their potential roles in peroxisomal function (Neuspiel *et al*, 2008; Braschi *et al*, 2010) and mitochondrial quality control (Soubannier *et al*, 2012a,b; McLelland *et al*, 2014). MDVs can be observed by monitoring multiple mitochondrial markers by confocal microscopy, particularly in cells where Drp1 function is lost (Box 1). With the resulting hyperfused reticulum, these small vesicles are much more noticeable using high-resolution imaging techniques. Technically, there are still some limitations, primarily in the limited assortment of antibodies. There are approximately 1,000 proteins in the mitochondria, and we can only follow a handful using immunofluorescence approaches. In addition, we have noted that MDVs are somewhat fragile and not as easily fixed compared with the mitochondrial tubules (we routinely fix with 5–6% paraformaldehyde, for example). This likely reflects differences in lipid and protein composition within MDVs. In future studies, it will be important to examine the functional contribution of MDVs in

the physiology of specific tissues, particularly neurons, given the apparent links to Parkinson's disease.

The mitochondria are the most recent eukaryotic organelle from which vesicle transport has been observed. However, there is some irony in the knowledge that this process has clearly been conserved from the organelle's humble origins as archaebacteria. It leads us to wonder what other fascinating and unexpected cellular pathways are waiting to be discovered as we dig deeper into the mechanisms and meaning of mitochondrial vesicles.

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Conflict of interest

The authors declare that they have no conflict of interest.

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