Molecular basis of local energy generation during mitochondrial and peroxisomal division

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Summary: GTPase dynamin-related protein (Dnm1)-mediated membrane fission is an important membrane remodeling event supporting the proliferation and housekeeping function of semiautonomous organelles such as the mitochondrion and peroxisome. Dnm1 is at the heart of the membrane fission machinery, which constricts the neck of the dividing organelles. Similar to classical dynamin protein, Dnm1 hydrolyzes GTP, an energy source, thereby generating a constriction force to sever the neck. To complete this process, replenishment of GTP to Dnm1 needs to be done in a regulated and timely manner. However, the molecular mechanisms that provide GTP to Dnm1 are not known. In this review, we present the evidence for emerging consensus on Dnm1 function and our recent work demonstrating that: (1) The ATP-GTP converting, nucleoside diphosphate kinase-like protein DYNAMO1 is present in the mitochondrial and peroxisomal membrane fission machinery. (2) DYNAMO1 facilitates enzyme kinetics of Dnm1 and locally provides GTP to Dnm1 on the membrane fission machinery. (3) The membrane fission machinery spends more GTP on constriction than on recruitment, as seen by the in vivo experiments and in vitro reconstitution of the Dnm1 structure. Summarizing these data, this review would help to understand the mechanism by which Dnm1 promotes membrane fission using GTP as an energy source. We also discuss how future research might solve the remaining open questions regarding the energy issues presently under discussion.

Key words: Dynamin-related protein Dnm1, local GTP generation, mitochondrial division, nucleoside-diphosphate kinase, peroxisomal division

INTRODUCTION

Cellular membranes constantly reshape for transporting materials within the cell by endocytosis and budding organelles, or for organelle division. For homeostatic maintenance of plasma membrane proteins, plasma membrane invaginates and forms endocytic vesicles. Likewise, transporting vesicles invaginate from the organelle membrane. For the proliferation of organelles such as mitochondria, chloroplasts or peroxisomes, a part of their membrane constricts to generate daughter organelles. As the membrane reshapes for vesicle generation or organelle division, the tubular membrane must close at the last step. However, this process leads to elastic stress and requires external force for completion. The fission reaction is carried out by the constriction of a ring- or cylindrical helix-shaped mechanochemical machinery wrapped around the neck of vesicles and organelles. The membrane fission machinery was first observed around the neck of synaptic vesicles endocytic pit in the fly neuro muscular junction, as an “electron-dense collar”, approximately 10 nm in thickness and 20–25 nm in diameter (Kosaka and Ikeda 1983, Koenig and Ikeda 1989). Similar electron-dense deposits or structures are also observed around the neck of the dividing organelles such as mitochondrion, chloroplast, and peroxisome in slime mold (Physarum polycephalum), red algae (Cyanidium caldarium and Cyanidioschyzon merolae), yeast, and mammalian cells (Kuroiwa et al. 1977, Mita et al. 1986, Kuroiwa et al. 1995, Yoon et al. 2003, Ingerman et al. 2005, Imoto et al. 2013). Several studies have shown that members of the dynamin family of GTPases localize around these electron dense regions at membrane fission sites (Takei et al. 1995, Miyagishima et al. 2003, Nishida et al. 2003). Dynamin (classical dynamin) was the first dynamin family protein to be identified (Shpetner and Vallee 1989), and was found to be the mammalian orthologue of the fly (Drosophila melanogaster) shibire gene product, which is known to mediate synaptic vesicle endocytosis (van der Bliik and Meyerowitz 1991). Moreover, a classical dynamin homologue, dynamin-related proteins Dnm1 (Dnm1 in yeast and algae, Drp1 in mammals, and DRP3 in land plants) regulate mitochondrial and peroxisomal division (Bleazard et al. 1999, Smirnova et al. 2001, Arimura and Tsutsumi 2002, Koch et al. 2003, Mano et al. 2004), and the plant orthologue Dnm2/DRP5B or ACCUMULATION AND REPLICATION OF CHLOROPLASTS5 (ARCS) regulates chloroplast division (Gao et al. 2003, Miyagishima et al. 2003). These proteins form a ring-like or helical structure (Hinshaw and Schmid 1995, Miyagishima et al. 2003, Ingerman et al. 2005, Ji et al. 2015), which is consisting with the shape of the electron-dense collar observed on the neck of dividing organelles, suggesting a role in membrane constriction (Switek and Hinshaw 1998, Kuroiwa et al. 2008, Antony et al. 2016).

Among members of the dynamin family proteins,
structure and function of classical dynamin and Dnm1 are well characterized (Chappie et al. 2011, Mears et al. 2011, Bui and Shaw 2013, Antonny et al. 2016). Classical dynamin and Dnm1 contain several domains (Figure 1): (1) GTPase domain (G-domain), which hydrolyzes GTP. G-domain forms dimer to bridge adjusting turns of the oligomerized ring or helix to maximize GTPase activity (Chappie et al. 2010, Mears et al. 2011). This domain is most important for the membrane fission reaction; (2) middle domain and GTPase effector domain (GED), which contain a-helical stalks and participate in assembly into highly ordered ring or helix structures (Chappie et al. 2010, Mears et al. 2011, Bohuszewicz and Low 2018, Kalia et al. 2018); (3) pleckstrin homology (PH) domain, which is conserved in classical dynamin, and has an amphipathic loop for interaction with phosphoinositide-4,5-bisphosphate (PIP2) (Fushman et al. 1995). Dnm1 has a variable domain (B-insert) instead of the PH domain as a functional analogue (Bustillo-Zabalbeitia et al. 2014); (4) proline-rich domain (PRD), which binds to Src homology 3 (SH3) domain in classical dynamin-adaptor proteins (Praefcke and McMahon 2004). In contrast to classical dynamin, Dnm1 does not contain the PRD. The orchestrated function of these domains results in the recruitment of classical dynamin and Dnm1 molecules to the membrane fission sites, which then polymerize into contractile structures to pinch off membrane tubules, upon GTP-binding and hydrolysis (Praefcke and McMahon 2004). These processes involve constriction of the neck of the vesicle or dividing organelles; the magnitude of constriction is remarkably different between classical dynamin and Dnm1. An in vitro study has shown that Dnm1 orthologues in yeast and mammals form helix structure around lipid tubes (Dnm1 helix structure) (Mears et al. 2011, Kalia et al. 2018). The magnitude of constriction of lipid tubes measured using Dnm1 orthologues in yeast, algae, or mammals is at least ~50 nm (Mears et al. 2011, Bohuszewicz and Low 2018, Ugarte-Uribe et al. 2019), which is ~5-fold higher than that of classical dynamin (~10 nm) (Mears et al. 2011). The diameter of the neck of a dividing mitochondrion and peroxisome is substantially larger in yeast, algae, and mammalian cells (100–300 nm) (Bleazard et al. 1999, Nishida et al. 2003, Yoon et al. 2003, Imoto et al. 2013, Soliman et al. 2018) than the neck of an classical dynamin-dependent endocytic pit observed in fly and mammalian cell (~20–50 nm) (Kosaka and Ikeda 1983, Raimondi et al. 2011), and could explain the higher magnitude of constriction of Dnm1 helix structure. However, studies using Dnm1 orthologues in yeast or mammals showed that Dnm1 has a weak affinity for GTP (Km of basal G-domain is at least 1 mM) (Ingerman et al. 2005, Macdonald et al. 2016). Moreover, Dnm1 orthologues in yeast, algae, and mammals have a relatively high rate of GTP hydrolysis on the membrane (Ingerman et al. 2005, Macdonald et al. 2016, Bohuszewicz and Low 2018)—at least 5,000-fold higher than small GTPases (Bourke et al. 1990). Indeed, a study using yeast Dnm1 demonstrated that significant constriction mediated by Dnm1 helix structure requires extraordinarily high levels of GTP concentration (~1 mM) (Mears et al. 2011), which is consistent with the idea that allosteric enhancer of G-domain is required to elevate the affinity of Dnm1 to GTP (Macdonald et al. 2016). Adaptor proteins of Dnm1 such as Mitochondrial division protein 1 (Mdv1) in yeast and, Mitochondrial fission factor (Mff) and Mitochondrial dynamics protein of 49 kDa and 51 kDa (Mfd49/51) in mammalian cells are known to enhance the function of the G-domain (Lackner et al. 2009, Koirala et al. 2013). As Dnm1 does not contain the PRD, these adaptor proteins directly bind to other domains such as G-domain, B-insert, and GED in yeast and mammalian cells (Bui et al. 2012, Liu and Chan 2015, Kalia et al. 2018). However, the interaction of Dnm1 orthologue Drp1 in mammals with Mfd49 itself requires nucleotide binding during the recruitment (Kalina et al. 2018), followed by polymerization with Mff on the membrane (Helle et al. 2017). Moreover, conservation of Dnm1 adaptor proteins across eukaryotes is limited, Mdv1 has not found in animal cells, and Mff and MiD49/51 are only found among animal cells and mammalian cells, respectively (Roy et al. 2015), even though Dnm1-mediated division of mitochondria and peroxisomes occurs in almost all eukaryotic cells, including those in animals, fungi, land plants, and algae (Kuroiwa et al. 2008). Thus, the role and identity of the factor that overcomes the energy barrier of GTP hydrolysis of Dnm1 is an important open question to be answered in the field. In this review, we will discuss our recent findings demonstrating that ATP-GTP converting enzyme facilitates Dnm1 function and local GTP generation is required for the function of the mitochondrial and peroxisomal division machinery in red algae C. merolae. Based

![Figure 1](image_url)  
*Figure 1. Domain structure of dynamin and Dnm1. Dynamin contains a GTPase domain, a middle domain, a pleckstrin homology (PH) domain, a GTPase effector domain (GED), and a proline rich domain (PRD). Dnm1 contains GTPase domains, middle domains, and GEDs. Dnm1 also contains a B-insert domain. Dnm1 does not possess PH or PRD domains.*
on recent research in this field, local GTP generation is thought to be well conserved between Dnm1 and classical dynamin (Boissan et al. 2014, Imoto et al. 2018). We will then introduce working models of local GTP generation and discuss about the caveats and remaining questions. We will also provide the future perspectives on this field.

**ADVANTAGES OF USING C. merolae AS A MODEL SYSTEM TO ANALYZE ORGANELLE DIVISION**

To challenge the question regarding the GTP issue described above, we used a proteomics approach to identify the novel enhancer of G-domain function of Dnm1, which required the isolation of the membrane fission machinery—containing functional Dnm1—in bulk; however, the isolation is not a trivial matter, due to the numerous mitochondria and peroxisomes per cell that divide randomly. To overcome this problem, we used the unicellular red algae, *C. merolae*, which contains only one mitochondrion, peroxisome, and chloroplast per cell (Figure 2A). The division of these organelles can be synchronized by the light/dark cycle (Suzuki et al. 1994). This feature enabled capturing snapshots of membrane fission events and demonstrated that the division of the chloroplast, mitochondrion, and peroxisome occurs once during mitosis, in this order (Miyagishima et al. 1999a, 1999b) (Figure 2B). These organelles divide by binary fission, and the Dnm1 forms a ring-shaped structure around the necks of the dividing mitochondrion and peroxisome and constricts them (Nishida et al. 2003, Imoto et al. 2013). In dividing chloroplasts, this function is mediated by Dnm2/DRP5B (Miyagishima et al. 2003). Thus, we can harvest the cells in bulk at the desired time point when these dynamin-related proteins are progressively constricting the neck. Mitochondrial constriction is mediated by the mitochondrial division (MD) machinery, consisting of an outer ring—formed around the cytoplasmic side of the mitochondrial outer membrane—and an inner ring—formed around the matrix side of the inner membrane (Kuroiwa et al. 2008). The outer ring is composed of the Dnm1 ring structure (Nishida et al. 2003) and the MD ring, containing a bundle of polyglucan filaments, ~5 nm in width (Yoshida et al. 2017). The inner ring is a remnant of the bacterial cell division apparatus containing filamenting temperature sensitive mutant Z (FtsZ) (Takahara et al. 2001) that has been lost before the diversification of the Opisthokonts (Purkanti and Thattai 2015). The MD machinery can be isolated; however, it is physically associated with the plastid dividing (PD) machinery, containing Dnm2/DRP5B (Yoshida et al. 2009). Therefore, separation of these two structures seemed to be difficult. In contrast, the division machinery of peroxisomes is formed at times and sites different than the MD and PD machineries (Imoto et al. 2013). The peroxisome dividing (POD) machinery is composed of dynamin-based (DB) rings and skeletal filamentous ring formed at the cytoplasmic side of peroxisomal membranes (Imoto et al. 2013, 2017). The DB ring

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**Figure 2.** Timings of organelle divisions are highly coordinated in *Cyanidioschyzon merolae* cell. (A) Fluorescent images of DNA and each organelle in *C. merolae* cell are shown. Cn, cell nucleus; Mn, mitochondrial nucleoid; Chl-n, chloroplast nucleoid; Mito, mitochondrion; Chl, chloroplast; ER, endoplasmic reticulum; Golgi, Golgi body; Lyso, lysosome, Ps, peroxisome. Bar = 1 μm. (B) Schematic image represents time course of organelle divisions during the cell cycle. Divisions of organelles can be synchronized by 12-h light/12-h dark cycles. Mitochondrial and chloroplast divisions occur during G2-early mitotic phase followed by peroxisomal division during late mitotic phase. Divisions of mitochondrion and chloroplast is regulated by the mitochondrial-dividing (MD) and plastid-dividing (PD) machineries, respectively. Division of peroxisome is regulated by peroxisome-dividing (POD) machinery. After mitosis, respective daughter organelles are partitioned to the daughter cells and physically separated by cytokinesis. G1, G1 phase; S, synthesis phase; G2, G2 phase; M, mitotic phase; C, cytokinesis. Images were modified from Imoto and Yoshida (2017).
contains Dnm1 and can be physically separated from the other components of the POD machinery, which enables mapping of candidate proteins to specific structures.

CHARACTERIZATION OF NUCLEOSIDE-DIPHOSPHATE KINASE LIKE PROTEIN DYNAMO1 AS AN ENHANCER OF DNM1 FUNCTION

Using isolated POD machinery fractions from C. merolae culture (Figure 3A, B), we investigated the molecular identity of the DB-ring. We analyzed the isolated POD machinery fraction using liquid chromatography and mass spectrometry, and peptide mass fingerprinting using the complete genomic sequence of C. merolae (Matsuzaki et al. 2004, Nozaki et al. 2007). We found that a ~17 kDa protein was enriched with Dnm1 in the fraction. It was the product of CML110C, an orthologue of nucleoside-diphosphate kinase (NDPK) that catalyzes GTP generation by transient phosphorylation of the catalytic histidine residue while transferring g-phosphate from ATP to GDP (Norman et al. 1965, Kim et al. 2003) (Figure 3C, D). Indeed, electrospray ionization mass spectrometry analysis showed that CML110C generates GTP upon addition of ATP and GDP. CML110C contains the consensus catalytic motif of NDPK, NxxHxSD; when the histidine residue was substituted with aspartic acid (H116D), GTP generating activity was abolished. Thus, CML110C has NDPK function. Moreover, CML110C binds Dnm1 directly and the interaction elevates the kinetics of GTPase activity of Dnm1. These results suggested that CML110C had two functions—enhancement of G-domain function and generation of GTP.
We hypothesized that CML110C has an important function in the GTPase activity of Dnm1 during organelle division and named this protein dynamin-based ring motive-force organizer 1 (DYNAMO1).

In the cell, DYNAMO1 showed cytoplasmic localization from interphase to G2 phase, and localized to the mitochondrial division site, together with Dnm1, during early mitosis (Figure 4A). After mitochondrial division, DYNAMO1 relocated to the neck of dividing peroxisome with Dnm1 (Figure 4B). Suppression of DYNAMO1 disrupted Dnm1 localization to the neck of the dividing mitochondrion (Figure 4C). Previous study suggested that Dnm1 is recruited from cytoplasm to the neck of the dividing mitochondrion (Nishida et al. 2003). Thus, DYNAMO1 is likely involved in the recruitment of Dnm1. This recruitment is independent of its enzymatic activity of DYNAMO1 since dominant negative expression of H116D mutation (DYNAMO1-H116D) does not inhibit the recruitment. Therefore, DYNAMO1 binding, and not GTP generation promoted the recruitment of Dnm1 from the cytoplasm to the division site. We further tested this hypothesis by performing an in vitro reconstitution assay with Dnm1 and DYNAMO1. We found that the interaction between Dnm1 and DYNAMO1 promoted the formation of a higher order structure (Figure 4D; Dnm1-DYNAMO1 filament). A similar sized structure was also observed in solution with Dnm1 alone (Figure 4D; Dnm1 filament), but the number of Dnm1 filaments was much less than when DYNAMO1 was also present. The formation of Dnm1-DYNAMO1 filament does not require GTP. A previous study has shown that the formation of higher order structures of yeast Dnm1 improves its GTP-binding ability (Ingerman et al. 2005). Thus, the results of our in vitro experiment suggested that the enhancement function of DYNAMO1 is caused by promoting oligomerization of Dnm1, which is required for the recruitment of Dnm1 from the cytoplasm to the mitochondrial membrane.

Based on the results from the in vivo and in vitro experiments, we concluded that DYNAMO1 functioned in recruiting Dnm1 from the cytoplasm to the membrane fission sites. This process is mediated by Dnm1 and DYNAMO1 binding and is independent of GTP generation. Then, how is the role of GTP generation activity? As DYNAMO1 was localized to the neck of the dividing mitochondrion and peroxisome throughout the division process, we hypothesized that the GTP generating reaction is important during membrane fission.
LOCAL GTP GENERATION FOR THE MD AND POD MACHINERIES THROUGH DYNAMO1

GTP generating activity is most likely irrelevant for Dnm1 recruitment but has an important function once Dnm1 is assembled around the neck of the mitochondrion or the peroxisome. After the recruitment, DYNAMO1 and Dnm1 colocalized along with the ring of the MD and POD machineries (Figure 5A). Immunoelectron microscopy of the isolated POD machinery showed that DYNAMO1 predominantly localized on the same string as Dnm1 (Figure 5B). Thereby, DYNAMO1 most likely generates GTP on Dnm1-based structures in the MD and POD machineries. Further, the GTP generating activity of DYNAMO1 was tested using a dominant negative form of DYNAMO1 (DYNAMO1-H116D). In the cells, upon expression of DYNAMO1-H116D, membrane fission of either the mitochondrion or the peroxisome or both was stalled during

Figure 5. Localization of DYNAMO1 in MD and POD machineries, and functional analysis of DYNAMO1 by gene suppression. (A) Phase contrast and immunofluorescence microscopy images of DYNAMO1 and Dnm1 on an isolated MD/PD machinery and POD machinery. Localizations of FtsZ1 (marker protein for MD machinery) and PDR1 (marker protein for PD machinery) are also shown. Bar = 500 nm. (B) Whole-mount negative-staining immunoelectron microscopy images of DYNAMO1 (10 nm immunogold particles) and Dnm1 (15 nm immunogold particles) on isolated POD machinery. Right panel shows a magnified image of the red-boxed area in the left panel. White arrowhead, filamentous ring; green arrowhead, DB ring. Bar = 200 nm, 50 nm (magnified image). (C) Profiles of mitochondrial and peroxisomal division based on morphological changes during division. n = 3, at least 50 cells were counted in each experiment. P, p-value (Mann–Whitney test). Bar = 1 μm. (D) Localization of expressed DYNAMO1-HA and DYNAMO1 H116D-HA, Dnm1, and mitochondria or peroxisomes. Bar = 1 μm. Images were modified from Imoto et al. (2018).
constriction (Figure 5C, D). Remarkably, Dnm1 and DYNAMO1 colocalized on the neck of the dividing mitochondrion and peroxisome. Thus, local GTP generation is important once the Dnm1-DYNAMO1 complex accumulates around the neck of the dividing mitochondrion and peroxisome. DYNAMO1 is also localized within the cytoplasm; thus, we cannot exclude the possibility that impaired mitochondrial and peroxisomal fission is due to the reduction of basal GTP level of the cell (global GTP level). However, inhibition of GTP generation activity of DYNAMO1 does not affect protein translation, one of the major GTP consuming events. Thus, inhibition of organelle division is most likely caused by the abrogation of localized GTP generation at the organelle division site rather than the depletion of global GTP levels. C. merolae possesses another NDPK homologue, DYNAMO2, which exclusively presents within cytoplasm (Imoto et al. 2019). DYNAMO1 and DYNAMO2 may have disparate functions in division of mitochondrion and peroxisome, and global GTP regulation, respectively.

GTP generating activity of DYNAMO1 during organelle division was further tested by in vitro reconstitution assay, which demonstrated that the Dnm1-DYNAMO1 filaments undergo constriction when DYNAMO1 generates GTP (Figure 6A). This conformational change was stalled when DYNAMO1 was replaced with the DYNAMO1-H116D, indicating that GTP generated by DYNAMO1 is used for constriction. The question arising here is the impact of GTP generation just by the side of Dnm1, as demonstrated in vivo and in vitro. Further, we compared the magnitude of constriction between Dnm1 filaments and Dnm1-DYNAMO1 filaments. The Dnm1-DYNAMO1 filaments showed a larger magnitude of constriction (Figure 6B), suggesting that the enhancement function of DYNAMO1 is also important for constriction, in addition to the formation of the Dnm1-DYNAMO1 filaments. Thus, enhancement of GTPase activity of Dnm1 is probably due to increase in number of the highly ordered structure and upregulation of GTPase activity. However, we cannot rule out the possibility that the larger magnitude of constriction of the Dnm1-DYNAMO1 filament is caused by conformational differences between the

Figure 6. Conformational changes of Dnm1-DYNAMO1 filaments. (A) Negative staining electron micrographs represent structural dynamics of Dnm1-DYNAMO1 or Dnm1-DYNAMO1-H116D filaments upon addition of ATP and GDP, or GTP. Bar = 500 nm. (B) Typical structures of Dnm1-DYNAMO1 filament with or without addition of ATP and GDP are shown. Schematic image represents measurement of constriction lengths of the filaments under the various conditions (n = 50). Bar = 200 nm. Images were modified from Imoto et al. (2018).
Dnm1 filament and the Dnm1-DYNAMO1 filament. Higher resolution imaging is required to interpret molecular details of the enhancement.

We demonstrated that DYNAMO1-binding enhanced the magnitude of the constriction. However, DYNAMO1-H116D, which binds to Dnm1 and does not disrupt Dnm1 localization, localized on the membrane fission sites. Thus, in addition to the Dnm1-DYNAMO1 interaction, GTP generation itself is also important for the function of Dnm1 in the MD and POD machineries. A previous study has shown that GTPase kinetics in classical dynamin protein is highly regulated by GTP concentration (Morlot et al. 2012). We hypothesized that local GTP generation elevates the local concentration of GTP around the neck of the dividing mitochondria and peroxisomes to promote Dnm1 mediated membrane fission. To test this hypothesis, we compared the magnitude of constriction under two conditions; Dnm1-DYNAMO1 filaments with ATP and GDP, and Dnm1-DYNAMO1 filaments with GTP. However, the magnitude of constriction under these two conditions was not significantly different (Figure 6B), although mitochondrial and peroxisomal division was stalled in the absence of local GTP generation in vivo. Caveat between in vivo and in vitro experiment is due to different diffusion coefficients in vivo and in vitro. The diffusion coefficient of the free nucleotide in water is ~360 μm^2/s (Hubley et al. 1996), but is thought be 10 times slower in vitro because of molecular crowding, as seen in calcium signaling (Bless et al. 2011, Guerrier and Holcman 2018). However, our in vitro experiment lacked time resolution, and it is possible that we missed conformational intermediates of the Dnm1-DYNAMO1 filaments. Moreover, we cannot rule out the other model based on the impact of local GTP generation on dynamin family proteins, currently being discussed in the field (see discussion in the next section).

WHAT WE KNOW AND WHAT WE NEED TO KNOW ABOUT LOCAL GTP GENERATION DURING MEMBRANE FISSION EVENTS

How dynamin family GTPases are energetically supported during membrane fission, is an intriguing question. We demonstrated that a novel protein component of the MD and POD machineries, NDPK-like protein DYNAMO1, functions as a Dnm1 enhancer and a local GTP generator on the Dnm1 mediated membrane fission site of the mitochondrion and the peroxisome in C. merolae (Figure 7). These two functions are needed to complete membrane fission. Importantly, local GTP generation is most likely a conserved phenomenon among dynamin family proteins. The functional relationship between dynamin family proteins and NDPK was first reported in the fly neuro muscular junction. A genetic approach using Drosophila melanogaster identified enhancer of shibire (orthologue of mammalian classical dynamin mediating synaptic vesicle endocytosis) mutant, called abnormal wing disc (Awd) (Krishnan et al. 2001). Awd was found to code for nucleoside diphosphate kinase protein (NDPK); thus, NDPK is important for classical dynamin dependent endocytosis of synaptic vesicles in flies (Krishnan et al. 2001). In mammalian cells, NDPK has been reported to be involved during clathrin-mediated endocytosis (CME) of growth factors (Dammai et al. 2003, Boissan et al. 2014). NDPK function in classical dynamin-dependent and clathrin-independent endocytosis (CIE) of interleukin 2 has also

Figure 7. A working model of DYNAMO1 functions during the division of mitochondrion and peroxisome. Initially, DYNAMO1 is recruited to the division site of the mitochondrion, together with Dnm1 (Phase 1). DYNAMO1 is involved in Dnm1 recruitment to the division site. Next, DYNAMO1 converts ATP to GTP on the ring of the MD machinery. A cytosolic ATP source is used for this reaction. Upon GTP generation, the MD machinery is constricted and GTPase Dnm1 pinches off mitochondrion. Moreover, the DYNAMO1-Dnm1 structure effectively generates a strong motive force for the constriction (Phase 2). MD machinery containing DYNAMO1 and Dnm1 is immediately disassembled (Phase 3). After mitochondrial division, DYNAMO1 functions in the POD machinery in an analogous manner to that in mitochondrial division. Image was adapted from Imoto e et al. (2018).
been reported (Boissan et al. 2014). In all these cases, NDPK isoforms, Non-metastatic cells 1 and 2 (NME1 and NME2, respectively), colocalized with the clathrin adaptor, adaptor protein (AP)-2 (Boissan et al. 2014). Therefore, NDPK proteins are involved in both CME and CIE. Thus, NDPK function during classical dynamin-dependent membrane fission is promising. The function of dynamin-related proteins, including Dnm1, in our report is also supported by NDPK. The dynamin-related protein, optic atrophy-1 (OPA-1), is known to regulate inner mitochondrial membrane fusion, and the NME1 isoform NME4 provides GTP to OPA-1 in mammalian cells (Tokarska-Schlattner et al. 2008, Schlattner et al. 2013). Recently, the NME1 isoform NME3 was shown to regulate the dynamin-related protein mitofusion-1 and -2 (Mfn1 and Mfn2 respectively), during outer mitochondrial membrane fusion in mammalian cells. In this case, the GTP generating activity of NME3 was not required for the function of Mfn1/Mfn2 (Chen et al. 2019). Therefore, NDPK also function on these dynamin-related proteins.

In summary, NDPK activity is conserved across most of the dynamin family member-dependent membrane remodeling events. Therefore, referring knowledge of NDPK in the functions of other dynamin family proteins is important to understand how the MD and POD machineries is energetically supported by DYNAMO1. Currently, we are uncertain how local GTP generation is regulated and what is the impact of local GTP generation on mitochondrial and peroxisomal division. There are two models regarding regulation of local GTP generation; one suggests that GTP is channeled within the complex of dynamin family proteins and NDPK (Zala et al. 2017) (Figure 8A) and the other suggests enrichment of local GTP concentration around the neck of dividing organelles (Boissan et al. 2018, Imoto et al. 2018) (Figure 8B).

A. THE CHANNELING MODEL

In the channeling model of NDPK function, which was originally proposed for NME1/NME2-classical dynamin dependent endocytosis, GTP generation within the Dnm1-DYNAMO1 complex maximizes the efficiency of GTP delivery to the G-domain of Dnm1 (Figure 8A). The maximizing enzyme kinetics is usually occurring spatial proximity within the complex of multifunctional enzymes to be separated from the diffusion equilibrium, called “channeling” (Ovádi and Sere 2000). A well-known example of channeling is the glycolysis reaction between glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the phosphoglycerate kinase (PGK) complex (Weber and Bernhard 1982), and it is also proposed in the NDPK reaction (Zala et al. 2017). In our study, in vitro experiments done to compare the magnitude of constriction of the Dnm1-DYNAMO1 complex upon ATP+GDP and GTP addition, showed no significant difference (Figure 8A). Thus, local GTP generation and consumption of freely diffusive GTP exhibit no difference on the magnitude of constriction of the Dnm1-DYNAMO1 complex. This in vitro experiment suggested that channeling of GTP is less likely to occur. However, as we mentioned in the previous section, our in vitro experiment lacks time resolution, no significance in the magnitude of the constriction between the two conditions may be a result of the constriction reaching a plateau phase.

Problem in the channeling model is unbalanced kinetics between generation and hydrolysis of GTP. Turnover of NDPK is $K_{cat} = 600$ s$^{-1}$ (Lasca et al. 1997), which suggests $>$500–2000-fold higher enzyme kinetics than GTPase activity of membrane-bound classical dynamin or Dnm1 orthologues in yeast, algae and mammals (Warnock et al. 1996, Macdonald et al. 2016, Bohuszewicz and Low 2018). Thus, the catalytic domains of DYNAMO1 and G-domain of Dnm1 need to be in
close proximity to secure a high GTP concentration ratio and to promote GTP hydrolysis without diffusion of the nucleotides. However, efficient GTP hydrolysis requires the G-domain dimer between the adjusting helical turns in a contractile-ring or -helix of Dnm1 as well as classical dynamin (Chappie et al. 2010, Mears et al. 2011). In this case, interaction of NDPK domain and G-domain will compete with the formation of G-domain dimer. For the further analysis and discussion of the channeling model, structural analysis of the NDPK protein-dynamin family protein complex is needed.

B. THE ENRICHMENT MODEL

The enrichment model reconciles the fact that dynamin family proteins including Dnm1 have a low affinity for GTP and a high rate of GTP hydrolysis which cannot be supported by the physiological level of GTP. In our study, we saw that DYNAMO1 accumulated at a high level with Dnm1 at the neck of the dividing mitochondrion and peroxisome. Similar dynamics of NDPK protein has also been reported during classical dynamin and NME1/NME2 dependent CME in mammalian cells (Boissan et al. 2014). As mentioned above, the enzyme kinetics of NDPK is much higher than that of the GTPase activity of dynamin family proteins. Thus, a balance between GTP generation and consumption may not exist around the NDPK enriched membrane fission sites. In the cell, NDPK forms a tetramer-hexamer (Kim et al. 2003), and the functional membrane fission ring contains 26–40 molecules of classical dynamin during endocytosis (Cocucci et al. 2014) and ~100 molecules of Dnm1 orthologue Drp1 during mitochondrial fission in mammalian cells based on quantitation of endogenous GTP-tagged proteins (Michalska et al. 2018), which is ~200 nm in diameter (Ji et al. 2015) (diameter of Dnm1 positive MD machinery in vivo is <700 nm in C. merolae (Nishida et al. 2003)). Accurate modeling, including the number of G-domain dimers and number of helix turns, is required in the future. Given the parameters of enzyme kinetics between NDPK and Dnm1 or classical dynamin, excess amount of GTP may be generated around the membrane fission sites to increase the concentration of local GTP. However, the diffusion coefficient of nucleotides is ~360 μm²/s (Hubley et al. 1996), and it is uncertain whether GTP can be locally enriched in the environment. This could be understood by carefully considering the coupling between generation/consumption and diffusion of GTP. One way to locally enrich GTP is to modify the diffusion coefficient, as seen during the diffusion of calcium ions, whose diffusion coefficient is slowed down ~10 times because of molecular crowding or restriction of movement by the cytoskeleton and cellular organelles (Biess et al. 2011, Guerrier and Holcman 2018). Another possibility by which local enrichment can be achieved is liquid-phase separation/condensation. Recently, liquid-phase separation of cycling GMP-AMP synthase (cGAS), which converts GTP and ATP to cAMP, has been shown in mammalian cells (Du and Chen 2018). In this study, enrichment of ATP or GTP within the cGAS-DNA liquid droplets has been demonstrated. Thus, even small molecules such as ATP and GTP can be phase separated. However, there is only little information on buffering proteins/factors that can change the diffusion coefficient of GTP, or on intrinsically disordered proteins that help phase separation/condensation of GTP around membrane fission sites. For example, transmission electron microscopy images of platinum replicas of an unroofed cell showed that the mitochondria were surrounded with a dense cytoskeletal network in mammalian cells (Yang and Svvitkina 2019). During CME, early stage endocytic proteins have been shown to form phase separated droplets on the endocytic sites (Bergeron-Sandoval et al. 2017).

In summary, the field is left with the challenge of reconciling with two apparently opposing models; one in which the NDPK reaction and the GTP catalytic reaction are taking place within closed space to maximize enzyme kinetics, without the nucleotides diffusing away, and the other in which DYNAMO1 generates locally enriched concentration of GTP around the MD and POD machineries. Of course, the two models are not necessarily mutually exclusive. To understand the mechanism clearly, more information is needed on how Dnm1 and DYNAMO1 interact, and how GTP molecules are mobilized around the membrane fission sites.

IMPACT OF GTP GENERATING ACTIVITY ON DNM1 FUNCTION

Local GTP generation is promising and conserved among various NDPK protein isoforms and dynamin family members. Therefore, GTP generating reaction of NDPK and GTP hydrolysis of dynamin family proteins are most likely coupled. GTP is needed at two steps for Dnm1 function during mitochondrial and peroxiosomal division. First, GTP binding is required for the recruitment of Dnm1 from cytoplasm to the membrane. Second, GTP hydrolysis is required for membrane fission. In this section, we will discuss how DYNAMO1 function couples these two steps.

In mammals, GTP-binding drives conformational changes in Dnm1 orthologue Drp1 which further allows for the Drp1-MiD49 interaction (Kalia et al. 2018). The transmembrane protein MiD49 is anchored to the mitochondrion (Palmer et al. 2011) and Drp1-MiD49 interaction is the key for the recruitment of Drp1 (Losón et al. 2015). In our research, DYNAMO1 showed enhancement of G-domain, and was needed for the recruitment of Dnm1 from the cytosol to the mitochondrial membrane. Therefore, we propose that DYNAMO1 facilitates GTP-binding to Dnm1. C. merolae does not encode MiD49, however C. merolae Dnm1 shows similar conformational changes as well as mammalian Drp1 upon GTP binding (Bohuszewicz and Low 2018). This conformational change can expose B-insert region to interact with membrane. In C. merolae, the B-insert may play an important role in the recruitment instead of MiD49. It is also possible that adaptor proteins of Dnm1, such as WD40 repeat-containing protein, mitochondrial division apparaturus 1 (Mda1) (Nishida et al. 2007), may be involved. A previous study has shown that Mda1 accumulates on the equatorial plane of the mitochondrion where membrane fission occurs, prior to...
Dnm1 recruitment (Nishida et al. 2007). Thus, Mda1 is likely to be an important candidate as the functional analogue of MiD49. To understand how the Dnm1-DYNAMO1 complex interacts with Mda1 on the neck of the dividing mitochondrion is an important question. In contrast, peroxisomal membranes do not contain MiD49 in mammalian cells (Palmer et al. 2013). In our study, peroxisomal division was not impaired by the inhibition of gene expression of DYNAMO1, although DYNAMO1-H116D expression strongly inhibited its division. Thus, it is unclear how cytosolic Dnm1 is recruited to the peroxisome in both of our study and mammalian cells in terms of GTP-related regulations. However, efficiency of DYNAMO1 gene suppression was observed to be ~80% in this study, and the remaining 20% may be sufficient to recruit DYNAMO1 to the peroxisome. To understand the mechanism further, we will need a Dnm1 unbound form of DYNAMO1 mutant which can act as a dominant negative, or identify unknown candidate proteins involved in the recruitment of Dnm1 to the peroxisome in C. merolae.

During membrane fission, GTP hydrolysis of Dnm1 drives pinching-off of the membrane. Although extensive research has been done for decades in the field of classical dynamin and Dnm1, how membrane pinching occurs is not known. One possible model is the ratchet model which is originally proposed in function of classical dynamin and also applied to Dnm1 (Mears et al. 2007, 2011, Chappie et al. 2010, 2011). In this model, constriction of the neck is achieved by sliding the adjusting turns of Dnm1 rings or helix upon GTP hydrolysis. One of the caveats of this model is the burning GTP during constriction. GTP is consumed during sliding and thus multiple cycles of GTP hydrolysis happen until the diameter of the neck reaches the limit for spontaneous membrane fission, at ~4 nm. Moreover, the basal rate of GTP hydrolysis of polymerized Dnm1 orthologues in yeast, algae and mammals on the membrane is dramatically elevated which is so-called assembly-stimulated GTPase (Ingerman et al. 2005, Macdonald et al. 2016, Bohuszewicz and Low 2018). Thus, how a cell provides sufficient amount of GTP is an important question. In our study, DYNAMO1 accumulated with Dnm1 on the neck of the dividing mitochondrion and peroxisome. DYNAMO1 locally generated GTP for Dnm1 function on the MD and POD machineries. As discussed in previous sections, we hypothesized that function of G-domain is maximized by channeling GTP to Dnm1 or by enriching the local GTP concentration. We are still uncertain on whether one of these models or some other model explains how GTP accumulates; however, we know for certain that local GTP generation supports Dnm1 energetically to generate the force required for the constriction of the membrane. We envisage that in addition to supplying sufficient GTP for Dnm1, local GTP generation regulates the coordinated action of GTP hydrolysis. For the proper constriction activity of dynamin family proteins, coordinated action of GTP hydrolysis is required (Liu et al. 2013, Antony et al. 2016). For example, if a given proportion of G-domain dimers are not hydrolyzing GTP while others are hydrolyzing GTP, or if adjusting G-domain dimers do not perform GTP hydrolysis synchronously, there would be no sliding. Thus, GTPase activity and mechanochemical cycles of classical dynamin/Dnm1-ring or -helix should be coupled. In vitro, GTP hydrolysis by the G-domain in the contractile structure is not coordinated in classical dynamin (Tuma and Collins 1994). In the mammalian cell, GTP-hydrolysis transition state specific nanobody of the classical dynamin has recently been developed (Galli et al. 2017). Live cell Total Internal Reflection Fluorescence (TIRF) microscopy of the nanobody showed transient bursts of GTP hydrolysis during CME; thus, timing of GTP hydrolysis is well coordinated in the case of classical dynamin. Classical dynamin and Dnm1 orthologues in yeast, algae, and mammals are sharing same mechanochemical features such as G-domain dimerization and assembly-stimulated GTPase activity (Ingerman et al. 2005, Macdonald et al. 2016, Bohuszewicz and Low 2018); therefore, GTP hydrolysis during membrane fission of the mitochondrion and peroxisome may be also coordinated in the cell. The mechanism that triggers the coordinated action of GTP hydrolysis is unknown. In both cases of Dnm1-mediated mitochondrial and peroxisomal divisions and classical dynamin-mediated CME, the local GTP generation is necessary (Boisson et al. 2014, Imoto et al. 2018). Local GTP generation and activation of G-domain through NDPK proteins may trigger the coordinated action of GTP hydrolysis.

CONCLUSIONS

In this review, we have summarized our study regarding NDPK-like protein DYNAMO1. DYNAMO1 was found to be involved in two important functions; 1) recruitment of Dnm1 to the neck of the dividing mitochondrion, and 2) promoting constriction of the MD and POD machineries. We have also discussed other issues in our study and the field to understand the molecular mechanism by which NDPK proteins and dynamin family proteins function. To solve the issues, we will need to develop various approaches in the future. For understanding the GTP channeling and local GTP enrichment models, information about the structure of the Dnm1-DYNAMO1 interface and visualization of GTP concentration is needed. Recently, a circularly permuted YFP (cpYFP)-based GTP sensor has been developed (Bianchi-Smiraglia et al. 2017). GTP imaging using the cpYFP may open another door in the field of NDPK protein and dynamin family protein research. The working model of NDPK protein and dynamin family proteins is also unknown currently. When does local GTP generation start? What is the minimum functional unit for efficient fission, is it a single ring or helix? How many NDPK proteins are required per membrane fission machinery and what is the geometry of the NDPK proteins in the membrane fission machinery? To address these questions, ultimately visualization of the structure of the membrane fission machinery is required using in situ cryo-electron tomography and subtomogram averaging techniques. Although these are considerably challenging, it will provide a new direction to understand membrane fission events conserved across eukaryotic cells.
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