A dimeric equilibrium intermediate nucleates Drp1 reassembly on mitochondrial membranes for fission

Patrick J. Macdonalda,b,*, Natalia Stepanyantsa,b,*, Niharika Mehrotrab, Jason A. Mearsb,c, Xin Qic,d, Hiromi Sesakia, and Rajesh Ramachandran a

aDepartment of Physiology and Biophysics, bDepartment of Pharmacology, and cCenter for Mitochondrial Diseases, Case Western Reserve University School of Medicine, Cleveland, OH 44106; dDepartment of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD 21205

INTRODUCTION

The mitochondria of eukaryotic cells form elaborate networks of branched, tubular structures that constantly undergo fission and fusion (Chan, 2012). A delicate balance of these counteracting processes is essential for the maintenance of mitochondrial size, distribution, homogeneity, and inheritance (Okamoto and Shaw, 2005; Youle and van der Bliek, 2012; Friedman and Nunnari, 2014). In addition, mitochondrial dynamics are intricately linked to apoptosis (Hoppins and Nunnari, 2012). Emerging evidence indicates that defects in mitochondrial dynamics precipitate neurodegenerative diseases such as Alzheimer’s, Huntington’s, and Parkinson’s (Itoh et al., 2013) and may be critically involved in cardiac dysfunction (Ong and Haunsey, 2010).

Dynamin-related protein 1 (Drp1; Figure 1A) mediates mitochondrial membrane fission (Bui and Shaw, 2013; Elgass et al., 2013). Located predominantly in the cytosol, Drp1, in response to a variety of physiological cues, is recruited to the outer mitochondrial membrane (OMM), where it presumably organizes into a large helical polymer that wraps around the organelle at discrete, predetermined division sites (Friedman et al., 2011; Korobova et al., 2013). Here Drp1 is believed to exert its mechanochemical activity by constricting the mitochondrial double membrane to the point of fission. Drp1 targeting to potential mitochondrial division sites is facilitated through interactions with OMM-anchored adaptor proteins such as fission factor 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/51; Loson et al., 2013). Here Drp1 is believed to exert its mechanochemical activity by constricting the mitochondrial double membrane to the point of fission. Drp1 targeting to potential mitochondrial division sites is facilitated through interactions with OMM-anchored adaptor proteins such as fission factor 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins of 49 and 51 kDa (MiD49/51; Loson et al., 2013).

Our mechanistic understanding of how Drp1 mediates membrane fission stems primarily from in vitro studies of its yeast homologue, Dnm1p (Lackner and Nunnari, 2009). Purified Dnm1p, which

ABSTRACT

The GTPase dynamin-related protein 1 (Drp1) catalyzes mitochondrial division, but the mechanisms remain poorly understood. Much of what is attributed to Drp1’s mechanism of action in mitochondrial fission parallels that of prototypical dynamin in endocytic vesicle scission. Unlike the case for dynamin, however, no lipid target for Drp1 activation at the mitochondria has been identified. In addition, the oligomerization properties of Drp1 have not been well established. We show that the mitochondria-specific lipid cardiolipin is a potent stimulator of Drp1 GTPase activity, as well as of membrane tubulation. We establish further that under physiological conditions, Drp1 coexists as two morphologically distinct polymeric species, one nucleotide bound in solution and the other membrane associated, which equilibrate via a dimeric assembly intermediate. With two mutations, C300A and C505A, that shift Drp1 polymerization equilibria in opposite directions, we demonstrate that dimers, and not multimers, potentiate the reassembly and reorganization of Drp1 for mitochondrial membrane remodeling both in vitro and in vivo.

Monitoring Editor

Thomas D. Fox
Cornell University

Received: Feb 7, 2014
Revised: Apr 14, 2014
Accepted: Apr 21, 2014

Monitoring Editor

Thomas D. Fox
Cornell University

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E14-02-0728) on April 30, 2014.

*These authors contributed equally to this work.

Address correspondence to: Rajesh Ramachandran (rxr275@case.edu).

Abbreviations used: CL, cardiolipin; Drp1, dynamin-related protein 1; Fis1, fission factor 1; FRET, Förster resonance energy transfer; GUV, giant unilamellar vesicle; MALS, multiangle light scattering; MiD 49/51, mitochondrial dynamics proteins of 49 and 51 kDa; Mff, mitochondrial fission factor; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SEC, size-exclusion chromatography; WT, wild type.

© 2014 Macdonald, Stepanyants, et al. This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).

“ASCB,” “The American Society for Cell Biology,” and “Molecular Biology of the Cell®” are registered trademarks of The American Society for Cell Biology.
exists as multimers in solution, propagates into helical polymers of diameter comparable to mitochondrial division sites in vivo when assembled onto membrane tubules derived from negatively charged liposomes (Ingerman et al., 2005). Cooperative GTP hydrolysis triggers a large, concerted conformational rearrangement in the preassembled Drp1 polymer, which mechanistically constrains the underlying membrane tubule and poises it for fission, similar in principle to prototypical dynamin in endocytic vesicle scission (Mears et al., 2011). Mutations that limit Dnm1p self-assembly to a terminal dimer in solution also impair helical propagation and cooperative GTPase stimulation (Ingerman et al., 2005).

By contrast, the oligomerization properties of mammalian Drp1 appear distinct. However, these have not been unambiguously established. Mammalian Drp1 reportedly exists in solution as stable dimers (Koirala et al., 2013), tetramers (Yoon et al., 2001; Bossy et al., 2010), or in dynamic dimer–tetramer equilibrium (Chang et al., 2010; Frohlich et al., 2013). Furthermore, Drp1 helices formed in the presence of GTP analogues in solution (~30–50 nm diameter), unlike those of Dnm1p (Ingerman et al., 2005), appear morphologically distinct from the corresponding helices formed on negatively charged membrane templates, which can vary greatly in diameter from 30 to >150 nm, depending on the Drp1 isoform and/or conditions used (Yoon et al., 2001; Bossy et al., 2010; Frohlich et al., 2013; Koirala et al., 2013). Whether these differences reflect isoform-specific properties of Drp1 (Strack et al., 2013) and/or the inherent property of the pure phosphatidylserine (PS) liposomes used to undergo variable shape transformations (Hauser and Phillips, 1973; Fuller et al., 2003) is yet to be determined. Moreover, although the functional significance of distinct Drp1 polymerization in solution versus that on membranes remains to be established, it is unclear whether Drp1 multimerization in solution is essential for nucleation of Drp1 higher-order self-assembly on membranes (Ingerman et al., 2005; Bhar et al., 2006).

Here we report that the mitochondria-localized lipid cardiolipin potently activates Drp1 and supports membrane tubulation. With the identification and characterization of two new mutants, we further reveal that Drp1 exists in dynamic equilibrium between two morphologically distinct oligomeric states in the cell, one in the cytosol and the other localized on mitochondrial membranes, which equilibrate via a dimeric assembly intermediate. We show that dimers, and not multimers, potentiate the assembly and reorganization of Drp1 on mitochondria for membrane remodeling and fission.

RESULTS

Cardiolipin is a potent stimulator of Drp1 GTPase activity

Although pure PS liposomes are routinely used as templates for assaying Drp1 activity in vitro, this lipid is essentially absent (~1 mol%) at the mitochondria (Kagan et al., 2005; van Meer et al., 2008; Horvath and Daum, 2013). OMM-mimetic liposomes composed of a variety of negatively charged lipids were shown to bind and enzymatically stimulate yeast Dnm1p, but the activating lipid target(s) were obscure (Lackner et al., 2009). Through an extensive lipid

![Figure 1](image-url)
screen (Supplemental Figure S1A), we identified the negatively charged, mitochondria-specific lipid cardiolipin to be the most potent stimulator of Drp1’s cooperative GTPase activity on membranes (Figure 1, B and C). Cardiolipin (CL), found predominantly in the inner mitochondrial membrane (IMM), is reportedly enriched at "contact sites" between the IMM and OMM at concentrations up to 25 mol% (Ardail et al., 1990; Schlattner et al., 2014; Tatsuma et al., 2014). This corresponded to the typical CL content of our "contact site" biomimetic liposomes. No stimulation observed for Drp1 at CL concentrations ≤10 mol% suggested that "CL-enriched microdomains" at the OMM may indeed function as "recruitment platforms" for Drp1 activation (Sorice et al., 2009; Figure 1D). At an equivalent mole fraction, PS did not substantially increase Drp1 GTPase activity (Supplemental Figure S1A). Other negatively charged lipid species present at the OMM, namely phosphatidic acid (PA) and phosphatidylglycerol (PG), enhanced Drp1 GTPase activity, although not nearly to the same extent as CL (Supplemental Figure S1A).

We next investigated Drp1 in the presence of various phosphoinositides to determine whether the extent of Drp1 GTPase stimulation was positively correlated with the negative charge density of lipid headgroups. A recently proposed role for Drp1 in synaptic vesicle endocytosis also suggests a likely interaction with phosphoinositides at the plasma membrane (Li et al., 2013). Remarkably, no such correlation was evident (Supplemental Figure S1B). Indeed, phosphatidylinositol-4-phosphate (PI4P) was substantially more effective than the more negatively charged phosphatidylinositol-4,5-bisphosphate (PI4,5P2) in stimulating Drp1 GTPase activity. Phosphatidylinositol (PI) of negative charge density lower than PI4P was, on the other hand, ineffective. Although the physiological relevance of these findings remains to be established, our data nevertheless indicate that CL is a mitochondria-specific, lipid stimulator of Drp1 GTPase activity and that Drp1 recognition of CL is not solely based on headgroup negative charge density.

**Drp1 tubulates CL-containing liposomes**

A previous study postulated that Drp1 mediates mitochondrial membrane fission by catalyzing the formation of a hemi-fusion/fission intermediate in a CL-dependent manner (Montessuit et al., 2010). However, no membrane constrict or tubulation necessary for such an event was apparent in that study. Instead, Drp1 activity was limited to the tethering and hemifusion of freely suspended liposomes. Moreover, under the conditions used, neither higher-order Drp1 self-assembly nor assembly-dependent stimulation of GTPase activity on CL-containing liposomes was evident. We therefore reevaluated the behavior of Drp1 on CL-containing liposomes.

To this end, we used a system of surface-immobilized giant unilamellar vesicles (GUVs) as model membrane templates to visualize Drp1-mediated membrane remodeling in real time. We indeed found that Drp1 was recruited to the GUV membrane surface in a CL-dependent manner (Figure 2A). Extensive remodeling of the membrane into narrow, protein-coated membrane tubules, however, further ensued (Figure 2B and Supplemental Figure S2). This phenomenon was not observed previously (Montessuit et al., 2010).

Electron microscopy (EM) examination of the membrane tubule ultrastructure revealed a uniform pattern of helical Drp1 self-assembly that approximated 100–150 nm in diameter (Figure 2C). These dimensions were comparable to the diameter of mitochondrial constrict sites observed in vivo (Ingerman et al., 2005; Friedman et al., 2011).

On pure PS liposomes, Drp1 formed helices of mostly comparable diameter (Supplemental Figure S3A). However, helical regions of highly variable diameter were also evident (Supplemental Figure S3A), indicating that use of this inherently transformable membrane template may partially account for the observed differences in membrane tubule diameters for the same, or between different, Drp1 isoform(s) in independent studies (compare our results with those of Frohlich et al., 2013, and Koirala et al., 2013). That Drp1 could form curvature-adaptable polymers on membranes was further evident from its helical assembly on preformed CL-containing lipid nanotubes of ~30 nm diameter (Supplemental Figure S3B).

**Drp1 constitutes two morphologically distinct polymers**

Drp1 reportedly forms helical polymers of uniformly narrow diameter (~30 nm) on PS membrane tubules when either GTP/PS, a slowly hydrolyzable GTP analogue (Yoon et al., 2001), or GTP (Koirala et al., 2013) is added. Drp1 can indeed form polymers of similar diameters in solution even in the absence of a membrane template when either GMP-PCP, a nonhydrolyzable GTP analogue (Figure 3A), or GTP S (Yoon et al., 2001; Bossy et al., 2010; Koirala et al., 2013) is present. To determine whether GTP binding causes a pronounced constriction of the proportionally large, membrane-bound helical polymer to approach the very narrow diameters found for Drp1 helices in solution, we incubated Drp1 with CL-containing liposomes in the presence of GMP-PCP. Remarkably, GMP-PCP did not elicit any such constriction (Figure 3B). This result was indeed similar to that previously observed for yeast Dnm1p (Mears et al., 2011). Instead, we observed the emergence of a substantial fraction of the tightly wound, helical polymers described earlier for Drp1 in solution (Figure 3B). The coexistence of these morphologically distinct species under steady-state conditions suggested that Drp1 populates two polymeric states, one in solution and the other membrane associated, which relate via dynamic equilibrium. To ascertain this possibility, we next investigated the polymerization equilibria of Drp1.

**Drp1 displays complex polymerization equilibria in solution**

Size-exclusion chromatography (SEC) under physiological ionic strength conditions (150 mM KCl in buffer solution) revealed a very broad elution spectrum for Drp1 wild type (WT; Figure 4A, top). These data indicated that Drp1 exists in a fast dynamic equilibrium between multiple oligomeric states in solution. The existence of such equilibria was evident from consequent shifts in the Drp1 WT SEC elution profile as a function of loaded protein concentration (Figure 4B, top).

In a screen for mutants that slightly altered Drp1 polymerization equilibria but were otherwise comparable to WT in enzymatic activity (Supplemental Figure S4), we identified Drp1 C345A and C607A. Native Cys residues were substituted, as their electrophilic modifications were shown to alter Drp1 oligomerization (Cho et al., 2009; Mishra et al., 2010). Fortuitously, these mutants exhibited discernible peaks within their broad SEC elution profiles (Figure 4A, middle). Coupled multangle light scattering (MALS) measurements (see Materials and Methods for a description) of the various peaks within revealed that Drp1 exists in a dynamic, dimer–oligomer equilibrium in solution and propagates in quantized dimeric increments (Figure 4A, middle, right axis). Thus the minimal functional assembly subunit of Drp1 under physiological conditions is a dimer, and not a tetramer, as previously suggested.

We also identified two additional mutants of Drp1, C300A and C505A (Figure 1A), that exhibited dramatically altered oligomerization properties relative to WT (Figure 4A, bottom). SEC-MALS analyses revealed that Drp1 C300A was supra-oligomeric in solution, with a greater propensity to form higher-order structures even at low

---

Volume 25 | June 15, 2014 | 1907

Polymerization equilibria in Drp1
protein concentrations (Figure 4B, middle). Drp1 C505A, on the other hand, was predominantly dimeric in solution even at relatively high protein concentrations (Figure 4B, bottom). Nevertheless, both mutants exhibited robust polymerization equilibrium dynamics with respect to protein concentration.

In the presence of GMP-PCP, both Drp1 C300A and Drp1 C505A constituted helical polymers in solution (Figure 4C), albeit with varied efficiencies relative to WT. In centrifugation assays that reliably sediment these high-density protein polymers, both mutants appeared partially impaired relative to WT (Figure 4D), indicating that altered local structural dynamics caused by the mutations (Figure 1A) also affect Drp1 helical propagation in solution. In the constant presence of

**Figure 3:** Morphologically distinct polymers of Drp1. (A) Tightly wound helical polymers of Drp1 formed in the presence of GMP-PCP in solution. (B) Coexisting solution-suspended (slender arrows) and membrane-associated (block arrows) helical polymers of Drp1 found in the presence of GMP-PCP and CL-containing liposomes. Representative EM images. Scale bar, 100 nm.
the nucleation of its higher-order self-assembly on membranes (Chang et al., 2010).

In a marked departure from dynamin, we found that dimeric Drp1 C505A bound CL-containing liposomes equally well as Drp1 C300A and Drp1 WT (Figure 5A). Because the conventional centrifugation assay cannot reliably distinguish between solution-suspended oligomers of Drp1 and its membrane-bound polymers, we developed a Förster resonance energy transfer (FRET)–based approach to assay directly for Drp1–membrane association. To this end, we used the lone B-insert tryptophan (Trp) residue, W552, as a FRET energy “donor” for “acceptor” 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (Dansyl-PE) lipid molecules distributed randomly across the CL-containing membrane surface. Identical increases in Dansyl emission intensity upon Trp excitation for all three species revealed comparable membrane association (Figure 5A, left), a property also reflected in spin-sedimentation profiles (Figure 5A, right).

Remarkably, however, Drp1 C505A was significantly more active in tubulating CL-containing membranes than both Drp1 C300A and Drp1 WT (Figure 5B). Consistent with this observation, the assembly-stimulated GTP hydrolysis rate of Drp1 C505A was also significantly higher than that of both Drp1 C300A and WT (Supplemental Figure S5). From these data, we conclude that mutations C300A and C505A, under physiological conditions in solution, differentially alter Drp1 polymerization equilibria by shifting them in opposite directions to supra-oligomers and dimers, respectively.

Dimers potentiate Drp1-mediated membrane remodeling both in vitro and in vivo

Our current impressions of how Drp1 functions in mitochondrial division stem largely from parallels drawn to dynamin in endocytic vesicle scission (Bui and Shaw, 2013). For instance, Drp1 multimerization in solution (tetramerization or higher), by analogy with dynamin (Ramachandran et al., 2007), is believed to be essential for the nucleation of its higher-order self-assembly on membranes (Chang et al., 2010).

In a marked departure from dynamin, we found that dimeric Drp1 C505A bound CL-containing liposomes equally well as Drp1 C300A and Drp1 WT (Figure 5A). Because the conventional centrifugation assay cannot reliably distinguish between solution-suspended oligomers of Drp1 and its membrane-bound polymers, we developed a Förster resonance energy transfer (FRET)–based approach to assay directly for Drp1–membrane association. To this end, we used the lone B-insert tryptophan (Trp) residue, W552, as a FRET energy “donor” for “acceptor” 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (Dansyl-PE) lipid molecules distributed randomly across the CL-containing membrane surface. Identical increases in Dansyl emission intensity upon Trp excitation for all three species revealed comparable membrane association (Figure 5A, left), a property also reflected in spin-sedimentation profiles (Figure 5A, right).

Remarkably, however, Drp1 C505A was significantly more active in tubulating CL-containing membranes than both Drp1 C300A and Drp1 WT (Figure 5B). Consistent with this observation, the assembly-stimulated GTP hydrolysis rate of Drp1 C505A was also

GTP, however, the two mutants behaved very differently. Drp1 C505A, similar to WT, did not propagate into sedimentable higher-order polymers, consistent with the role of GTP hydrolysis in limiting Drp1 self-assembly (Figure 4D, bottom). Drp1 C300A, on the other hand, retained a significant fraction of sedimentable higher-order polymers, which appeared resistant to disassembly by GTP hydrolysis (Figure 4D, middle). Consistent with this observation, Drp1 C300A exhibited a significantly higher basal GTPase activity than both Drp1 C505A and WT (Supplemental Figure S5). From these data, we conclude that mutations C300A and C505A, under physiological conditions in solution, differentially alter Drp1 polymerization equilibria by shifting them in opposite directions to supra-oligomers and dimers, respectively.
If the foregoing model for Drp1 function holds true, Drp1 C505A should be considerably more active than Drp1 C300A in supporting mitochondrial division, when each is expressed to a comparable level in Drp1-knockout (KO) cells. Indeed, dimeric Drp1 C505A was significantly more effective than supra-oligomeric Drp1 C300A in rescuing the mitochondrial fission defect (Figure 6 and Supplemental Figure S6). Oligomerization morphology and equilibria thus modulate Drp1 function in mitochondrial membrane remodeling both in vitro and in vivo.

**DISCUSSION**

The data presented here significantly advance our understanding of Drp1’s mechanism in mitochondrial division and, of importance, reconcile some disparate findings that have been in the field since its inception. Early studies on purified yeast Dnm1p indicated that the minimal subunit of Dnm1p under physiological conditions is a dimer (Ingerman et al., 2005; Bhar et al., 2006). By contrast, mammalian Drp1 was shown to be a tetramer (Yoon et al., 2001; Bossy et al., 2010). This difference in oligomeric state was attributed to the variable requirements for OMM adaptor proteins that exist between the significantly greater than that of Drp1 WT when assayed at low limiting protein concentrations (Figure 5C). Lipid stimulation of Drp1 C300A, on the other hand, was dramatically impaired (Figure 5C). Thus the extents of Drp1-mediated membrane remodeling and lipid-dependent GTPase stimulation appeared anticorrelated to Drp1 oligomerization propensity in solution. In other words, the larger the Drp1 oligomer in solution, the lower was its potential to constitute remodeling-competent higher-order polymers on membranes.

From these data, we conclude that Drp1 exists in dynamic equilibrium between two morphologically distinct oligomeric states, one in solution and the other on the membrane, which equilibrate via a dimeric assembly intermediate (Figure 5D). Drp1 C505A, stabilized as minimal dimers in solution, can thus readily reorganize on the membrane to form the distinct membrane-associated polymers required for membrane remodeling. By contrast, in both Drp1 WT and C300A, this reorganization presumably entails, first, disassembly of the preexisting solution oligomers into dimers and, further, subsequent reassembly of the dimers into membrane-associated polymers to engage in membrane remodeling.

The data presented here significantly advance our understanding of Drp1’s mechanism in mitochondrial division and, of importance, reconcile some disparate findings that have been in the field since its inception. Early studies on purified yeast Dnm1p indicated that the minimal subunit of Dnm1p under physiological conditions is a dimer (Ingerman et al., 2005; Bhar et al., 2006). By contrast, mammalian Drp1 was shown to be a tetramer (Yoon et al., 2001; Bossy et al., 2010). This difference in oligomeric state was attributed to the variable requirements for OMM adaptor proteins that exist between the...
two types of organisms (Bhar et al., 2006; Chan, 2012). We show that human Drp1 under physiological conditions exists in dynamic equilibrium between multiple oligomeric states in solution and propagates in quantized dimeric increments. A similarly broad SEC elution profile observed previously for Dnm1p (Ingerman et al., 2005) may indeed reflect similar dynamics. We conclude that Dnm1p and Drp1 are both minimal dimers in solution.

Our data suggest that CL-enriched microdomains constitute lipid targets that specifically recruit Drp1 to the OMM. However, Drp1 is also recruited to peroxisomal membranes, whose CL content in mammalian cells, although considered negligible, is uncertain (Schumann and Subramani, 2008; Schrader et al., 2012). Enriched in the peroxisomes of yeast, CL has not been biochemically detected in the peroxisomes of mammalian cells (Fujiki et al., 1982; Zinser et al., 1991; Leber et al., 1994; Wriessnegger et al., 2007). However, the recent discovery of mitochondria-derived vesicles that deliver selective protein cargo from the OMM to peroxisomes suggests that CL may indeed be cotransported to peroxisomes via these vesicular membrane carriers (Neuspiel et al., 2008; Schumann and Subramani, 2008). In the absence of irrefutable evidence that supports a role for CL in Drp1 peroxisome recruitment, we speculate that PA, a negatively charged lipid enriched in maturated peroxisomes, with biophysical properties similar to CL and also capable of stimulating Drp1 (Supplemental Figure S1A), plays a corresponding role (Athenstaedt and Daum, 1999; Guo et al., 2007; Mileykovskaya and Dowhan, 2009; Mileykovskaya et al., 2009). Containing a significant fraction of membrane cholesterol, peroxisomal membranes may similarly constitute PA-enriched “rafts,” which in turn recruit Drp1 (Woudenberg et al., 2010).

If a direct lipid interaction permits Drp1 association with mitochondrial and peroxisomal membranes as suggested, how can we reconcile the essential roles of membrane-anchored Drp1 adaptors Fis1, Mff, and MiD49/51 in Drp1 recruitment in vivo? It is plausible that a “coincidence detection” of both membrane CL and adaptors at mitochondrial division sites may enhance Drp1 binding to the mitochondria in a synergistic manner and at lower CL concentrations generally found at the OMM (McMahon and Gallop, 2005). It is also plausible that adaptors serve a regulatory role in the Drp1 mechanism by functioning across various checkpoints in the Drp1 oligomerization and GTP hydrolysis cycles (Figure 5D), either upstream or downstream of direct Drp1-membrane interactions. Interestingly, Fis1 and Mff localize to both mitochondrial and peroxisomal membranes and may function in a similar capacity at these distinct membrane compartments to recruit and differentially regulate Drp1 (Schrader et al., 2012; Shen et al., 2014). MiD49/51, restricted to the mitochondria, on the other hand, may exhibit functions that are considerably different from those of Mff and Fis1, as suggested by recent studies (Palmer et al., 2011, 2013; Koirala et al., 2013; Loson et al., 2014; Richter et al., 2014). Elucidation of the specific roles of adaptor proteins in the Drp1 mechanism awaits further examination.

Our studies reveal that under physiological conditions, Drp1 constitutes two morphologically distinct helical polymers. One is a tightly constricted polymer ~50 nm in diameter stabilized by GTP binding in solution, and the other is a relaxed polymer ~100–150 nm in diameter that circumscribes constricted mitochondrial membranes. A lack of pronounced membrane constriction in the membrane-bound polymer upon GMP-PCP addition (i.e., from a starting diameter of ~100–150 nm down to ~30–50 nm found for Drp1 helices in solution) indicates that GTP binding has differential effects on Drp1 molecules suspended in solution versus those bound to lipid. The coexistence of these distinct polymeric populations in vitro, however, correlates well with early observations of punctate yet mobile Dnm1p-GFP structures in the yeast cytosol present alongside discrete Dnm1p foci on the mitochondrial surface (Naylor et al., 2006).

With the characterization of two newly identified mutants, Drp1 C300A and Drp1 C505A, that exhibit dramatically altered oligomerization propensities relative to WT, we further demonstrate that solution dimers, and not multimers, potentiate the reorganization and reassembly of Drp1 on mitochondrial membranes for remodeling and fission. Of interest, the respective locations of the two mutations in the Drp1 crystal structure (Figure 1A) correlate well with their differential effects on Drp1 oligomerization. C505 is located at self-assembly interface 3 of the Drp1 “stalk” involved in stabilizing lateral dimer–dimer contacts in the helical polymer (Frohlich et al., 2013). C300, on the other hand, is positioned at a critical flexion point in the G domain–BSE interface that is responsive to nucleotide-dependent conformational changes originating at the opposite G domain dimerization interface (Wenger et al., 2013). Not
surprisingly, mutations C300A and C505A alternately stabilize or destabilize self-assembly in Drp1, respectively.

In contrast to C505A, all other mutations that reportedly stabilize Drp1 as a dimer in solution also significantly impair function. It is important to note that unlike the charge neutral C505A substitution, all such mutations involve either elimination of a critical surface charge (R403A in Drp1) or introduction of a new surface charge (E490R in Drp1, G385D and G436D in Dnm1p; Ingerman et al., 2005; Ford et al., 2011; Frohlich et al., 2013) that disrupts key ionic interactions necessary for higher-order Drp1 self-assembly. Regardless of such differences, the unaffected membrane binding of Drp1 C505A observed in vitro is in general agreement with the uninhibited mitochondrial recruitment of dimeric Dnm1p G385D reported previously (Bhar et al., 2006). These results further establish that multimerization in the cytosol is not essential for, and is indeed counterproductive to, Drp1 mitochondrial recruitment.

On the basis of a direct correlation between the differential biochemical/biophysical properties of Drp1 C300A and Drp1 C505A on CL-containing membranes in vitro and their corresponding efficacies in supporting mitochondrial fission in vivo, we propose that CL-enriched “contact sites” constitute specific lipid targets for Drp1 activation, as well as the concerted remodeling of the mitochondrial double membrane. Coincidence detection of OMM adaptor proteins at these “contact sites” may accelerate Drp1 retention and reorganization on the OMM for fission. We speculate that CL-enriched “contact sites” exist spatially overlapped with mitochondrial constriction sites generated by tubular endoplasmic reticulum membrane contact, previously shown to recruit Drp1 (Friedman et al., 2011).

On the basis collectively of the foregoing data, we propose that Drp1 polymers found in the cytosol function primarily as “reservoirs” primed to generate “fission-competent dimers” for mitochondrial recruitment and remodeling. The priming transitions presumably occur either through the action of basal GTP hydrolysis and/or via the effect of Drp1 adaptor proteins localized at the OMM. We further speculate that the various posttranslational modifications of Drp1 (phosphorylation, S-nitrosylation, etc.) that govern function in vivo (Chang and Blackstone, 2010) do so via regulation of Drp1 polymerization dynamics.

MATERIALS AND METHODS
Expression, purification, and fluorescent labeling of Drp1
Full-length human Drp1 isoform 3 (699 amino acid residues) subcloned in pRSET C (Life Technologies, Carlsbad, CA) was expressed as an N-terminal polyhistidine-tagged recombinant protein in Escherichia coli and purified to apparent homogeneity using a combination of nickel-nitriloacetic acid metal ion affinity and DEAE anion exchange chromatographies as described in detail in the Supplemental Methods. Mutations were introduced by PCR-based QuickChange mutagenesis (Agilent Technologies, Santa Clara, CA) and confirmed by automated DNA sequencing. Protein concentration was determined by absorbance using a molar absorptivity coefficient ε280 of 42,860 M⁻¹ cm⁻¹ for Drp1.

Drp1 was labeled with a thiol-reactive BODIPY-Fl derivative as previously described for dynamin (Ramachandran et al., 2007). The stoichiometry of labeling, determined using a molar absorptivity coefficient of 76,000 M⁻¹ cm⁻¹ at 502 nm for BODIPY-Fl, was ~2 mol dye/mol protein. Unlabeled and BODIPY-Fl-labeled Drp1 were aliquoted and stored at ~80°C in buffer containing 10% (vol/vol) glycerol.

Preparation of liposomes and lipid nanotubes
All lipids were purchased from Avanti Polar Lipids (Alabaster, AL). Liposomes composed of 25 mol% bovine heart CL, 35 mol% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; PE), and 40 mol% 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC; PC) were prepared by extrusion (21 times) through polycarbonate membranes of 400-nm pore diameter according to standard protocols

GTPase assay
Lipid-stimulated hydrolysis of GTP by full-length Drp1 and mutants (0.5 μM final) preincubated on CL-contaminating liposomes (150 μM final) for 15 min at room temperature before GTP addition was monitored at 37°C over time using a malachite green–based GTPase assay as described earlier (Leonard et al., 2005). GTP (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 1 mM. MgCl₂ and dithiothreitol (DTT) were present at 2 and 1 mM final, respectively. Basal GTP hydrolysis rates for Drp1 WT and mutants were measured similarly but in the absence of lipid.

Preparation of GUVs and confocal light microscopy
GUVs were electroformed under physiological salt conditions (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.5, 150 mM KCl) using home-built, platinum wire electrode chambers as described elsewhere (Montes et al., 2007). The lipid mixture contained either 25 mol% CL, 35 mol% PE, and ~40 mol% PC (PC/PE/CL) or 25 mol% CL and ~75 mol% PC (PC/CL). Both 0.1 mol% rhodamine-PE and 0.5 mol% biotin-PE replacing equivalent mole fractions of DOPC were included, respectively, for fluorescence imaging and GUV immobilization on streptavidin (Sigma-Aldrich)-coupled, biotin-LC-bovine serum albumin–coated (Thermo Scientific, Rockford, IL) LabTek II chambered coverslides prepared according to published protocols (Yildiz et al., 2003). Still images were captured using a 60x oil-immersion objective mounted on an Olympus FV1000 IX81 confocal microscope (Olympus USA, Melville, NY). For visualization of Drp1-mediated membrane remodeling, unlabeled and BODIPY-Fl–labeled Drp1 were premixed at a molar ratio of 3:2 and diffused into the imaging chamber to achieve a final protein concentration of 0.5 μM. The relative efficiency of membrane tubulation by Drp1 WT and mutants was quantified and represented as percentage GUVs tubulated. More than 25 GUVs were scored for each sample from three independent experiments. All procedures and experiments were conducted at room temperature.

EM
Drp1 WT and mutants (2 μM final) were incubated with GMP-PCP (1 mM final), CL-containing liposomes (50 μM total lipid), or both, in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, 2 mM MgCl₂, and 1 mM DTT for 30 min at room temperature before deposition on carbon-coated copper EM grids for 2% uranyl acetate staining. Negative-stain EM images were obtained using a Tecnai Spirit BioTwin transmission electron microscope (FEI, Eindhoven, Netherlands) operated at 100 keV with a LaB₆ filament. Images were captured on a Gatan US4000 UHS charge-coupled device
Drp1 WT and mutants were fractionated on a Superose 6 10/300 GL SEC column (GE Healthcare, Piscataway, NJ) in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, and 1 mM DTT and analyzed in-line using tandem miniDAWN Treos MALS and Optilab rEX differential refractive index detectors (Wyatt Technologies, Santa Barbara, CA). Data analysis was accomplished using the ASTRA 6.1 software package (Wyatt Technologies). Drp1 WT and mutants were each loaded at 10 μM in a total volume of 500 μl for molar mass determinations. Protein concentration was varied as indicated for examining concentration-dependent shifts in elution profile.

Molar mass determination by SEC alone relies on calibration against protein reference standards (typically globular proteins) for size and the relative elution position of the molecule of interest, with inherent assumptions of globular shape for the molecule and inertness to interactions with the sizing matrix (column resin) that may ultimately influence its elution profile. This can often lead to erroneous molar mass determinations, especially for nonglobular (asymmetric, elongated), reversibly oligomerizing protein species such as Drp1 and dynamin. However, in combination with MALS (SEC-MALS), measurements are absolute in which the average molecular mass of each eluted species (defined by peaks) is determined in-line based on a direct relationship between protein concentration (measured by in-line refractive index detection), molar mass (protein stoichiometry), and the intensity of scattered light, without calibration against reference standards or assumptions of molecular shape and size (for detailed explanation of this now widely used technique, see Wen et al., 1996; Andersson et al., 2003; Ye, 2006; De et al., 2010).

**Trp-Dansyl FRET assay for Drp1 membrane binding**

Binding of Drp1 WT or mutants to CL-containing liposomes was detected by monitoring FRET between a Trp residue in Drp1 (W552 in the B insert) and Dansyl-PE lipid molecules randomly distributed on the membrane surface using a SX20 stopped flow spectrometer (Applied Photophysics, Leatherhead, United Kingdom). The final concentrations of Drp1 and lipid after mixing were 0.4 and 20 μM, respectively. Drp1 Trp was excited at 280 nm, and Dansyl emission intensity was monitored between 460 and 540 nm, using a 500FS40-25 standard bandpass filter (Andover Corporation, Salem, NH). The FRET-dependent increase in Dansyl emission intensity was recorded at 0.1-s time intervals. Experiments were maintained at 25°C using a circulating water bath.

It is important to note that the remaining two Trp residues present in Drp1, W90 in the G domain and W699 as the terminal residue of isoform 3, are positioned too far from the membrane surface to be able to participate in Trp-Dansyl FRET (R0 = 21 Å, where R0 is the characteristic distance for a FRET pair at which the efficiency of energy transfer [E] is 50%; Gustiananda et al., 2004). Owing to the inverse-sixth-power distance dependence of FRET, E rapidly approaches zero as the distance separating the FRET pair increases to 1.5R0, or ~32 Å for the Trp-Dansyl pair (Piston and Kremers, 2007).

**Sedimentation assay for Drp1 oligomerization and membrane binding**

Drp1 WT and mutants (5 μM protein final) were incubated with or without CL-containing liposomes (500 μM lipid final) in buffer containing 20 mM HEPES, pH 7.5, 150 mM KCl, 2 mM MgCl2, and 1 mM DTT in the absence or presence of nucleotide (1 mM final) for 30 min at room temperature. Supernatant (S) and pellet (P) fractions were obtained by high-speed centrifugation of the samples at 20,800 × g in a refrigerated microcentrifuge maintained at 4°C. Densitometry of S and P fractions after SDS–PAGE and Coomassie staining was performed using ImageJ (National Institutes of Health, Bethesda, MD). Drp1 pelleted is presented as percentage of total.

**Cell culture and immunocytochemistry**

Drp1-KO mouse embryonic fibroblasts (MEFs; Wakabayashi et al., 2009) were maintained in DMEM supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 1% (vol/vol) penicillin/streptomycin at 37°C in 5% CO2/95% air. Cells were transfected with 2 μg of plasmid DNA encoding either Myc-tagged Drp1 WT or mutants (pCMV-Myc; Clontech, Mountain View, CA) using TransIT-T2020 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer’s protocol.

Cells cultured on coverslips were washed with cold phosphate-buffered saline (PBS), fixed in 4% formaldehyde, and permeabilized with 0.1% Triton X-100. After incubation with 2% normal goat serum (to block nonspecific staining), fixed cells were incubated overnight at 4°C with rabbit anti-Tom20 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-Myc (1:500; Santa Cruz Biotechnology) primary antibodies. Cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 568-conjugated anti-mouse secondary antibodies (1:500; Invitrogen, Carlsbad, CA) for 60 min at room temperature. This was followed by incubation with Hoechst dye (1:10,000; Invitrogen) for 10 min. Coverslips were mounted on glass slides and imaged by confocal fluorescence microscopy using an Olympus FV1000 IX81 confocal microscope (Olympus USA).

To quantify mitochondrial fragmentation, cells were immunostained with anti-Tom20 and anti-Myc antibodies as described. Mitochondrial morphology was then examined in >100 Myc-positive cells for each sample from three independent experiments. The percentage of Myc-Drp1–expressing cells displaying fragmented, punctiform mitochondria, as exemplified by Myc-Drp1 WT–expressing cells in Figure 6A, relative to the total number of Myc-expressing cells was quantified.

**Western blotting**

Drp1 KO MEFs were transfected with the indicated plasmids as described. Total protein was harvested 24 h after transfection, and protein concentration was determined by Bradford assay. From each sample 30 μg of total protein was resuspended in Laemmli buffer, resolved by SDS–PAGE, and transferred onto nitrocellulose membranes. Membranes were probed with anti-Myc and anti-actin (Sigma-Aldrich) antibodies (1:1000 dilution for both) followed by visualization using enhanced chemiluminescence.


