The complex dance of organelles during mitochondrial division

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Abstract

Mitochondria are dynamic organelles that undergo cycles of fission and fusion events depending on cellular requirements. During mitochondrial division, the large GTPase Dynamin-related protein-1 (Drp1) is recruited to endoplasmic reticulum (ER)-induced mitochondrial constriction sites where it drives fission. However, the events required to complete scission of mitochondrial membranes are not well understood. Here, we emphasize the recently described roles for Golgi-derived PI(4)P-containing vesicles in the last steps of mitochondrial division. We then propose how trans-Golgi network (TGN) vesicles at mitochondria-ER contact sites and PI(4)P generation could mechanistically execute mitochondrial division, by recruiting PI(4)P effectors and/or the actin nucleation machinery. Finally, we speculate on mechanisms to explain why such a complex dance of different organelles is required to facilitate the remodelling of mitochondrial membranes.

Highlights

- TGN-vesicles are recruited to mitochondria-ER contacts and mitochondrial fission sites.

- PI(4)P-generated by the Arf1/PI(4)KIIIβ axis on TGN vesicles drives mitochondrial division downstream of the recruitment and activity of the main actor of fission, Drp1.

- TGN vesicles and lysosomes are found at ER-induced mitochondrial constrictions leading to 4-way organelle contact sites during mitochondrial membrane remodelling.

- The molecular mechanisms of how TGN vesicles containing-PI(4)P induce mitochondrial division is unknown. PI(4)P directly localized at the OMM or on TGN vesicles could recruit specific effectors that, subsequently, activate cytoskeleton-related machinery required to execute the final scission of mitochondrial membranes.

- Metabolic flux, particularly active lipid exchange and modification at mitochondria-organelle contacts sites could promote specialized microdomains that coordinate complex membrane remodelling events.

Mitochondrial dynamics

Mitochondria play a central role in cellular bioenergetics, housing hundreds of essential biochemical reactions[1]. However, the concept of mitochondria as a signalling **organelle (see Glossary)** has emerged, with mitochondria being implicated in numerous cellular functions[1]. Mitochondria have a characteristic double-membrane structure, with an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM), which encompasses and protrudes into the mitochondrial matrix, forming the so-called structure, cristae[2]. Mitochondria form an elaborate network in cells, which is constantly being remodelled by a tight balance of opposed mitochondrial division and fusion events, known as **mitochondrial dynamics**. Indeed, cells regulate their mitochondrial network from a tubular network to a hyperfused or fragmented morphology to adapt to the cellular metabolic state, and in response to cellular cues[3]. Mitochondrial fusion allows the efficient diffusion of metabolites, proteins and sharing of mitochondrial DNA (mtDNA) and is considered as a safeguard mechanism to enhance cell survival[3]. The molecular mechanisms and contribution of other organelles involved in mitochondrial fusion are summarized in ***Box 1***.

On the other hand, **mitochondrial division** appears to be involved in an increasing number of cellular functions including critical roles in mitochondrial quality control and the regulation of immunity[4]. Mitochondrial fission is a multi-step process regulated by the mitochondrial recruitment of the large cytosolic GTPase **Dynamin-related protein-1** (Drp1) interacting with OMM localised adaptor proteins such as mitochondrial fission factor (Mff)[5] and mitochondrial dynamics protein of 49 and 51kDa (MiD49/51)[6]. Drp1 is recruited to mitochondria-endoplasmic reticulum (ER) contact sites (MERCs), where it oligomerizes forming a ring-like structure around the mitochondrial tubule[7]. GTP hydrolysis drives a conformational change in Drp1 oligomers leading to a tight constriction of the tubule[8]. However, the steps occurring downstream of Drp1 oligomerization have been debated and remain elusive. Although *in vitro* reconstitution experiments suggest that Drp1 could mediate mitochondrial division[9], multiple publications in the last years have highlighted a more complex process *in cellulo*[10]. This raises the question as to how the fission process is completed. For example, it has been recently proposed that not only the ER but also multiple organelles, including lysosomes and **trans-Golgi network (TGN)** vesicles are also found at mitochondrial fission sites[11, 12]. In this opinion, we describe a recently identified role for TGN vesicles in this process and propose mechanisms as to how they could drive the last step

of division. Furthermore, we also discuss the functional contribution of multiple organelle contact sites in the fission process.

Mitochondria-organelle contact sites and mitochondrial division

Mitochondria form an elaborate network of organelle interactions within the cell by forming mitochondria-organelle contact sites (MOCS) with the ER (called mitochondria-ER contact sites, MERCs), peroxisomes, endosomes, lysosomes and lipid droplets (LDs)[13]. The architecture and functions of MERCs and other MOCS have recently been reviewed[13]. Here, we focus on the role of MOCS in mitochondrial division, which has been recently put to the forefront of mitochondrial dynamics research.

Membrane contact sites (MCS) between the ER and mitochondria are essential during the initial steps of the fission process. MERCs not only induce Ca^{2+}-dependent IMM constriction[14, 15], but they also initiate the constriction of mitochondrial membranes, at sites of mtDNA replication[16]. Indeed, with the Inverted-formin 2 (INF2) at the ER and Spire1C at the OMM, MERCs serve as a scaffold for cytoskeletal components to assemble and to generate actin cables, thought to provide the mechanical force required to induce initial membrane constriction and mark the sites for Drp1 recruitment [17-19]**(Figure 1)**. Recently, several studies have highlighted an intricate functional interconnection between mitochondria and lysosomes[20]. Indeed, lysosomes establish dynamic MCS with mitochondria, which also mark sites of mitochondrial fission[11]. The release of MCS between mitochondria and lysosomes occurs following Rab7 GTP hydrolysis, mediated by Fis1-dependent recruitment of the Rab7 GTPase activating protein TBC1D15 to mitochondria, a process required for mitochondrial division[11] **(Figure 1)**. Moreover, Rab7 seems to be involved in the tethering of both organelles, not only for mitochondrial fission but also to regulate the communication and the spatio-temporal distribution of both organelles within the cell[21]. These dynamics may provide increased membrane plasticity required for membrane remodelling driven by Drp1 and other downstream factors.

Although essential for mitochondrial homeostasis, the role of other organelles in mitochondrial fission is still poorly understood. Peroxisomes and LDs establish MCS and work in synergy with mitochondria to carry-out processes such as lipid metabolism, β-oxidation or

steroid synthesis[22, 23]. To date, a direct role for LDs in mitochondrial fission has not been proposed. While peroxisomes share some of the mitochondrial division machinery[24], recent studies have shown that deficiencies in certain peroxisomal proteins induce mitochondrial fragmentation in a Drp1-dependent manner, suggesting that peroxisomes may also regulate mitochondrial fission[25]. Plasmalogens derived from peroxisomes were also shown to be required for cold-induced mitochondrial fission, suggesting a functional role for lipid exchange between peroxisomes and mitochondria[26]. Finally, while it has been reported that perinuclear mitochondria establish MCS with the Golgi apparatus to regulate Ca^{2+} gradients in pancreatic cells[27], their contribution to mitochondrial division is unknown.

Unveiling the secrets of the late steps of mitochondrial division

Drp1 oligomerizes in a spiral-like structure whose minimum inner diameter has been shown down to 15 nm[28, 29]. Whether this constriction is enough to trigger both outer and inner membranes fission is debated, but it has been proposed that additional factors may be required to execute the final stage of this process. Recently, the endocytic-related GTPase dynamin 2 (Dyn2) has been suggested to trigger these final events[30]. At the plasma membrane (PM), the GTPase-dependent membrane scission activity of Dyn2, together with actin polymerization, allows the pinching of endocytic cargoes[31]. Since actin polymerization is also involved in mitochondrial dynamics[32], it has been hypothesized that the physical principles regulating both endosomal and mitochondrial fission could be analogous. In addition, a Drp1 isoform can localize to the PM and endosomal compartments, further suggesting similarities between both fission mechanisms[33]. Indeed, it has been proposed that Dyn2 activity, with its intrinsic membrane constricting properties, was the final step of mitochondrial membranes scission[30]. In the original study, not only fluorescently tagged Dyn2 was observed to be recruited at ER-induced mitochondrial fission sites by confocal live-cell imaging, but silencing of Dyn2 by siRNA in HeLa and Cos-7 cells was also associated to mitochondrial hyperfusion and resistance to cell death, similar to Drp1-depleted cells[30]. However, it has been recently proposed that the absence of Dyn2 does not lead to a dramatic mitochondrial hyperfusion phenotype in all cellular models tested suggesting that Dyn2 function is not essential for the mitochondrial fission process[9, 12, 34]. In these new studies, it has been reported that Dyn2 knock-out (KO) or Dyn1-Dyn2-Dyn3 triple KO mouse embryonic fibroblasts (MEFs) and HeLa cells do not exhibit a drastic mitochondrial elongation

phenotype, neither the number of observed fission events were decreased[9, 34]. Nevertheless, it is still possible that Dyn2 function in mitochondrial division can be cell-type dependent or required only in response to certain conditions. It has been recently suggested that different Drp1-associated machineries could regulate mitochondrial fission depending on the fate of the dividing mitochondria[35]. Therefore, it could be hypothesized that there is not one unique pathway driving the last steps of mitochondrial division. Finally, an alternative pathway based on TGN vesicles-mitochondria contact sites and **phosphatidylinositol 4-phosphate (PI(4)P)** signalling has been proposed to facilitate these final steps of mitochondrial division[12].

The Arf1/PI(4)KIIIβ axis at the TGN

ADP-ribosylation factor-1 (Arf1) belongs to the family of Arf small GTPases of the RAS superfamily, which is composed of six members. Arfs mediate membrane remodelling and vesicle formation by controlling the recruitment of protein effectors to multiple membranes[36]. At the Golgi apparatus, the GTP-bound form of Arf1 regulates formation of either COPI vesicles at the cis-Golgi or TGN vesicles at the trans-Golgi network[36]. At the TGN, Arf1 recruits the PI(4)P kinase, **phosphatidylinositol 4-kinase IIIβ (PI(4)KIIIβ)**, which phosphorylates phosphatidyl inositol (PI) forming PI(4)P (see ***Box 2***), facilitating vesicle formation and subsequent release[37]. In addition, multiple PI(4)P-binding proteins are recruited to TGN membranes where they coordinate the budding and scission of these vesicles[38]. Therefore, both Arf1 and newly synthesized PI(4)KIIIβ-dependent pools of PI(4)P are essential components of the machinery required for the generation, scission and targeting of TGN vesicles to other cellular compartments.

New roles for Arf1/PI(4)KIIIβ-generated PI(4)P on TGN vesicles during mitochondrial division

Although Arf1 and PI(4)KIIIβ have not been directly localized to mitochondria, previous studies have shown a role for Arf1 in the regulation of mitochondrial dynamics. Indeed, deficiencies in Arf1 or its effector Golgi-specific brefeldin A resistance guanine nucleotide exchange factor-1 (GBF1) lead to mitochondrial morphology defects in mammals, *C. elegans* and *S. cerevisiae*, and have been mechanistically associated to aberrant clusters of the yeast mitofusin, Fzo1, accumulating at mitochondria[39]. More recent work has also identified the mitochondrial kinesin adaptor, Miro1, as a GBF1-interacting protein, which

contributes to the spatial positioning of mitochondria[40]. Finally, the specific role for PI(4)P generated by the Arf1/PI(4)KIIIβ axis in mitochondrial division has been recently shown, where the silencing of either Arf1 or its effector, PI(4)KIIIβ, leads to a drastic mitochondrial elongation phenotype[12]. In this context, a recent publication has confirmed the pro-fission activity of PI(4)KIIIβ in *Drosophila*[41]. These data suggest an essential and evolutionarily conserved role for PI(4)KIIIβ in mitochondrial dynamics regulation.

Mechanistically, it has been shown that PI(4)P is required downstream of Drp1 recruitment to terminate mitochondrial division. Mitochondrial elongation induced by PI(4)P loss was neither due to a defect in Drp1 recruitment to mitochondria, nor increased fusion activity, and was resistant to stress-induced fragmentation. Inhibition of Arf1 and PI(4)KIIIβ resulted in a hyperfused phenotype characterized not only by mitochondrial elongation, but also by extreme mitochondrial interconnectivity and branching[12]. Indeed, while Drp1-depleted cells display few but very elongated mitochondria, loss of Arf1 and PI(4)KIIIβ induced a significant increase of the number of junctions and branches emerging from the mitochondrial network compared to control and Drp1-silenced cells, suggesting that both machineries mediate different steps of mitochondrial division[12]. However, the mechanism regulating this particular mitochondrial network is currently unknown. Arf1/PI(4)KIIIβ deficiency also induced the formation of mitochondrial super-constrictions sites, characterized by a long and narrow neck closely apposed to ER tubules where both IMM and OMM could be observed by transmission electronic microscopy. These results indicate that Arf1 and PI(4)KIIIβ regulate mitochondrial fission downstream of Drp1-driven mitochondrial constriction[12]. Using quantitative live-cell imaging, more than 70% of mitochondrial fission events were shown to be positive for Arf1. Importantly, Arf1 was recruited to MERCs on TGN vesicles downstream of Drp1, but just prior to division, thus revealing the existence of a 4-way contacts between mitochondria, TGN, ER and lysosomes[12]. Exploiting artificial probes to detect intracellular PI(4)P pools, it was confirmed that TGN vesicles enriched in PI(4)P were recruited to ER-induced mitochondrial constrictions and fission events downstream of Drp1[12]. Although it has been proposed that TGN-PI(4)P vesicle recruitment are Drp1-dependent[12], how the PI(4)P-loaded TGN vesicles are recruited to the mitochondrial fission site is unknown.

Some components of the mitochondrial fission machinery, Drp1 and its receptor Mff, are also involved in peroxisomal division[24]. Peroxisome morphology was not affected in the

absence of Arf1 or PI(4)KIIIβ[12] suggesting either that the recruitment of TGN vesicles is not required for the scission of single membrane bound organelles, or perhaps reflecting additional signalling roles or metabolic flux requirements for this contact in regulating mitochondrial dynamics. As mentioned, *in vitro* approaches have proposed that Drp1 activity is enough to execute membranes scission[9]. In this sense, it could be hypothesized that Drp1 could be sufficient to mediate peroxisomal, but not mitochondrial fission. This could be explained by the different lipid membrane composition and availability of both organelles, raising the possibility that the generation of PI(4)P pools may be exclusively required for mitochondrial division.

Regarding the role of PI(4)P in mitochondrial fission, other reports have highlighted the importance of **phosphoinositides** in mitochondrial dynamics. For example, it has been reported that the PI(5)P phosphatase, synaptojamin2 (Synj2), could be recruited to the OMM by interacting with OMP25, whose overexpression leads to changes in mitochondrial morphology and distribution, presumably by altering OMM phosphoinositide homeostasis[42]. It has also been shown that the loss of mitochondrial pools of $PI(4,5)P_2$, achieved by targeting the phosphatase Skip to the OMM, promotes mitochondrial fragmentation[43]. In this context, it can be hypothesized that the hydrolysis of $PI(4,5)P_2$ by Skip leads to PI(4)P generation and enhancement of mitochondrial division.

Together, these studies emphasize the intimate and functional interplay between different organelles and add a further layer of complexity to mitochondrial division. However, the precise molecular mechanism by which Arf1/PI(4)KIIIβ on TGN vesicles regulates the late stages of the fission process is still unknown. In the next paragraphs, we will postulate potential mechanisms that could ensure mitochondrial division. We propose that two sequential steps may be required: 1) generation of transient PI(4)P pools on TGN vesicles or on the OMM, and 2) the recruitment of PI(4)P-effectors and/or actin-related proteins to mechanically drive the scission of mitochondrial membranes.

Mitochondria and PI(4)P trafficking

To date, stable PI(4)P pools have not been documented at mitochondria, although the presence of its precursor PI has been described[44, 45]. Recent studies have identified different PI(4)K isoforms at mitochondrial membranes suggesting that minor and fluctuating PI(4)P

pools could be synthesized[12, 46]. In fact, rapamaycin-induced recruitment of the PI(4)KIIIα catalytic fragment to the OMM results on PI(4)P production further confirming the capacity of mitochondrial membranes to sustain PI(4)P generation[44].

The Arf1/PI(4)KIIIβ complex transported on TGN vesicles could be responsible for PI(4)P pools at the OMM **(Figure 2A)**. It has been shown that a small, but detectable proportion of PI(4)KIIIβ can be detected in pure isolated mitochondrial fractions[12]. Such a small amount of PI(4)KIIIβ on mitochondrial membranes suggest a labile and transient association that would be only required during the fission event. Once at the OMM, PI(4)KIIIβ could synthesize discrete but functional PI(4)P pools to drive the final stages of mitochondrial division. In this connection, a similar mechanism has been recently described during autophagy. Indeed, Atg9-Arfaptin2-PI(4)KIIIβ positive vesicles traffic from TGN to the autophagosome formation site to deliver PI(4)KIIIβ to pre-autophagosomal membranes and thus facilitate PI(4)P synthesis during autophagosome biogenesis[47]. Alternatively, PI(4)P could traffic directly to mitochondria by *non-vesicular* mechanisms driven by lipid transfer proteins (LTPs) and lipid gradient between two membranes[48] **(Figure 2A)**. Therefore, the PI(4)P-enriched microenvironment found at TGN membranes make TGN vesicles suitable candidates to deliver PI(4)P to other organelles, including mitochondria, that act to increase or alter membrane curvature and recruit specific lipid protein effectors. Also, PI(4)P-enriched TGN vesicles could form ternary complexes with other organelles, such as the ER, in order to regulate phospholipid trafficking at MOCS (discussed in ***Box 2***).

PI(4)P-dependent recruitment of membrane-remodelling effectors to fission sites

PI(4)P-dependent recruitment of mechanical effectors is well characterized in organelle shaping and vesicle-scission[38]. Even if the mitochondria cannot be considered a PI(4)P enriched organelle, it can be hypothesized that transient PI(4)P pools directly at the OMM or at TGN vesicles could be sufficient to recruit specific effectors to execute mitochondrial division.

An interesting PI(4)P effector candidate is Golgi phosphoprotein-3 (GOLPH3), also named MIDAS, or VPS74 in yeast, a peripheral TGN protein controlling Golgi architecture and anterograde trafficking[49]. GOLPH3 is a PI(4)P effector[50] and once recruited,

GOLPH3 inserts into membranes inducing a high membrane curvature that promotes the scission and separation of vesicles from the TGN[51]. GOLPH3 shuttles between Golgi and mitochondrial membranes[52], further suggesting the existence of PI(4)P pools at the OMM. Moreover, GOLPH3 modulates mitochondrial mass by regulating mitochondrial lipid synthesis, indicating a direct role of this protein in mitochondrial homeostasis[52]. While at the TGN GOLPH3 requires Myosin-18a to drive the final scission event[53], the non-muscular Myosin II has been involved in mitochondrial division[19, 54]. Therefore, the GOLPH3/myosin complex represents an interesting pathway, which could regulate PI(4)P-dependent mitochondrial membrane remodelling **(Figure 2B)**. Another PI(4)P effector on mitochondrial membranes is the small GTPase Rab11, which is also recruited to PI(4)P-enriched membranes where it regulates the final step of membrane scission during vesicle release[55]. Rab11 has also been found at mitochondrial fission sites where it re-organizes the actin cytoskeleton and regulates Drp1 trafficking[56]. Thus, future investigations are required to elucidate the molecular contribution of these and others PI(4)P effectors during the last stage of mitochondrial division **(Figure 2B)**.

PI(4)P-dependent recruitment of the actin-related machinery to fission sites

The polarized forces generated by the actin machinery serves to regulate the tension of cellular membranes resulting in membrane deformation and scission[57]. Most of the PI(4)P effectors, as described for GOLPH3, require the cytoskeleton machinery to mechanically terminate membrane scission[53]. For example, it has been proposed that endosomal PI(4)P is able to recruit the Wiskott-Aldrich syndrome protein and scar homolog (WASH) complex to sorting endosomes, where in combination with Arp2/3-complex-dependent **actin nucleation**, it regulates the scission and segregation of endosomal cargoes, specifically at ER-endosome contact sites[58]. The localization of the PI(4)P synthesizing machinery at MERCs suggests that a similar mechanism may also occur during mitochondrial division[12]. Thus, mitochondrial or TGN-PI(4)P could serve as a signaling molecule to mediate the recruitment of actin-nucleating proteins to the mitochondrial fission site. A dynamin-independent endocytic pathway has also been highlighted and reported to be largely dependent on Arf1[59]. Indeed, Arf1 can directly orchestrate membrane scission via CDC42 and N-WASP-dependent actin polymerization[60]. Thus, it is tempting to speculate that Arf1/PI(4)KIIIβ-generating PI(4)P pools could regulate actin polymerization to accomplish mitochondrial scission **(Figure 2B)**. In accordance with this hypothesis, silencing of Arp2/3 and other actin-nucleating

regulators leads to a similar phenotype of PI(4)P pool depletion, characterized by not only mitochondrial hyperfusion but also branching, with no decrease in mitochondrial Drp1 recruitment[61].

Therefore, it can be proposed that the transient generation of PI(4)P pools at TGN vesicles could be involved in the recruitment of effectors, which working together with Arp2/3-dependent actin nucleation, execute the final scission of mitochondrial membranes **(Figure 3)**.

Multiple organelles at the mitochondrial fission sites

MCS have added another layer of complexity to the mitochondrial division process. While the role of MERCs is well established (i.e. actin recruitment, **lipid trafficking**, Ca^{2+} homeostasis), the mechanistic contribution of the other organelles in mitochondrial division is not fully understood. Several studies based on advanced microscopy techniques have described the promiscuity of mitochondrial interactions with different endomembranes, highlighting the existence of ternary or even quaternary organelle complexes[62, 63]. Indeed, MCS consisting of ER, mitochondria, Golgi, peroxisomes and LDs have been observed and quantified[62]. However, while the physiological role and the mechanisms underlying the formation of MCS to drive mitochondrial division remain unknown, MCS could provide a functional platform allowing the propagation of signals, which may coordinate responses to particular cellular cues, such as organelle remodelling events[64].

It can be speculated that MOCS could serve as hotspots for lipid trafficking. Organelle-shaping processes imply the generation of new membranes or the biophysical adaptation of pre-existing ones requiring intrinsic lipid biosynthetic capability. Therefore, newly synthesized lipids at the ER must be delivered to different endomembranes, where they are converted into distinct species to preserve organelle identity. For example, phosphatidic acid (PA) transported from the ER to mitochondria is used as a precursor for cardiolipin (CL) biosynthesis, with both phospholipids regulating mitochondrial morphology[65, 66]. It has recently been suggested that lipid trafficking at MCS is mediated by VPS13 proteins, a group of LTPs[67]. Interestingly, both VPS13A[68] and VPS13D[69] have been involved in mitochondrial dynamics regulation, further suggesting that lipid trafficking at MOCS could modulate mitochondrial morphology. PDZD8, a Synaptotagmin-like Mitochondrial lipid-binding

Proteins (SMP) containing protein, which localizes at multiple MCS[70, 71], has recently been involved in the generation of three-way contacts between ER, lysosomes and mitochondria[72], and represents another interesting candidate. Finally, the members of OSBP-related proteins (ORPs), ORP5 and ORP8, have also been found at multiple contact sites between the ER and the PM[73], endosomes[74], LDs[75] and mitochondria[76], where they can control mitochondrial architecture and function[76]. Together, this suggests the existence of specialized microdomains where LTPs can be rapidly recruited to mediate inter-organelle lipid trafficking required for organelle membranes remodelling.

MOCS could serve as specialized platforms not only for lipid trafficking, but also for ion signalling such as Ca^{2+}. Ca^{2+} fluxes at MCS have been well defined, particularly at MERCs where Ca^{2+} transfer to mitochondria induces IMM constriction and mitochondrial division[14, 15]. However, lysosomes are also intracellular Ca^{2+} stores[77]. It has been recently reported that Ca^{2+} can be directly transported from lysosomes to mitochondria at MCS[78]. Since lysosomes, ER and mitochondria are involved in three-way contacts and due to the roles of both the ER and lysosomes in mitochondrial division, it would be interesting to analyse if coordinated Ca^{2+} transfer from both organelles regulate IMM constriction to initiate mitochondrial division[14, 15]. Otherwise, it can be hypothesized that proton transfer between lysosomes and mitochondria could modulate mitochondrial membrane remodelling by regulating mitochondrial depolarization[79].

MOCS could also be assimilated as microdomains where membrane remodelling events of multiple organelles are mechanistically and spatio-temporally coupled within the cell. For example, it has been reported that both endosomal and mitochondrial fission events could be mechanistically related. Indeed, the retromer subunit VPS35 plays a role during mitochondrial dynamics by regulating the levels of fission and fusion factors at the OMM[80, 81]. Translocation of VPS35 from endosomes to the mitochondria requires an early endosomal fission event driven by the endosomal proteins rabankyrin-5 and EHD1[82]. Knockdown of VPS35 or EHD1 leads to mitochondrial elongation suggesting a functional interconnection between both endosomal and mitochondrial fission events[82]. Thus, the functional cross-talk between the remodelling machineries of different organelles at MOCS should be addressed to fully understand the coordinated regulation of membrane organelle dynamics**.**

Finally, most endomembranes require a pre-fission step to facilitate the engulfment of the damaged organelle by autophagic structures. Therefore, it can be hypothesized that organelle communication at MOCS could be crucial to modulate organelle quality control. Indeed, not only MERCs have been involved in mitophagy[83], but endosomal Rabs can also be targeted to mitochondria during Parkin-dependent mitophagy[84] and damaged mitochondria can be sequestered into Rab5-positive early endosomes before lysosomal degradation[85]. Moreover, recent studies have shown the contribution of Golgi membranes during mitophagy where they could serve as a donor membrane for autophagosomes[86] or be required for the transport of the mitophagy receptor Optineurin to damaged mitochondria in order to regulate mitochondrial lipid remodelling[87].

Concluding remarks

While the role of Drp1 and the related machinery during mitochondrial division has been widely described, the molecular mechanisms underlying the final scission steps are still under debate. A new mechanism has described the roles for Golgi-derived PI(4)P-vesicles driving the last steps of mitochondrial division. In this opinion, we have highlighted TGN vesicles as signaling platforms that could ensure PI(4)P availability and/or the recruitment of membrane remodelling effectors together with the actin-related machinery to execute mitochondrial membranes scission. Further experiments based on proteomic analysis will be required to elucidate the interactome at the level of the TGN vesicle and mitochondrial interface. It will be essential to elucidate the identity of PI(4)P effectors and the downstream events to mechanistically understand how the final scission event is achieved.

The colocalization of TGN vesicles with lysosomes at MERCs[12] emphasizes the importance of understanding how inter-organelle communication drives mitochondrial division, and the contribution of lipid and metabolite fluxes in these events. Since mitochondria share part of the fission machinery with other endomembrane systems, it will also be interesting to determine if membrane fission of different organelles could be spatio-temporally coordinated. To address these questions, biochemical methods to induce MOCS[88] coupled to high-resolution microscopy[62, 63] will shed light on the physio-temporal dynamics underlying the remodelling and fission of the different cellular compartments.

Outstanding questions

- How are TGN vesicles recruited to mitochondrial fission sites? Does a tethering complex exist between TGN vesicles and mitochondria?

- Does PI(4)P generation imply the translocation of the ARF1/PI(4)KIIIβ complex from the TGN to the OMM or is PI(4)P transported directly to the mitochondria by *non-vesicular* trafficking from other organelles?

- Which and how are PI(4)P effectors involved in the final mitochondrial scission event? Are PI(4)P and its effectors required to recruit the cytoskeletal machinery during the late steps of mitochondrial division?

-Why are multiple organelles localized at the mitochondrial fission site? What is the precise sequence of organelle recruitment during mitochondrial division?

-Do MOCS define specialized cellular regions for inter-organelle communication? What is the physiological relevance of MOCS? Could the loss or gain of function of one type of contact at MOCS affect the others?

Text boxes

Box 1 Mitochondrial fusion at MCS

Mitochondrial fusion is a two-step mechanism, whereby each membrane undergoes an exclusive fusion event[89]. During the first step of mitochondrial fusion, the dynamin GTPases Mitofusin-1 and -2 (Mfn1/2) engage in an *in trans* homo- or hetero-typic interaction, tethering the opposing OMMs together. Subsequent conformational changes of MFNs and GTP hydrolysis lead to the OMM fusion, forming a contiguous compartment[89]. IMM fusion occurs downstream of OMM fusion and is regulated by the heterotypic interaction between the dynamin-like GTPase OPA1 and cardiolipin in opposing membranes, which requires OPA1-driven GTP hydrolysis[89]. In this sense, it is also well established that IMM fusion depends on the balance between both short (pro-fission) and long (pro-fusion) OPA1 isoforms[90].

Moreover, it has been proposed that OPA1 requires Mfn1 to drive IMM fusion suggesting that the fusion of both membranes are functionally connected[91].

Recent publications have highlighted that, similarly to mitochondrial fission, mitochondrial fusion also occurs at MERCs[63, 92]. The presence of ER tubules enhance the mitochondrial fusion activity, likely by stabilizing the opposing mitochondria, restricting the motility and therefore accelerating the fusion of both membranes[60, 63]. Moreover, it has been recently reported that the main actors of both the fission and fusion machineries colocalize at MERCs, further emphasizing MOCS as hotspot platforms for efficient mitochondrial membranes remodelling[92]. In addition, state of the art super-resolution live microscopy techniques detailed that mitochondrial fusion events can be stimulated by motile late endosomes/lysosomes forming MCS with mitochondria, which leads to collisions with distal mitochondria and induces fusion[63]. Thus, the functional interaction between mitochondria and other organelles during mitochondrial fusion is an emerging field that will require further attention in the coming years.

Box 2 PI and PI(4)P transport at MCS

Phosphoinositides are a family of phospholipids in cellular membranes that are involved in many cellular processes including recruitment of proteins, membrane remodelling or lipid exchanges. Although phosphoinositides are not major components of mitochondrial membranes, PI and PI(4,5)P2 have been identified at mitochondria[44, 45]. Nevertheless, even if mitochondrial PI(4)P pools have not yet been described, they can be induced by the artificial recruitment of the PI(4)KIIIα catalytic domain, confirming that mitochondrial PI can serve as precursor for PI(4)P synthesis at the OMM[44]. Although the exact mechanism by which PI reaches the mitochondria is unknown, it can be hypothesized that PI-transfer proteins (PITP) could facilitate PI trafficking from the ER to the OMM, as described at ER-PM[93] and ER-TGN MCS[94]. Alternatively, it has been also suggested that ER-derived vesicles enriched in the PI synthesizing enzyme PIS could supply PI to multiple organelles including mitochondria by *kiss-and-run* events[95].

Non-vesicular trafficking of lipids between mitochondria and other organelles has been largely documented. For example, the trafficking of PtdSer and PA from the ER to the mitochondria is well established in both yeast and mammals[96]. The transport of cholesterol from endosomes to mitochondria through START-domain containing proteins has been also reported[97]. Therefore, it is also plausible that PI(4)P could directly traffic to mitochondrial

membranes from other closed organelles at MCS. In this sense, several proteins from the oxysterol-binding proteins (OSBP) and OSBP-related proteins (ORPs) family have been shown to counter-exchange PI(4)P with other phospholipids between multiple endomembranes including ER-endosome[58, 74], ER-PM[73], ER-TGN[98] and ER-LD[75]. OSBP/ORPs proteins harbour a pleckstrin homology (PH) domain which interacts with PI(4)P in the donor membrane and a FFAT motif which binds to VAP proteins at the ER[99]. Once recruited to MCS, OSBP/ORPs extract PI(4)P lipid monomers from the donor membrane and transport them in hydrophobic pockets to the acceptor membrane in exchange to other lipids. Interestingly, some members of these families have been detected at the mitochondrial interface in different mitochondrial datasets[46]. Therefore, it can be hypothesized that PI(4)P could be transfer directly from TGN vesicles to the mitochondria by an unidentified machinery. Alternatively, the enriched proportion of PI(4)P in TGN vesicles and their localization at MERCs raise the possibility that TGN membranes could participate in a ternary complex with the ER to transfer PI(4)P to the mitochondria **(Figure 2A)**. Finally, a physical localization of the ORPs members, ORP5 and ORP8, has been recently documented at MERCs to regulate mitochondrial architecture and PtdSer transfer to the mitochondria[76, 100]. Therefore, it is plausible to think that MERCs-localized ORPs could transfer back the putative mitochondrial PI(4)P pool to the ER in exchange of PtdSer.

Glossary Box

Actin nucleation: The polymerisation of soluble actin monomer forming actin filaments, which can have multitude of geometries to modulate membrane shape and promote membrane deformation.

ADP-ribosylation factor-1 (Arf1): A small GTPase of the Ras superfamily of GTPase, which is involved in membrane remodelling events such as the generation of COPI-coated vesicles or TGN vesicles.

Dynamin-related protein-1 (Drp1): A large GTPase of the dynamin family, which oligomerizes in ring-like structures around different endomembranes, leading to their constriction and facilitating membrane scission events.

Lipid trafficking: Inter-organelle lipid distribution can occur through both *vesicular* or *non-vesicular* mechanisms. While *vesicular* trafficking requires the formation and targeting of protein-coated vesicles to the receptor organelle, *non-vesicular* trafficking relies on LTPs, which use the driving force of lipid gradients at MCS to mediate lipid transport between different organelles.

Membrane contact sites (MCS): The close juxtaposition (typically 30-50 nm) of two opposing membranes generating a platform that favours the exchange of molecules without membrane fusion.

Mitochondrial dynamics: The modulation of the mitochondrial network through fission and fusion processes to control mitochondrial number, area and length.

Mitochondrial division: The multi-step process of dividing a mitochondrion into two daughter mitochondria.

Organelle: Membranous compartment found within eukaryotic cells, which compartmentalize biochemical reactions to coordinate their specific functions.

Phosphoinositide: An acidic phospholipid present in cellular membranes, which can be modified via phosphorylation or dephosphorylation of its inositol headgroup, generating a variety of products.

Phosphatidylinositol 4-kinase IIIβ (PI(4)KIIIβ): A lipid kinase predominately localized to the Golgi apparatus. It phosphorylates the 4th hydroxyl group of the inositol headgroup of PI forming PI(4)P.

Phosphatidylinositol 4-phosphate (PI(4)P): The most abundant monophosphorylated PI with a major role in Golgi homeostasis. In addition, PI(4)P works like a signalling molecule that recruit multiple effectors to distinct organelles to regulate membrane morphogenesis.

Trans-Golgi network (TGN): The terminal body of the Golgi apparatus, which regulates the packaging and sorting of vesicles to destined membranes (including lysosomes and the PM) or to extracellular secretion (exocytic process).

Figures and legends

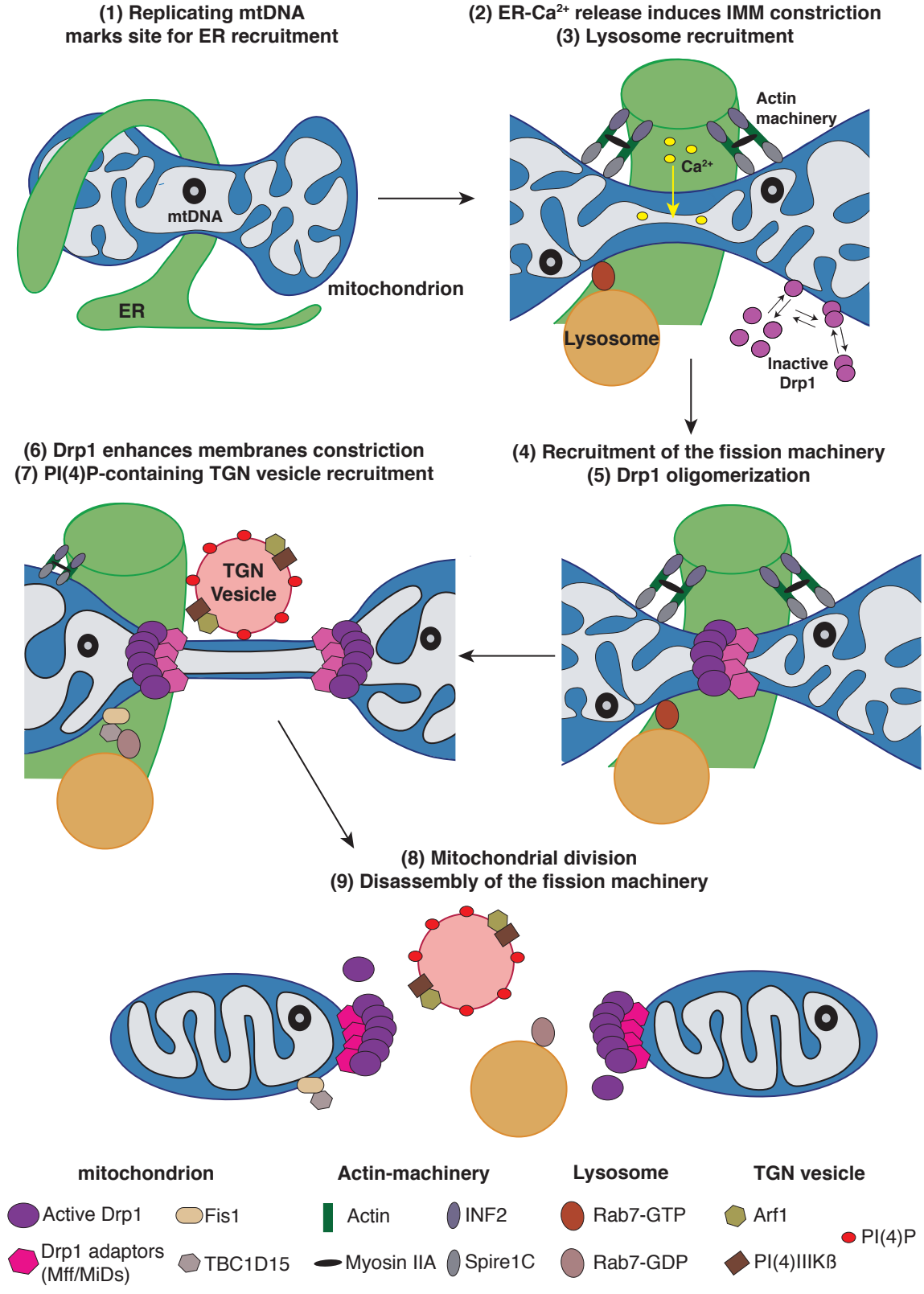

Figure 1: Proposed model for mitochondrial division.

(1) mtDNA replication marks the site for the recruitment of the endoplasmic reticulum (ER). (2) At these sites, the ER-bound inverted-formin 2 (INF2) and mitochondrial Spire1C establish actin cables between the two organelles, with Myosin II allowing actin contraction, to provide the mechanical force to induce mitochondrial pre-constriction. ER-Ca^{2+} release into mitochondria via mitochondrial calcium uniporter leads to inner mitochondrial membrane (IMM) constriction upstream of Dynamin-related protein-1 (Drp1) recruitment. (3) Lysosomes are recruited and tethered to mitochondria by Rab7 in a guanosine triphosphate (GTP)-bound state. If Ca^{2+} release from lysosomes to mitochondria contributes to IMM constriction is unknown. In parallel, Drp1 monomers and oligomers are in balance between the cytosol and mitochondria. (4) At ER-induced mitochondrial pre-constriction sites, the adaptors of Drp1, mitochondrial dynamics proteins, MiD49 and MiD51, and mitochondrial fission factor (Mff), can accumulate, and (5) recruit Drp1, which oligomerizes in a ring-like structure around the mitochondrial tubule. (6) Upon GTP hydrolysis, Drp1 changes conformation and enhances mitochondrial constriction. (7) Then, trans-Golgi network (TGN) vesicles containing the kinase phosphatidylinositol 4-kinase IIIβ (PI(4)KIIIβ), the GTPase ADP-ribosylation factor-1 (Arf1) and their lipid product, phosphatidylinositol 4-phosphate (PI(4)P), are recruited to extended mitochondrial constriction sites, which lead to (8) membranes scission and the generation of two new mitochondria. (9) Finally, the fission machinery disassembles, with Mff and Drp1 remaining at mitochondrial tips, the release of TGN vesicles, and the untethering of lysosomes from mitochondria, which is mediated by the recruitment to mitochondria of TBC1 domain family member 15 (TBC1D15) by mitochondrial fission 1 protein (Fis1), driving lysosomal Rab7 GTP hydrolysis.

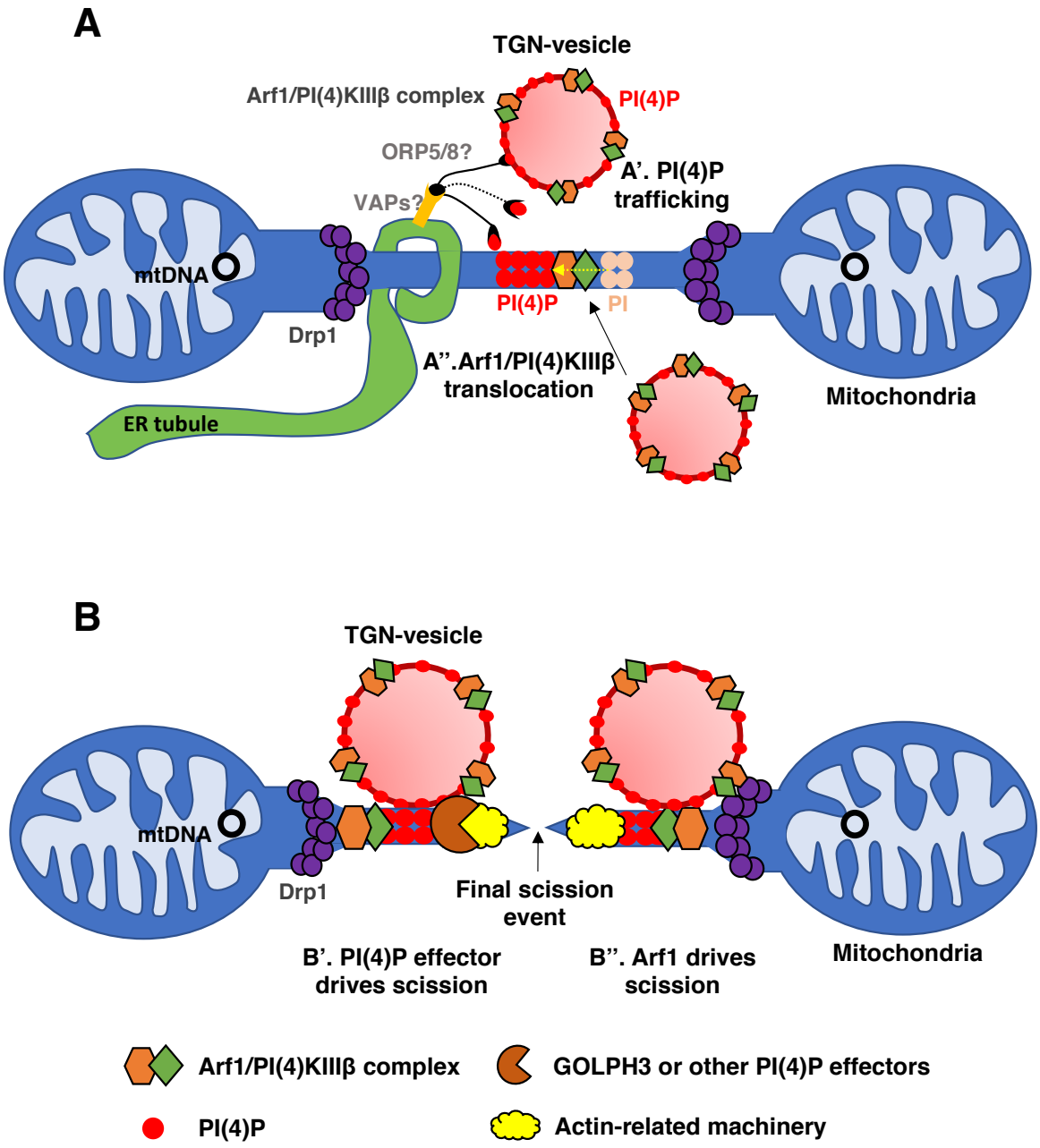

Figure 2. Proposed models for potential PI(4)P localization on mitochondrial membranes and mechanisms regulating final membranes scission.

(A) Two mechanisms to explain the potential transient localization of mitochondrial phosphatidylinositol 4-phosphate (PI(4)P) are depicted. **(A')** PI(4)P-enriched trans-Golgi network (TGN) vesicles could serve as PI(4)P reservoirs/donors for mitochondrial membranes at mitochondria-ER contacts (MERCs). OSBP-related proteins (ORPs) could facilitate PI(4)P trafficking from TGN-membranes to the outer mitochondrial membrane by forming a ternary

complex with VAP proteins at ER membranes. **(A'')** Residual mitochondrial phosphatidylinositol 4-kinase IIIβ (PI(4)KIIIβ) or transferred PI(4)KIIIβ from TGN vesicles to mitochondria could use mitochondrial PI to synthesize transient PI(4)P pools required during mitochondrial division. **(B)** TGN vesicles-containing PI(4)P at MERCs or mitochondrial PI(4)P could **(B')** recruit different effectors leading to actin nucleation, or **(B'')** promote Arf1-mediated actin polymerization, which both could generate the mechanical forces required to stretch the mitochondrial membranes and execute the final scission event.

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Competing interest

The authors declare that there are no competing interests.

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