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Lipid Signaling on the Mitochondrial Surface

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Summary

Regulated production and elimination of the signaling lipids phosphatidic acid (PA), diacylglycerol (DAG), and phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$) creates a complex and interconnected signaling network that modulates a wide variety of eukaryotic cell biological events. PA production at the plasma membrane and on trafficking membrane organelles by classical Phospholipase D (PLD) through the hydrolysis of phosphatidylcholine (PC) has been studied widely. In this chapter, we review a newly identified, non-canonical member of the PLD superfamily, MitoPLD, which localizes to the mitochondrial surface and plays a role in mitochondrial fusion via the hydrolysis of cardiolipin (CL) to generate PA. The role of PA in facilitating the mitochondrial fusion event carried out by proteins known as Mitofusins is intriguing in light of the role classic PLD-generated PA plays in facilitating SNARE-mediated fusion of secretory membrane vesicles into the plasma membrane. In addition, however, PA on the mitochondrial surface may also trigger a signaling cascade that elevates DAG, leading to downstream events that affect mitochondrial fission and energy production. PA production on the mitochondrial surface may also stimulate local production of $PI4,5P_2$ to facilitate mitochondrial fission and subcellular trafficking or facilitate Ca^{2+} influx.

Keywords

phosphatidic acid; MitoPLD; mitochondrial fusion; fission; insulin signaling; calcium homeostasis

1. Introduction

The lipid second messenger phosphatidic acid (PA) plays pleiotropic roles in the regulation of a variety of cell functions including receptor signaling, membrane vesicle trafficking, and cytoskeletal organization [1–4]. Although the bulk of cellular PA is synthesized via acylation pathways, PA is also produced via other types of lipid-modifying enzymes on a much faster time scale, and this latter pathway serves to generate PA that functions in a signaling context in many types of cell biological processes. There are two major families of enzymes involved in generation of PA during signaling events - Phospholipase D (PLD1 and PLD2 in mammals), classical members of which hydrolyze phosphatidylcholine (PC) to yield choline and PA, and diacylglycerol kinases (DAGKs), which generate PA by phosphorylating DAG [this issue, see 5]. Conversely, PA can be converted into DAG through dephosphorylation, via the action of Type I or Type II phosphatidic acid phosphohydrolases [this issue, see 6]. Finally, one of the actions undertaken by PLD-generated PA is to stimulate the recruitment and activation of phosphatidylinositol 4-phosphate 5 Kinases [PIP5Ks, this issue, see 7] to generate

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Phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), which can be hydrolyzed by Phospholipase C (PLC) as another way to generate DAG (Fig. 1). Thus, the lipids PA, DAG, and $PI4,5P_2$ form a complex and interconnected signaling network that regulates a diverse set of cell biological pathways.

While there have been hundreds of reports that have described PLD, PIP5K, and DAGK activities on the plasma membrane, secretory apparatus, nuclear envelope, and trafficking membrane vesicles, relatively little is known regarding lipid signaling at the surface of mitochondria. Mitochondria are stereotypically viewed as kidney-shaped organelles scattered throughout the cytosol, as a result of the images obtained from electron microscopy thin sections that are frequently presented in textbooks and reviews. In fact, however, many of these images are actually presenting oblique slices of long mitochondrial tubules that are part of a network-like structure that extends from the perinuclear region to as far as the edge of the cell (Fig. 2). Mitochondria are constantly undergoing fission and fusion, and their morphology is ultimately regulated by the fusion to fission balance, which can vary in different cell types [8]. The balance is dynamically controlled – mitochondrial tubules undergo massive fission during cytokinesis and then rapidly fuse together again in the synthesis phase of the cell cycle [9,10]. Insulin and other growth factor stimulation increases the fusion to fission balance [11, 12], which may reflect the fact that larger mitochondria are more efficient at generating energy. In contrast, induction of apoptosis triggers mitochondrial fragmentation by increasing the rate of fission, although the fission in itself does not cause cell death [13]. Directed subcellular localization of mitochondria is also important to ensure that they function properly in the context of generating energy where it is needed in the cell, in particular for cells with localized high energy requirements, such as neurons, at axonal sites of synaptic transmission, which are distant from the cell body [14,15], and lymphocytes, at regions of myosin dynamics during chemotaxis [16]. In pancreatic acinar cells, mitochondria form a belt surrounding the granule-rich region to confine Ca^{2+} signaling within the apical pole [17]. Subcellular mitochondrial trafficking is affected by mitochondrial morphology [18], and genetic mutations that lead to fission and fusion dysfunction not only cause mitochondrial aggregation or fragmentation in cultured cells, but cause diseases such as type 2A Charcot-Marie-Tooth, an inherited peripheral neuropathy [19,20] characterized by blunted synaptic transmission and axonal die-back from their distal target sites.

Some of the proteins important for mitochondrial fusion and fission have been identified, as shown in Figures 3 and 4. The proteins tend to function specifically for mitochondria, but in a manner analogous to how similar types of proteins function in fusion and fission of cytosolic membrane vesicles as they bud from and fuse into subcellular membrane compartments. For example, the proteins called Mitofusins (Mfns) that mediate mitochondrial fusion perform a function similar to that undertaken by SNARE complex proteins for other types of membrane fusion [21, 22], although there are distinct aspects to the fusion mechanism [8, 23]. Similarly, fission is mediated by a dynamin-related protein, Drp1 [10, 24], which functions analogously to dynamin during the process of endocytosis. Mitochondria translocate through the cell via microtubules and the actin cytoskeleton, using both unique tethering proteins and universal components of the cytoskeleton [18, 25], and this movement is promoted by the mitochondrial Rho GTPases (Miro 1 and Miro 2) [26, 27]. As described in other chapters in this issue, PA, DAG, and $PI4,5P_2$ regulate numerous aspects of cytosolic membrane vesicle fusion, fission, and trafficking through effects on the recruitment and function of protein partners. Similar types of roles for lipid signaling on the surface of mitochondria are just beginning to be reported and appreciated [28].

Reports of PLD activity in association with mitochondria have been relatively sparse. A PLD activity with unique biochemical properties (metal or calcium ion-stimulated, phosphatidylethanolamine-preferring) was described more than a decade ago [29], but has

never been cloned. The classic isoform PLD1 has also been reported as being co-enriched with mitochondrial fractions of cell lysates prepared from Alzheimer's disease patients [30], although this may reflect association of PLD1 with amyloid precursor protein (APP) [31], rather than specific interaction with the mitochondria *per se*, and other organelles significantly co-fractionate in those fractions as well (e.g. endoplasmic reticulum and nuclei). More recently, PLD1 overexpression was demonstrated to trigger translocation of a DAG-binding protein, Protein Kinase D1 (PDK1), to the mitochondria [32]. In addition, it was shown that mitochondrial stress results in increases in DAG levels on the mitochondrial surface. The PLD1 connection is intriguing and could ensue from several possible mechanisms. PLD1 overexpression could initiate mitochondrial stress, causing increases in DAG and subsequent PKD1 translocation. Alternately, PA produced by PLD1 at the Golgi or in the ER could be converted to DAG by a PA Phosphatase such as Lipin 1 and transferred to mitochondria through Mitochondrial-Associated ER Membranes (MAM) [33]. Finally, PLD1 could produce the PA at the mitochondrial surface, although there is no evidence for PLD1 localization or PLD1-generated PA or DAG production there at present.

As described in further detail below, our research group has described a divergent and intriguing PLD superfamily member, denoted MitoPLD, which anchors into the cytoplasmic surface of mitochondria and operates there *in trans* on the surface of other mitochondria brought into close proximity during the process of fusion [23]. Possessing a typical HKD catalytic motif, MitoPLD produces PA similar to classical PLDs, although it does so using a mitochondrial-specific lipid, cardiolipin (CL), instead of PC, as the substrate. A role for PA production on the mitochondrial surface in mitochondrial fusion has been identified; however, as is the case for classic PLD isoforms in other membrane compartments, there may be additional functions for MitoPLD as well, making the study of PA signaling on the mitochondrial surface a topic of emerging interest.

2. A PA production pathway on the mitochondrial surface regulates mitochondrial fusion

A BLAST search of the human genome for additional members of the PLD superfamily uncovered a protein with a single HKD half-catalytic site, later named MitoPLD, which was predicted to localize to mitochondria based on an N-terminal leader sequence [23]. There are at least half a dozen currently uncharacterized proteins that encode HKD (PLDc) domains in the mammalian genome, although many are quite divergent and it is not clear whether they retain enzymatic capability. Sequence analysis revealed that MitoPLD is more similar to ancestral prokaryotic PLD superfamily members such as Nuc, which is a DNA endonuclease, and cardiolipin synthase, than to classical mammalian PLD family members [23]. Although seemingly odd, similar types of conservation relationships (greater similarity to prokaryotic superfamily members than to other mammalian ones) are found for many other proteins that localize to mitochondria, potentially reflecting the fact that mitochondria arose from eukaryotic capture of a prokaryotic organism, and although many of the genes initially encoded by the mitochondrial genome have since relocated to the eukaryotic genome, they still retain evolutionary signatures of their origin.

Virtually all PLDs, prokaryotic and eukaryotic, encode two half-catalytic HKD domains [34] that fold together to form the functional enzymatic unit [this issue, 35]. The sole prior exception to this was the protein Nuc, which encodes only one half-catalytic site and dimerizes to achieve a functional enzymatic site [36]. Split-Venus complementation and co-immunoprecipitation approaches were thus employed to demonstrate that MitoPLD also dimerizes to generate an active enzymatic complex. Surprisingly though, MitoPLD displayed neither endonuclease nor CL synthase activity, but rather the reverse of CL synthase activity, i.e. hydrolysis of CL to generate PA. Even more surprisingly, protease surface digestion of intact mitochondria

revealed MitoPLD to be an outer membrane-anchored protein with its amino-terminus serving as a transmembrane segment and the carboxy-catalytic-terminus protruding into the cytosol. Since cardiolipin is primarily an inner membrane-specific lipid, this physical arrangement raised issues of how MitoPLD could possibly access its substrate. However, the outer mitochondrial membrane does contain 10–20% of the total mitochondrial cardiolipin [37,38], in particular at sites at which the outer and inner membranes come into contact [37], which are thought to represent locations at which mitochondrial fusion takes place. Intriguingly, this also suggests that MitoPLD most likely generates PA at the contact sites and functions there.

With respect to function, manipulation of MitoPLD expression was found to phenocopy gain and loss of proteins involved in mitochondrial fusion. Three proteins were previously known to mediate mitochondrial fusion: Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2) anchor in the outer membrane and tether adjacent mitochondria together via SNARE complex-like coiled-coil domains to promote outer membrane fusion [21]. Another protein, OPA1, is located in the inner membrane and intermembrane space and mediates inner membrane fusion [20]. Overexpression of MitoPLD caused aggregation of mitochondria [23], similar to overexpression of Mfn1 [21], whereas knock-down of MitoPLD or expression of a MitoPLD dominant-negative isoform led to mitochondrial fragmentation [23], similar to what is observed for cells lacking Mfn proteins [21], indicating that MitoPLD functions as a vital component of the fusion machinery by generating PA. How PA facilitates mitochondrial fusion remains to be determined, but the requirement for PA is surprisingly similar to its requirement in the fusion of secretory vesicles into the plasma membrane through SNARE-complex mediated action [39–42].

In both cases, the actual fusion event is mediated by protein machinery that triggers complex formation of coiled-coil domains *in trans*. For SNARE complex proteins, in the classical setting of fusion of secretory vesicles into the plasma membrane, the vesicles contain a v-SNARE protein, and the target membrane a t-SNARE protein. Each SNARE has a coiled-coil domain. The v-SNARE and t-SNARE proteins align their coiled-coil domains in a head-to-head orientation, which, through interacting, pull the membrane vesicle into contact with the plasma membrane to initiate the fusion reaction. Precisely how PA facilitates the fusion event is not understood, although a direct effect on the SNARE mechanism has been demonstrated [41], which may involve recruitment of the v-SNARE towards the plasma membrane through positively-charged amino acids in the v-SNARE interacting with PA on the plasma membrane. The Mfn-mediated mitochondrial fusion reaction also involves dimerization of coiled-coil domains, but there are differences. In this case, a homodimer is formed, since each mitochondrial surface expresses Mfn, and the coiled-coil domains align head-to-tail, which would be a non-functional orientation for classic SNARE proteins. How Mfn causes the fusion process to proceed beyond this point and what role PA plays in it is unknown, save that the progression of fusion also requires the activity of the Mfn GTPase. Nonetheless, the two distinct protein fusion machineries are facilitated by local PA production, suggesting that PA may play a common role in the fusion events.

Two questions that arose from this study were whether the fusion event is regulated by PA production, or by depletion of the substrate, CL, and would MitoPLD-triggered CL depletion affect other processes, since CL is important for efficient oxidative phosphorylation [43,44] and mediates apoptotic pathways [45]. As reported [23], no obvious perturbations in normal cell function were observed even with substantial MitoPLD overexpression. The cells were viable and proliferated normally. Cytochrome c was still localized to the mitochondria and MitoPLD overexpression didn't alter mitochondrial membrane potential. This agrees with another study on CL synthase RNAi knockdown that reported that cells with only 25% of the normal amount of CL can function properly in the absence of apoptotic signals [46]. MitoPLD

is not an abundant protein; hence its effects on total CL are unlikely to be significant during fusion events, although substantial changes in localized microregions can not be ruled out.

The findings raise many questions. Does MitoPLD-generated PA recruit signaling proteins, as do PLD1 and PLD2 at other membrane surfaces? How is PA signaling terminated at the mitochondrial surface? Are other lipid signaling pathways activated as a consequence of MitoPLD-generated PA production, and what processes might they impact on?

3. PA signaling pathways on the mitochondrial surface

3.1 PA signaling and diabetes

As a potent bioactive signaling lipid, PA generated on the plasma membrane by classic PLD isoforms is well-known to be turned over rapidly to terminate PA-mediated signaling events through the action of PA phosphatases (PAPs), which convert the PA to DAG [this issue, 6]. Interestingly, several studies have reported that there is a dynamic pool of DAG generated on the mitochondrial surface that can be observed using fluorescent sensors, in particular when DAG-metabolizing enzymes are blocked [47,48] or under conditions of mitochondrial stress [32]. In the latter case, the DAG has been shown to recruit Protein Kinase D1 to mitochondria. The mechanism responsible for this DAG production and its physiological significance have not been established, but there are several intriguing possibilities. The first is suggested by studies on insulin signaling in muscle and liver cells. Among many other actions, insulin stimulates shifts in mitochondrial energy production via the activation of pyruvate dehydrogenase (PDH), which is located in the mitochondrial interior. PDH is activated as a consequence of translocation of protein kinase Cδ (PKCδ) from the cytosol to the mitochondria [49]. However, it is not currently understood how PKCδ at the external face of the mitochondria elicits changes in PDH phosphorylation – presumably there are intervening steps in the signaling pathway. DAG is the only signal known to be able to recruit PKCδ to membrane surfaces [50–52]. The origin of the pool of DAG that could function to recruit PKCδ to the mitochondrial surface is not known. The generation of DAG could occur either from dephosphorylation of PA or from hydrolysis of phosphoinositides by phospholipase C (PLC). Although PLC signaling is best known for taking place on the plasma membrane and in the nucleus, and the activation of PLC requires G-protein subunits [53], which have not been found at mitochondria, one isoform of PLC has been observed to localize to mitochondria [54], as discussed below. Production of DAG could also be mediated by dephosphorylation of PA via Type I or Type II PAPs [this issue, 6]. Type 2 PAPs are multi-pass transmembrane proteins that place their catalytic sites on the external surface of the cell or in the lumen of organelles [55], making them unlikely candidates for mediating conversion of PA on cytoplasmic-facing membrane leaflets to DAG. Recently however, Type I PAPs were identified as a pre-existing gene family known as the Lipins [56,57]. Loss of activity of Lipin 1 causes a Type-II related diabetic lipodystrophy known as fatty liver dystrophy (fld) [58–60]. The syndrome is also characterized by abnormal mitochondrial handling of glucose and fatty acid metabolism [61], suggesting that PA or DAG on the outer mitochondrial surface might serve as a sensor for regulating mitochondrial energetics. These newly-found Type I PAP enzymes thus represent possible candidates for converting PA to DAG on the mitochondrial surface.

Placing these observations together, one possibility would be that insulin signaling stimulates mitochondrial fusion, resulting in increased MitoPLD-generated PA as fusing mitochondria come into contact. Increased PA on the mitochondrial surface would then recruit a Type I PAP such as Lipin, which would terminate the PA signaling by generating DAG. The DAG would recruit PKCδ, which in turn would active PDH and increase mitochondrial energy production. Exploration of this topic represents an area for future investigation.

3.2 PA, DAG, and potential roles in mitochondrial fusion and fission

PA and DAG have each been demonstrated to facilitate both fusion and fission of cytoplasmic membrane compartments in specific settings, for example, endocytosis from the plasma membrane [62,63], budding of vesicles from the Golgi [64–70], peroxisome division [71], insertion of membrane vesicles into the plasma membrane [40,72,73], and fusion of nuclear envelope membrane precursor vesicles [74]. PA generation is required for mitochondrial fusion [23], but it has not been determined whether PA directly facilitates fusion, or whether the fusion event is promoted by DAG subsequent to metabolism of the PA by a PAP such as Lipin. Even more intriguingly, a link between fusion and fission has been described. Mitochondria undergo fusion on average every 24 minutes, but at seemingly random times within that average time frame [75]. Once fusion occurs, though, a fission event rapidly follows (on average, within 1.3 minutes). Thus, some consequence of the fusion event triggers a subsequent fission event. This is an attractive finding from the perspective of a pro-homeostasis mechanism evolved to prevent excessive fusion in the cell and to target fission events onto the largest of the mitochondria. The mechanism underlying the linkage of fission to fusion is not known, but an intriguing possibility would be that early stages of the fusion reaction lead to generation of PA and the completion of fusion, following which the PA is metabolized to DAG, elevated levels of which promote fission. Such a function would be consistent with roles for DAG in membrane fission at the trans-Golgi [66,68] and in peroxisome division [71]. Examination of the linkage of fusion and fission in cell lines lacking MitoPLD or PAPs such as Lipin should provide insight into this possibility.

3.3 PI4,5P_2 signaling pathways and potential roles on the mitochondrial surface

PLC-δ1 has been reported to localize to mitochondria and to facilitate mitochondrial calcium uptake [76,77] through the mitochondrial calcium uniporter. PI4,5P_2 hydrolysis generates both phosphatidylinositol 3,4,5-triphsophate (IP_3) and DAG; the latter in this case is thought to be the relevant lipid signal that stimulates the Ca^{2+} uptake [54]. The requirement for biologically active PLC at the mitochondrial surface implies that PI4,5P_2 should be found there, which has been reported [78]. Approximately 5% of the cellular PI4,5P_2 is found at the mitochondrial outer membrane, resulting in a density for PI4,5P_2 of approximately 25% of that found at the plasma membrane [79]. A PI4,5P_2 -metabolizing enzyme, Synaptojanin 2a, has also been observed at the mitochondrial surface [79], and a second mitochondrial-localized PI4,5P_2 phosphatase elicits changes in mitochondrial shape when overexpressed or targeted to the outer mitochondrial surface [80], suggesting that the endogenous levels of PI4,5P_2 on the mitochondrial surface are physiologically significant. PI4,5P_2 regulates F-actin assembly, which has been linked to recruitment of Drp1, a component of the mitochondrial fission apparatus, to future sites of cleavage [80], and F-actin interaction has been reported to regulate mitochondria subcellular movement [reviewed in 18]. PI4,5P_2 is also key to linking membrane vesicles to dynein/kinesins to enable microtubule-based trafficking, and it may play a similar function for mitochondria, since overexpression of a PI4,5P_2 -sequestering PLC-PH domain sensor disrupts the directed mitochondrial movement observed in neurons in response to nerve growth factor extracellular stimulation [14,15,25]. Mitochondria become redistributed in migrating cells [16], suggesting possible roles for PI4,5P_2 in that process. Intriguingly, the atypical Rho GTPase, Miro, has been shown to have essential roles in mitochondrial trafficking by residing on the mitochondrial outer membrane and interacting with kinesin-binding proteins [18,26,27]. Although compelling evidence suggests Miro acts as a calcium sensor using EF-hands to control mitochondrial mobility [81–83], as a GTPase, Miro can also be activated by guanine nucleotide exchange factors (GEFs) that remain to be identified. However, given the recent findings that DAG and PA can function at other subcellular membrane surfaces to recruit GEFs that activate the small GTPases Ras [84,85] and Rac [86], it is possible that Miro could be regulated by mitochondrial lipid signals, such as DAG, PA, or PI4,5P_2, via recruitment of

its GEF. Moreover, microtubule motors (Dynein) have also been linked to Drp1 recruitment, again suggesting a role for $PI4,5P_2$ [87].

Finally, the Phosphatidylinositol 4-phosphate 5-Kinase that generate $PI4,5P_2$ are well known to be recruited and regulated by PLD-generated PA [88]; hence most of the components that would be necessary for a signaling network involving MitoPLD-generated PA stimulation of $PI4,5P_2$ synthesis leading to regulation of mitochondrial fission, movement, and Ca^{2+} homeostasis have been described, and the stage is set for conducting studies to establish their cell biological significance.

4. Conclusions

This review discusses a newly found pool of PA generated by MitoPLD and how this pool of PA on the mitochondrial surface might serve as a signaling lipid and function in diverse cell biological settings related to mitochondrial biology. MitoPLD mirrors its classic family members PLD1 and 2 by producing one or more potent lipid second messengers on the mitochondrial surface. Besides promoting mitochondrial fusion, MitoPLD-generated PA may also regulate mitochondrial fission, translocation, calcium homeostasis, and energy production. This field represents a novel area for future study and may identify even more players involved in signaling pathways on the mitochondrial surface.

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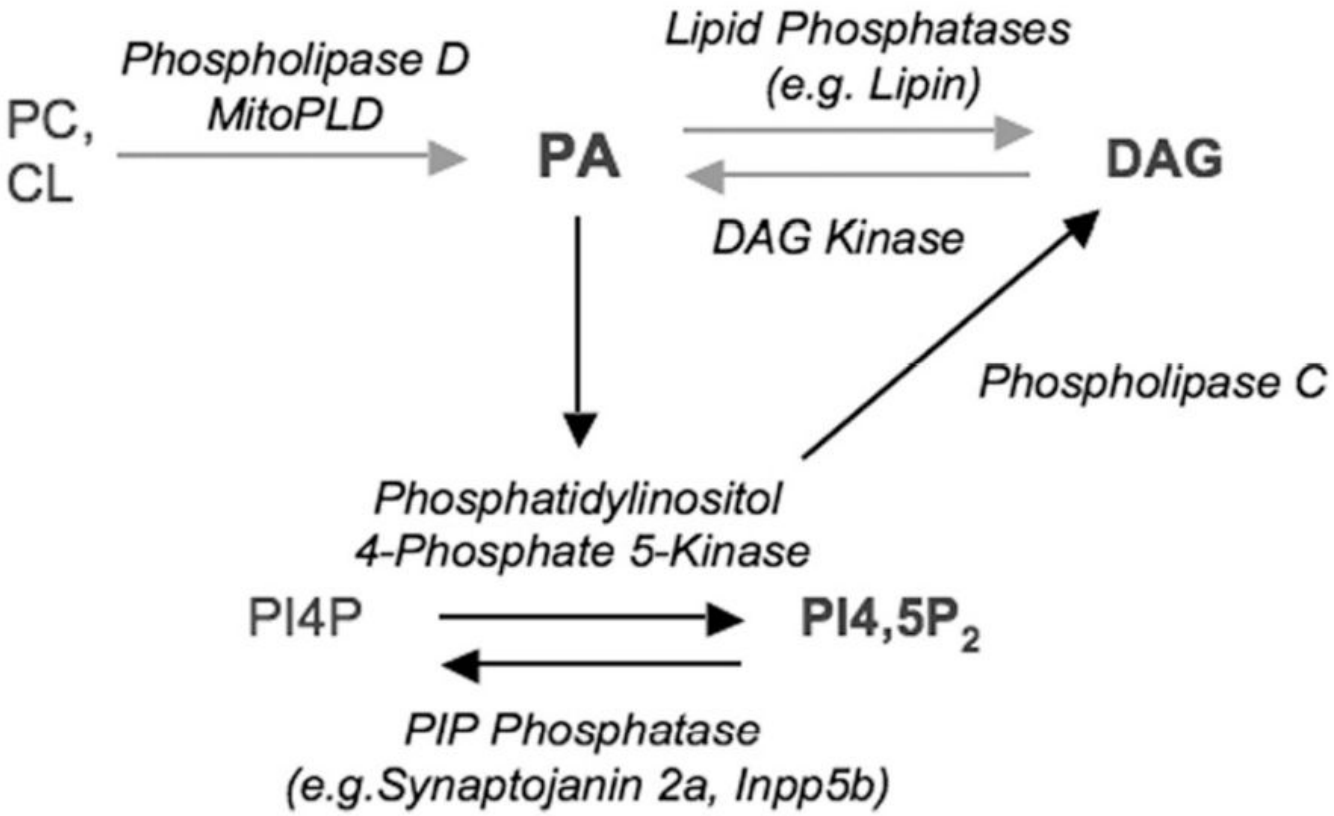

Figure 1. Signaling Lipids

Phosphatidylcholine (PC) and Cardiolipin (CL), which are major phospholipids in the plasma membrane and mitochondria, respectively, can be converted by members of the Phospholipase D superfamily into PA, which in turn can be used to generate DAG via action of PA Phosphatase. PA also stimulates the enzyme Phosphatidylinositol 4-phosphate 5-Kinase to generate $PI4,5P_2$, which can be converted to DAG via the action of Phospholipase C. Several of these actions are reversible, for example conversion of DAG back to PA by DAG Kinase.

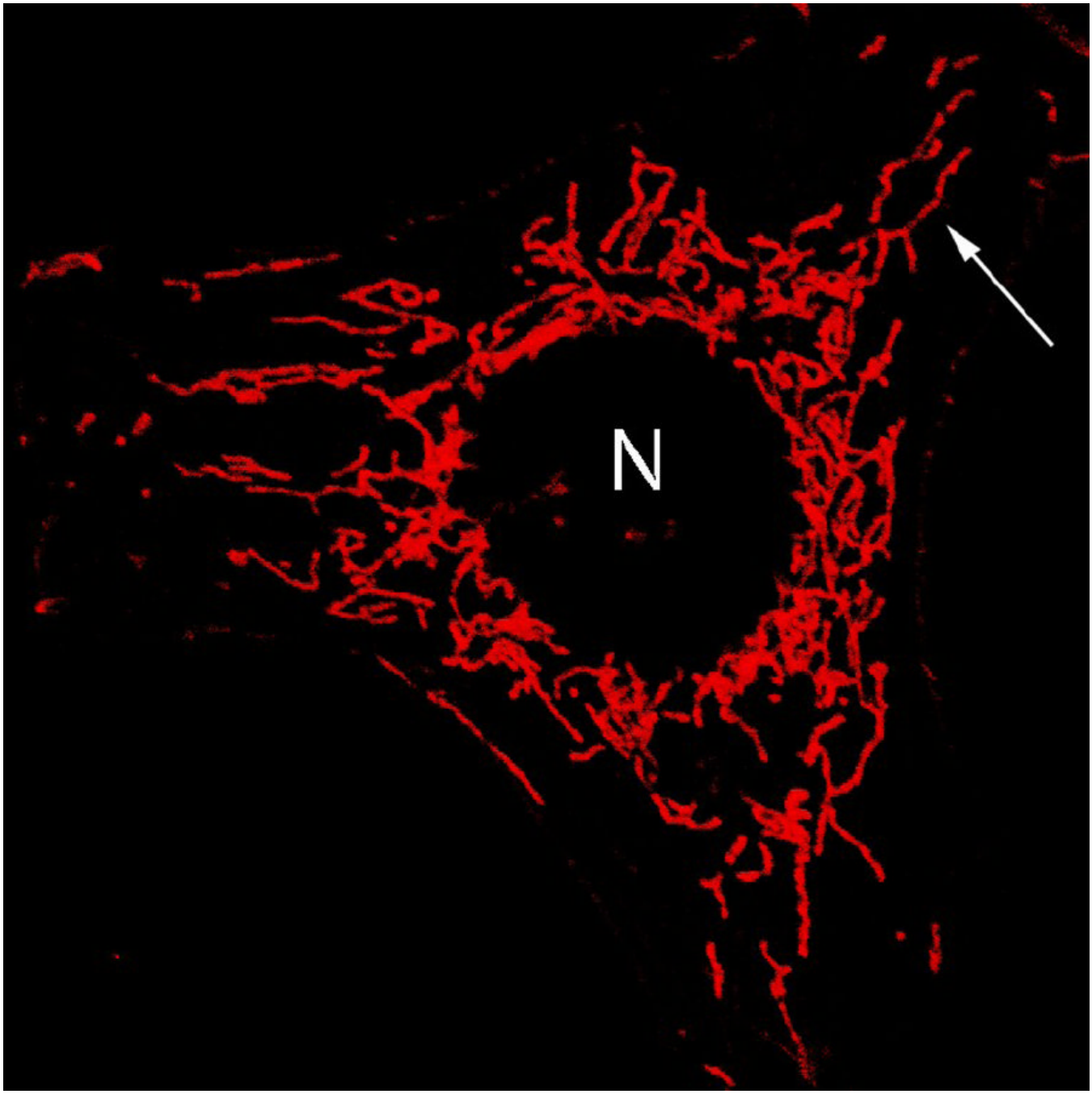

Figure 2. Tubular mitochondrial morphology

HeLa cell expressing mitochondrially-targeted enhanced green fluorescent protein (EGFP) demonstrates tubular organization of mitochondria. N, nucleus. Arrow, typical branched, tubular mitochondria.

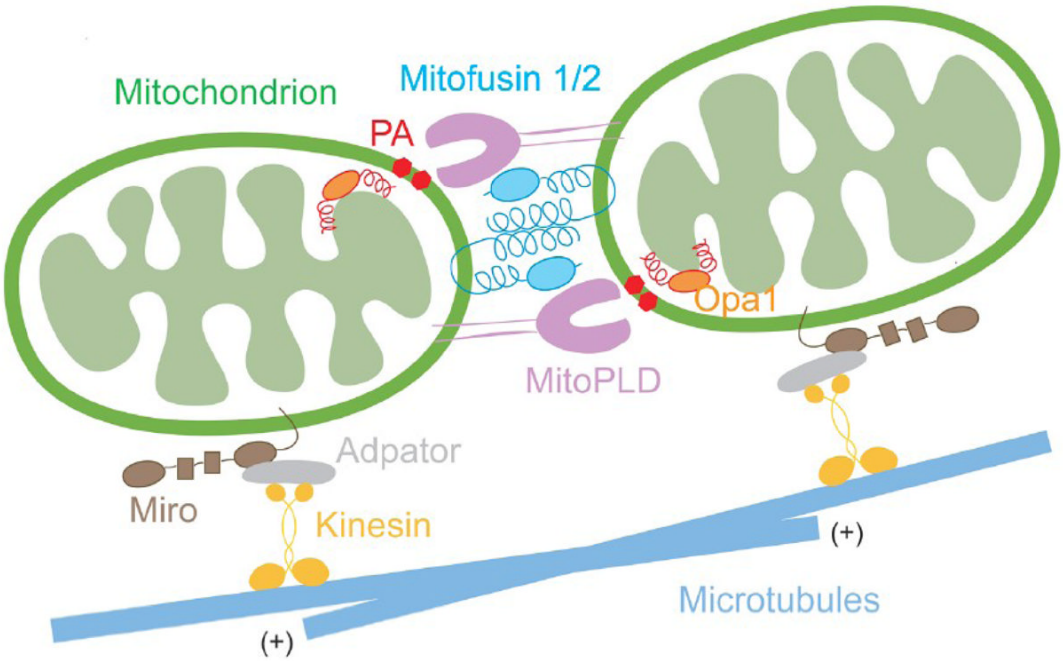

Figure 3. Mitochondrial fusion

Model for mitochondrial fusion and trafficking. During fusion, the mitochondrial outer membrane protein Mitofusin 1 and 2 tether adjacent mitochondria through their coiled-coil domains, bringing the MitoPLD dimer into close contact with its substrate, cardiolipin, at the opposing mitochondrial membrane. This generates PA, which facilitates outer membrane fusion. Inner membrane fusion is facilitated by an inter-membrane protein, Opa1. Mitochondrial movement along microtubules is promoted by the interaction of an outer membrane protein, Miro, with kinesin, through an adaptor protein.

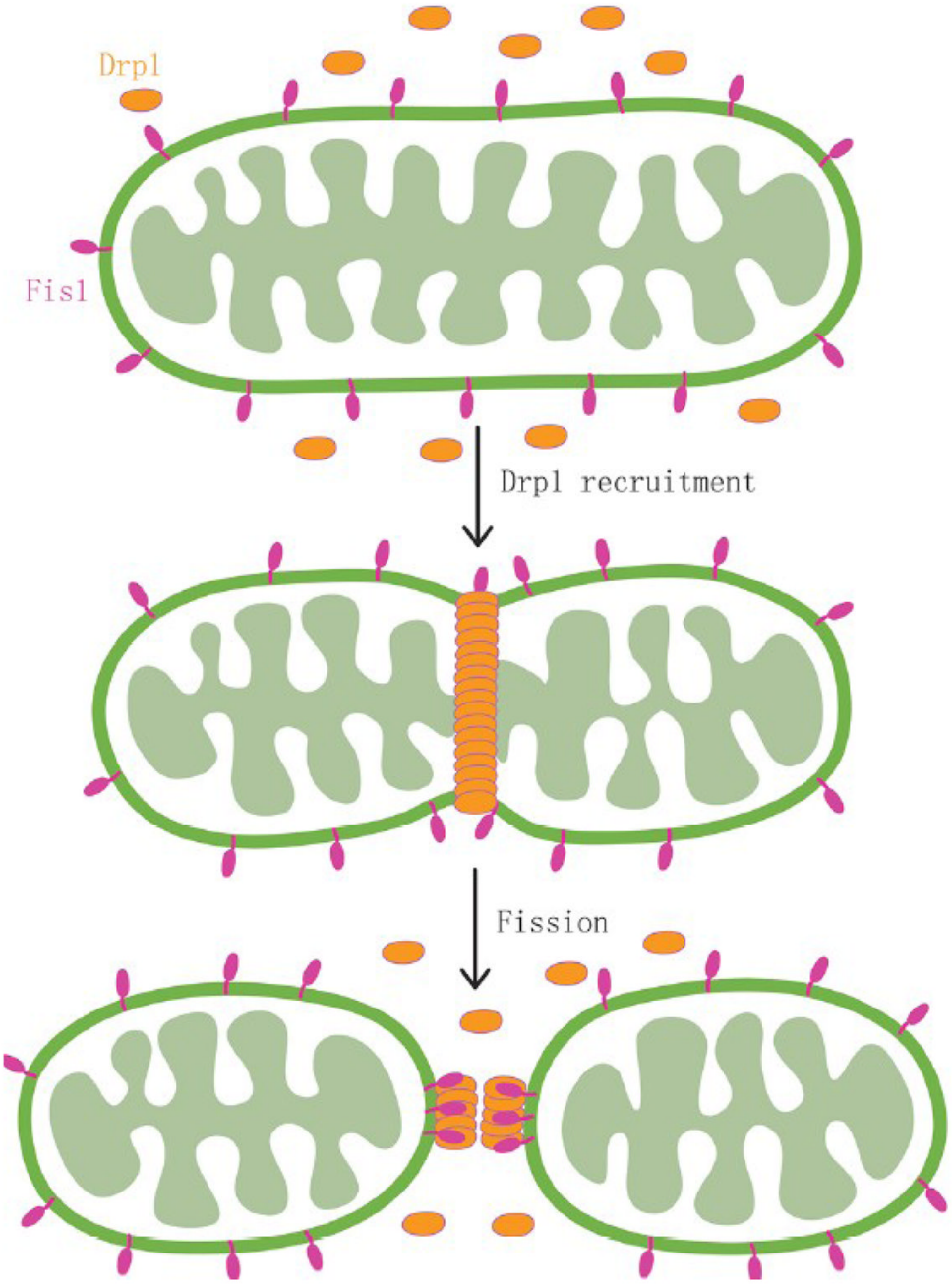

Figure 4. Mitochondrial fission

Model for mitochondrial fission. The fission protein, Fis1, resides in the mitochondrial outer membrane and recruits dynamin-like protein (Drp1) during mitochondrial fission. Drp1 assembles around the fission site to create membrane constriction.