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BAR Domain Scaffolds in Dynamin-Mediated Membrane Fission

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Biological membranes undergo constant remodeling by membrane fission and fusion to change their shape and to exchange material between subcellular compartments. During clathrin-mediated endocytosis, the dynamic assembly and disassembly of protein scaffolds comprising members of the bin-amphiphysin-rvs (BAR) domain protein superfamily constrain the membrane into distinct shapes as the pathway progresses toward fission by the GTPase dynamin. In this Review, we discuss how BAR domain protein assembly and disassembly are controlled in space and time and which structural and biochemical features allow the tight regulation of their shape and function to enable dynamin-mediated membrane fission.

Introduction

A defining feature of eukaryotic cells is the abundance of functionally and structurally distinct membranes. Rather than being stable entities, cellular membranes are in dynamic flux, as they are constantly changing their shape and exchanging material between them, e.g., via vesicular or tubular transport carriers. Dynamic membrane flux is essential for nearly all cell physiological functions, including secretion, cell signaling, migration, and development, among many other processes. Mechanistically, membrane flux involves the fission and fusion of transport carriers or even entire organelles (Bonifacio and Glick, 2004). During membrane fission, an initially continuous membrane is segregated into two separate entities (Kozlov et al., 2010). A prominent example of membrane fission is the internalization of parts of the plasma membrane and its surrounding fluid by clathrin-mediated endocytosis (CME), a process that requires membrane fission by members of the dynamin family of GTPases (Ferguson and De Camilli, 2006; Praefcke and McMahon, 2004). In this Review, we will focus on the role of membrane-associated BAR domain protein scaffolds (Frost et al., 2009; Peter et al., 2004; Qualmann et al., 2011) in promoting dynamin-dependent membrane fission. We will survey how bin-amphiphysin-rvs (BAR) domain proteins are recruited to distinct endocytic intermediates in a spatially and temporally well-defined sequence of events and how their assembly remodels the underlying membrane. Finally, we will discuss how these scaffolds co-operate with dynamin to promote membrane remodeling and fission in endocytosis.

Sequential Recruitment of Endocytic Proteins during Distinct Stages of Clathrin-Mediated Endocytosis

Endocytosis is an essential cellular process by which cells internalize parts of the plasma membrane along with extracellular material to regulate a variety of functions, ranging from neuro-transmission to pathogen entry and development (reviewed in McMahon and Boucrot, 2011; Saheki and De Camilli, 2012). Cells have evolved several and perhaps partially overlapping pathways of endocytosis that have been classified based on their differential requirements for the coat protein clathrin and for dynamin (Ferguson and De Camilli, 2012).

CME is initiated at the plasma membrane by the recruitment of early acting scaffolds (i.e., FCH domain only 1 and 2 [FCHO1/2] and its relative SGIP1 [Henne et al., 2010], epidermal growth factor receptor substrate 15 [Eps15], and intersectin [Henne et al., 2010; Pechstein et al., 2010] and adaptors [Cocucci et al., 2012], i.e., the heterotetrameric AP-2 complex, AP180, and clathrin assembly lymphoid myeloid leukemia [CALM]). These factors assemble at sites enriched in phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2] (Di Paolo and De Camilli, 2006) and generate a membrane template for clathrin protein assembly. During the following maturation process, the clathrin-covered plasma membrane area undergoes massive remodeling involving local deformation, resulting in a deeply invaginated spherical clathrin-coated pit (CCP). The late-stage CCP remains connected to the plasma membrane through a narrow stalk that finally undergoes dynamin-mediated fission to release a clathrin-coated vesicle (CCV), which concomitantly uncoats (Ferguson and De Camilli, 2012; McMahon and Boucrot, 2011) (Figure 1).

Endocytic membrane remodeling during CME is accompanied and presumably driven by changes in the local lipid composition. CCP initiation occurs at sites enriched in PIP2, a phosphoinositide (PI) synthesized on the cytoplasmic leaflet of the plasma membrane by type I PI 4-phosphate 5-kinases (PIPKis) (Di Paolo and De Camilli, 2006). PIPKIs associate with and are activated by

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AP-2 (Krauss et al., 2006). As clathrin displaces PIPKIs from AP-2 (Thieman et al., 2009), PI(4,5)P₂ synthesis is limited to early stages of CME. Late-stage CCPs contain the 5-phosphatase synaptojanin, an enzyme recruited to CCPs and activated by the BAR domain protein endophilin (Chang-Ileto et al., 2011; Milesevic et al., 2011). Synaptojanin converts PI(4,5)P₂ to PI(4)P, suggesting that PI(4,5)P₂ levels may decline as CCPs mature.

Recent work has shown that PI(4,5)P₂ hydrolysis to PI(4)P may be part of a PI conversion system that governs CME by converting PI(4,5)P₂ to PI(3,4)P₂. CCP maturation is accompanied by the clathrin-mediated recruitment of class II PI 3-kinase C2α (PI3KC2α), a large multidomain enzyme that synthesizes PI(3,4)P₂ from PI(4)P. Loss of PI3KC2α or enzymatic depletion of PI(3,4)P₂ stalls CME at the level of U-shaped CCPs, a stage prior to dynamin-mediated fission (Posor et al., 2013). These results suggest a mechanism whereby endocytic membrane remodeling during CCP maturation requires PI conversion from PI(4,5)P₂ to PI(3,4)P₂ (Figure 1).

PI(3,4)P₂ recruits assemblies of SNX9 and its close relative SNX18 to late-stage CCPs (Posor et al., 2013) (Figure 1). SNX9 and SNX18 are members of the sorting nexin family of BAR domain proteins found at the plasma membrane and throughout the endosomal system, where they execute key functions in membrane remodeling and protein sorting (Cullen and Korswagen, 2012). Unlike most other BAR domain proteins, which associate with acidic phospholipids with low specificity, SNXs (Figure 2) specifically bind to PIs 3-phosphates, including PI(3,4)P₂, and to some extent also to PI(4,5)P₂ via their phox-homology (PX) domain. Depletion of PI(3,4)P₂, either due to loss of PI3KC2α or through enzymatic hydrolysis by overexpression of a membrane-targeted PI(3,4)P₂-specific 4-phosphatase, interferes with the assembly of SNX9 at late-stage CCPs and with the formation or stability of ARP2/3- and BAR domain protein-coated tubular membrane invaginations, accumulating in dynamin-2-depleted cells (Posor et al., 2013). These data suggest a model whereby local PI conversion from PI(4,5)P₂ to PI(3,4)P₂ at maturing CCPs serves as an endocytic checkpoint at which the selective recruitment and assembly of SNX9/SNX18 prepares late-stage endocytic intermediates for dynamin-mediated fission and, hence, makes the pathway irreversible (Figure 1). We thus predict that this checkpoint (Aguet et al., 2013) plays a crucial role in regulating the fission process.

**BAR Domain Scaffolds Shape Different CCP Intermediates en route to Membrane Fission**

Numerous endocytic proteins have been identified and characterized that serve as protein scaffolds to regulate endocytic membrane remodeling, eventually leading to fission of late-stage CCPs. These scaffolds form rigid assemblies on the surface of membranes and impose their curvature onto the underlying lipid bilayer. Among these—as mentioned above—are several members of the BAR domain superfamily of membrane-molding molecules (Frost et al., 2009; Qualmann et al., 2011).

Consistent with the scaffolding concept, CME is characterized by the spatiotemporally defined recruitment and dissociation of distinct sets of BAR domain proteins (Posor et al., 2013; Taylor et al., 2012). BAR domains comprise dimeric α-helical coiled coils that bind to acidic membrane phospholipids with low affinity and stabilize or induce membrane curvature to achieve a geometry that corresponds to the overall shape of their membrane-binding surface (Peter et al., 2004). Cryo-electron microscopic studies have shown that the local curvature of the BAR domain matches the curvature of the underlying membrane, often without noticeable change from the crystal structure (Frost et al., 2008). Thus, dimeric BAR domains constitute rigid scaffolds for the generation of membrane domains with defined curvature. The PI(4,5)P₂-binding, shallowly curved FER and CIP4 homology (F)-BAR domain proteins FCHO1/2 arrive at nucleating CCPs and have been proposed to induce or stabilize the invagination of the initially flat plasma membrane into shallow early CCPs (Henne et al., 2010). Accordingly, FCHO1/2 are depleted from late-stage CCPs and from free CCVs. Maturation of shallow
CCPs to the pre-fission stage is accompanied by recruitment of more highly curved N-BAR proteins, such as amphiphysin and endophilin (Perera et al., 2006) (Figures 1 and 2). The inner diameter of N-BAR assemblies of endophilin or amphiphysin (Gallop et al., 2006; Peter et al., 2004) on membranes matches the diameter of the neck of late-stage CCPs undergoing dynamin-mediated fission and fits well with the ability of both proteins to recruit dynamin to late-stage CCPs (Meinecke et al., 2013; Ringstad et al., 1999; Shupliakov et al., 1997) (Figure 2).

BAR domain dimers contribute to scaffolding and curvature induction through higher-order interactions. When recruited to membranes, BAR domain dimers further self-assemble through low-affinity interaction sites (Mim and Unger, 2012) (Figure 2). For example, CIP4 or FBP17 use lateral contacts and/or contacts at the tips of their F-BAR domains to form helical oligomers at tubular membrane surfaces (Frost et al., 2008; Shimada et al., 2007). These secondary interactions are comparably flexible and allow the rigid F-BAR dimers to rotate along the membrane.
membrane surface. This results in differently spaced helical oligomers with different curvatures. The sequential transition between such oligomeric forms might in fact be a mechanism for how rigid F-BAR domain scaffolds can generate increasing membrane curvature. In contrast to CIP4, endophilin dimers lack prominent lateral interactions but employ their N-terminal amphipathic helices for crosslinking adjacent endophilin dimers (Mim et al., 2012) (Figure 2). The resulting open lattice of endophilin N-BAR assemblies may compartmentalize the membrane surface to accommodate interacting proteins, most notably dynamin.

The membrane-remodeling function of BAR domain proteins is modulated by additional sequence elements, in particular, amphipathic helices or hydrophobic membrane insertion wedges nested into or flanking the actual BAR domain (Frost et al., 2009; Qualmann et al., 2011) (Figure 2). Amphipathic helices have been found in an ever-growing list of membrane-remodeling proteins and are often unstructured until they insert in an asymmetric fashion into one leaflet of the membrane (Gallop et al., 2006). In N-BAR proteins such as amphiphysin or endophilin, the partial penetration of N-terminal amphipathic helices into one leaflet of the membrane bilayer locally induces curvature and may thereby potentially destabilize the bilayer to facilitate fission (Gallop et al., 2006; Peter et al., 2004). The propensity of amphipathic helices to induce membrane curvature also contributes to the ability of some BAR domain proteins to sense membrane curvature by detecting lipid packing defects (Bhatia et al., 2009). The interplay between curvature induction, stabilization of curved membrane domains by BAR domain protein scaffolds, and the destabilizing effects of amphipathic helix insertion are likely crucial for the spatiotemporal regulation of endocytic membrane remodeling and fission, as further discussed below.

In addition to these elements, BAR domain proteins often harbor additional structured domains that modulate membrane binding or regulate their assembly. For example, the PX domain of SNX9 forms an intramolecular interaction with the tip of its BAR domain. This interaction is stabilized by a small “yolk” domain, which ensures the parallel orientation of the basic lipid-binding sites within the PX and BAR domains (Pylypenko et al., 2007) (Figure 2). Similar to N-BAR proteins, an internal amphipathic helix of SNX9 was suggested to contribute to membrane remodeling.

Several BAR domain proteins, such as amphiphysin, endophilin, SNX9, or syndapin (also termed PACSIN), contain N- or C-terminal SH3 domains that coordinate scaffold assembly with dynamin recruitment (Figure 2). In syndapin 1, SH3 domains were shown to bind to the membrane interaction site of the BAR domain, therefore auto-inhibiting scaffold assembly and/or membrane remodeling (Rao et al., 2010). Similar auto-inhibitory roles were suggested for the SH3 domains of amphiphysin (Farsad et al., 2003), endophilin (Vázquez et al., 2013), and SNX9 (Meinecke et al., 2013; Yarar et al., 2007), though the exact mechanistic details are unclear. Auto-inhibition is thought to be relieved by binding of the SH3 domain to partially overlapping sites in the C-terminal PRD of dynamin (Rao et al., 2010) or, in case of SNX9, to actin regulatory factors such as N-WASP (Yarar et al., 2007).

**Dynamin Catalyzes the Rapid Fission of Clathrin-Coated Vesicles**

The actual fission reaction of late-stage endocytic intermediates in CME crucially depends on the GTPase dynamin, a protein that was originally discovered as a brain microtubule-binding protein and was later found to be involved in endocytosis by genetic loss-of-function studies in *Drosophila melanogaster* (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). Subsequent studies revealed a general requirement for dynamin in CME and several other forms of endocytosis, a function intimately linked to its ability to bind and hydrolyze GTP when assembled at appropriate templates (Ferguson and De Camilli, 2012; Ferguson et al., 2009; Hinshaw and Schmid, 1995; Takei et al., 1995).

Whereas invertebrate genomes, such as those of *C. elegans* or *D. melanogaster*, encode a single dynamin gene, mammals contain three dynamin genes with distinct expression patterns and multiple splice variants. Dynamin 1 is the major isoform in brain, whereas dynamin 2 is ubiquitously expressed. Dynamin 3 is found in brain and testis as well as in a few other tissues, including lung. These isoforms are similar overall, though differences exist with respect to lipid binding, affinities for SH3 domain-binding partners, oligomerization, membrane penetration (Liu et al., 2011), and GTPase activities (Ferguson and De Camilli, 2012). Whether these isoforms functionally overlap is unclear. Endocytic defects due to loss of function of dynamin 1 in knockout mice (see below) are fully rescued by re-expression of dynamin 1 or 3 but, surprisingly, not by dynamin 2 (Ferguson et al., 2007). This result suggests a functional specialization of dynamin isoforms in vivo that may relate to their differential curvature-generating and -sensing abilities (Liu et al., 2011).

Strong evidence from a variety of systems ranging from *Drosophila* mutants (Koenig and Ikeda, 1989), knockout mice, and cells derived from them (Ferguson et al., 2009) to minimal liposomal systems (Pucadyil and Schmid, 2008; Sweitzer and Hinshaw, 1998; Takei et al., 1999) indicates a key role for dynamin in membrane fission. *Shibire* mutant flies carrying a defective temperature-sensitive allele of dynamin display rapid onset paralysis, synaptic fatigue, and a neuronal activity-dependent depletion of synaptic vesicles (Koenig and Ikeda, 1989). At the nonpermissive temperature, the *shibire* mutant accumulates protein-coated endocytic intermediates at the presynaptic plasma membrane in larval neuromuscular synapses (Koenig and Ikeda, 1989). Also, neuronal synapses from mice lacking dynamin 1 (Ferguson et al., 2007) or both dynamins 1 and 3 (Raimondi et al., 2011) show a striking accumulation of clathrin-coated endocytic intermediates connected to the plasma membrane via long tubular extensions (or stalks). Endocytic fission deficits in these cells lead to a depletion of synaptic vesicles. Conversely, dynamin-GTP under conditions of longitudinal tension has been shown to be sufficient to mediate fission of liposomal templates (Roux et al., 2006).

In living cells and tissues, dynamin is recruited to CCPs in two phases. Initially, dynamin levels at CCPs are low (Aguet et al., 2013) and may depend on the association with dynamin-binding endocytic proteins such as intersectin (Koh et al., 2007), SNX9/SNX18 (Lundmark and Carlsson, 2003; Soulet et al., 2005), and amphiphysin (David et al., 1996; Owen et al., 1998; Shupliakov et al., 2003).
phase separation, as recently proposed for both clathrin-mediated and actin-mediated pathways (Ferguson et al., 2009; Posor et al., 2013), suggesting a cooperative interplay between dynamin, BAR domain proteins including endophilin and SNX9, as well as actin-polymerizing factors at the neck of late-stage CCPs (Taylor et al., 2011). Loss of SNX9/ SNX18, depletion of PIP(3,4)P2, or actin depolymerization all result in collapse of tubular endocytic intermediates induced by dynamin depletion (Ferguson et al., 2009; Posor et al., 2013), suggesting a cooperative interplay between dynamin, BAR domain proteins including SNX9/SNX18, and actin in membrane fission. Such cooperation may involve the ability of actin to aid fission by fostering lipid phase separation, as recently proposed for both clathrin-mediated (Yao et al., 2013) and clathrin-independent endocytosis (Römer et al., 2010).

A crucial aspect of endocytic membrane fission is its kinetics. In vitro studies of dynamin function on liposomal templates have shown that membrane fission requires seconds or even tens of seconds to complete, depending on membrane elastic properties and the concentration of GTP (Morlot et al., 2012). Though these timescales are grossly consistent with the kinetics of endocytic membrane fission of CCPs observed in nonneuronal cells (Taylor et al., 2011), they are much slower than the time course of fast endocytosis at neuronal synapses in some systems. Recent elegant work on C. elegans neuromuscular junctions, stimulated by single light pulses to induce exo- and endocytosis of synaptic vesicles and snap frozen within milliseconds, has revealed an ultrafast mode of endocytosis that operates within 50–100 ms (Watanabe et al., 2013a). Dynamin loss of function blocks fission of large, about 100 nm sized endocytic intermediates, that in wild-type animals would be consumed by membrane fission within 50–100 ms, e.g., much faster than the time required for dynamin alone to mediate scission in vitro. Ultrafast dynamin-dependent endocytosis has also been found in mouse hippocampal synapses, arguing for a conserved mechanism in invertebrates and vertebrates (Watanabe et al., 2013b). These data indicate that dynamin-mediated fission in neurons may be assisted by other factors (Kononenko et al., 2013), most notably actin (Watanabe et al., 2013b), BAR domain proteins such as endophilin, amphipathic helix-containing factors, or membrane lipids. These factors thus will need to be considered for a thorough understanding of the mechanism of membrane fission.

### Structural Insights into Dynamin Assembly

Dynamin assemblies into helical oligomers at membrane surfaces, leading to massive stimulation of its intrinsic GTPase activity and remodeling of the underlying membrane (Switzer and Hinshaw, 1998; Takei et al., 1998; Yoshida et al., 2004). A recent series of structural studies on dynamin and dynamin-related proteins has provided unprecedented mechanistic insights into this reaction (Chappie et al., 2010, 2011; Faelber et al., 2011, 2012; Ford et al., 2011). Besides the unstructured C-terminal proline-rich domain (PRD), dynamin comprises four domains: the GTPase domain, the bundle signaling element (BSE), the stalk, and the PH domain (Figure 2). A number of intra- and intermolecular interactions between these domains are required for dynamin’s helical assembly and for the regulation of its membrane-remodeling activity (Faelber et al., 2011; Ford et al., 2011).

The central domain mediating assembly into a helical filament is an antiparallel four-helix bundle called the stalk (Faelber et al., 2011; Ford et al., 2011). The architecture of this domain is somewhat reminiscent of that of the SNARE complex mediating membrane fusion; these helical bundles might provide the mechanical stability required for membrane-remodeling processes. The stalk stably self-associates in a criss-cross fashion via a symmetric hydrophobic interface. Such stalk dimers further assemble via two additional interfaces to form a filamentous oligomer (Faelber et al., 2011; Ford et al., 2011). Molecular dynamics simulations indicate that assembly of dimers provides for some flexibility, therefore allowing the dynamin helix to adapt to different tubular diameters (Faelber et al., 2011). The PH domain of dynamin is interspersed between the stalk and mediates binding to negatively charged membrane lipids (Burger et al., 2000; Ramachandran et al., 2009). Based on biochemical experiments and the localization of disease mutations, the PH domains were proposed to bind against a conserved surface of the stalk in solution. At this position, they may interfere with further assembly of dynamin subunits, thereby acting in an auto-inhibitory fashion (Faelber et al., 2011). In the presence of membranes, the PH domains can switch to a position below the stalk. It has been proposed that insertion of bulky hydrophobic residues of the PH domain of dynamin 1 into the outer monolayer of the membrane (Burger et al., 2000) might contribute to membrane curvature induction en route to membrane fission (Liu et al., 2011).

GTPase activity is not required for dynamin’s assembly on membranes but is required for membrane scission (Switzer and Hinshaw, 1998; Marks et al., 2001; Roux et al., 2006). The GTPase domains of dynamin can dimerize with low affinity in the presence of GTP and with higher affinity in the presence of a transition state analog of the GTPase reaction (Chappie et al., 2010, 2011). Following GTP hydrolysis, the GDP-bound GTPase domains are thought to dissociate into monomers. Cryo-electron microscopic studies have indicated that such GTPase domain dimers interlink adjacent dynamin filaments (Figure 2) (Mears et al., 2007). Nucleotide-hydrolysis-dependent rearrangements of such GTPase domain dimers might then pull neighboring stalk filaments along each other (Chappie et al., 2011; Faelber et al., 2011, 2012). In this way, the dynamin helix may act as a molecular sling that constricts the underlying membrane, thereby increasing membrane curvature.

How can this transition be achieved at the molecular level? Dimerization of the GTPase domains triggers conformational changes in catalytic residues and the positioning of a catalytic cation as a prerequisite for activation of the GTPase reaction (Chappie et al., 2010). The nucleotide-loading state, in turn,
was shown to control the twist of the central β sheet of the GTPase domains, which then directs the movement of a three-helical bundle, the BSE, at the backside of the GTPase domain (Chappie et al., 2011). The BSE is thought to function as a transmitter of conformational changes between the GTPase domain and the stalk. It adopts an open conformation toward the GTPase domain in the presence of a GTP analog and a closed conformation in the absence of nucleotide, the presence of GDP, or a transition state analog. The conversion between these two states might act as a GTPase-triggered power stroke.

**Cooperative Recruitment of Dynamin and BAR Domain Proteins**

In addition to their roles in establishing curvature, BAR domain proteins are also thought to recruit dynamin to CCPs. A role for BAR domain proteins, in particular amphiphysin (David et al., 1996) and endophilin (Ringstad et al., 1999), in recruitment of dynamin had originally been inferred from the ability of their SH3 domains to interact with the PRD of dynamin. Moreover, acute perturbation of complex formation between these proteins and dynamin in lamprey reticulospinal synapses in situ led to the accumulation of late-stage endocytic intermediates (Ringstad et al., 1999; Shupliakov et al., 1997), suggesting that amphiphysin or endophilin cooperate in the recruitment of dynamin to the vesicle neck of CCPs prior to membrane fission. An overlapping function of amphiphysin and endophilin isoforms in dynamin recruitment to the plasma membrane has recently been confirmed in nonneuronal cells (Meinecke et al., 2013).

Furthermore, binding of GST-tagged amphiphysin SH3 domains to dynamin was shown to prevent assembly of dynamin scaffolds, suggesting that BAR-SH3 domain proteins may negatively regulate dynamin assembly (Owen et al., 1999). Electron microscopic studies, on the other hand, provided evidence for the formation of amphiphysin-dynamin (Takei et al., 1999) and endophilin-dynamin complexes on the surface of tubulated liposomes, consistent with a role of BAR domain proteins as recruitment factors for dynamin (Farsad et al., 2001; Sundborger et al., 2011). These studies also revealed an increased pitch of the dynamin helix when coassembled with amphiphysin or endophilin. How coassembly affects the GTPase activity of dynamin and ultimately membrane fission remains controversial. Coassembly of dynamin with endophilin and amphiphysin on tubular lipid templates has been shown to reduce the self-assembly-stimulated GTPase activity of dynamin 1 (Farsad et al., 2001), suggesting that BAR domain protein scaffolds might prevent the GTPase domains of dynamin from reaching each other across neighboring rungs of the dynamin helical filament. A more recent study using liposomal templates with low-membrane tension (so-called SUPER templates) confirmed an inhibitory role of amphiphysin on dynamin’s GTPase stimulation but found little effect for endophilin (Neumann and Schmid, 2013). Yet another study reported stimulatory effects of amphiphysin on dynamin’s GTPase activity and on vesiculation that were dependent on liposome size and on the molar ratio of amphiphysin:dynamin (Yoshida et al., 2004). Thus, the effect of BAR-SH3 domain proteins on the enzymatic activity of dynamin in vitro appears to strongly depend on the exact experimental conditions (see also below).

In vivo, the BAR-SH3 domain proteins amphiphysin and SNX9 are thought to be recruited to CCPs via their clathrin- and AP-2-binding sites (Lundmark and Carlsson, 2003), i.e., prior to the arrival of dynamin. Recent data suggest that endophilin, a protein that apparently lacks the ability to associate with clathrin coat components, may undergo reciprocally cooperative recruitment with dynamin to the neck of CCPs. Depletion of endophilin reduced dynamin recruitment, whereas conversely, loss of dynamin interfered with endophilin accumulation at CCPs in nonneuronal cells (Meinecke et al., 2013). Such cooperative recruitment may depend on the auto-inhibition of BAR domain proteins by their SH3 domains, which is relieved upon binding to the PRD of dynamin (Rao et al., 2010; Vázquez et al., 2013). In contrast, fibroblasts lacking dynamins 1 and 2 display an accumulation of CCPs with elongated necks covered by BAR domain proteins, including endophilin, indicating that endophilin can also be recruited to the neck of CCPs independently of dynamin (Ferguson et al., 2009).

In addition to BAR-SH3 domain proteins, dynamin recruitment may be modulated by autoregulatory mechanisms. Although the precise molecular details are currently unresolved, such an autoregulatory mechanism appears to involve intra- and intermolecular interactions of the PH domain, the stalk and, possibly, the PRD (Faëber et al., 2012). Upon recruitment to the bud neck (i.e., by BAR-SH3 domain proteins), this auto-inhibitory clamp would be relieved and oligomerization may then proceed without further aid from BAR-domain-containing scaffolds. It is also currently unclear what determines the exact dimension of the dynamin oligomer at the lipid template. In vitro in the presence of nonhydrolyzable GTP analogs or GDP, dynamin can assemble into long filaments on tubular lipid templates comprising hundreds of turns (Mears et al., 2007). In vivo, the situation may be different. Once the growing dynamin filament has embraced the bud neck, the GTP-loaded GTPase domains of opposing turns face each other, and the GTPase activity and mechano-chemical function is triggered. This step may prevent further assembly of dynamin subunits to the growing filament. It may thus be envisaged that comparably small dynamin collars comprising only one helical turn mediate the fission of CCP necks, consistent with recent experimental data (Bashkirov et al., 2008; Shnyrova et al., 2013). In such a scenario, BAR domain proteins would be required for the initial recruitment of dynamin to the bud neck but would not interfere with nucleotide-dependent interaction of neighboring dynamin rings. Solving the mechanistic details of this puzzle is one of the important challenges for the near future.

**BAR Domain Proteins and Membrane Fission**

The direct involvement of BAR proteins in membrane fission remains controversial. Theoretical considerations suggest that rigid BAR domain scaffolds may promote tubulation but would not favor membrane fission (Boucrot et al., 2012), as they would fix the curvature of the membrane to the intrinsic curvature of the BAR scaffold. Thereby, they would block further constriction, a prerequisite for membrane fission. However, fission may be promoted by the ability of at least some BAR-domain-containing proteins to sequester PIs into stable lipid microdomains (Zhao et al., 2013), resulting in the generation of a phase boundary.
that could aid membrane fission (Liu et al., 2009). Similarly, hydrophobic membrane insertions of amphipathic helices or “wedges” often found within or adjacent to the BAR domain (Bhatia et al., 2009; Frost et al., 2009; Qualmann et al., 2011) could promote proper fission. Indeed, in vitro studies have provided evidence that at least the N-BAR-domain-containing protein amphiphysin can promote dynamin-dependent liposome vesiculation (Takei et al., 1999; Yoshida et al., 2004). Two recent studies have re-addressed this topic using high-tension giant unilamellar vesicles (GUVs) (Meinecke et al., 2013) or low-tension SUPER templates as substrates for dynamin (Neumann and Schmid, 2013). Meineke et al. observed vesiculation of GUVs only when dynamin, amphiphysin or endophilin, and GTP were added together. Furthermore, GUV shrinkage was strongly dependent on the interaction of these BAR domain proteins with dynamin, as dynamin mutants lacking the PRD were unable to vesiculate liposomes. Neumann et al. reported that the iso-

lated N-BAR domain of endophilin promotes dynamin-2-depen-
dent vesicle release from SUPER templates, as did full-length amphiphysin, whereas full-length endophilin was ineffective. By contrast, SNX9 even prevented dynamin-dependent vesiculation from SUPER templates, though it had been reported to stimulate dynamin-mediated membrane fission (Gehart et al., 2012). Furthermore, the formation of plasma mem-

brane association and dissociation (Schmid and Frolov, 2011) thus, is how the hydrolysis cycle of GTP and the resulting confor-
mational changes within assembled dynamin are coupled to its mem-

brane tube beneath would expand, leading to aborted fission. Clearly, the GTPase cycles of successive dynamin

imers have to be uncoupled to allow some to generate a confor-
mational change and constriction force (torque), whereas others are detached. It is tempting to speculate that BAR-domain scaf-
dles contribute to such a mechanism—for example, by selec-
tively anchoring one end of the helical dynamin filament to the membrane while the other would be free to move. The torque generated by dynamin in order to constrict a membrane below a diameter of 10 nm is huge, in the range of 1,000 pN·nm. As a comparison, proteins that unwind the DNA double helix usually exert torques of a few tens of pN·nm (Lee et al., 2013), and the F1-ATPase generates a maximal torque of 90 pN·nm. (Kinosa-
ta et al., 2000). The resulting torque may enable dynamin to
constrict the membrane even further than 10 nm in diameter (Morlot et al., 2012). It also makes dynamin one of the strongest mechano-enzymes known, as the strongest rotational motor described to date—the rotor of the bacterial flagellum—generates torques of 1,200–1,500 pN.nm.

Finally, how does the membrane actually break? At the edge of the constricted neck of dynamin, the membrane undergoes a dramatic change in curvature, resulting in local membrane stress at this location and a corresponding increase in elastic energy. The elastic energy accumulation is sufficient to reduce the energy barrier to fission, which may finally result from simple Brownian fluctuations of the membrane. The stochasticity of fission kinetics and the fact that fission occurs at the edge of the dynamin polymer support this mechanism (Morlot et al., 2012).

**Choreography toward Membrane Fission**

As described above, the order of endocytic protein recruitment to sequential stages of endocytic intermediates appears at least in part to be regulated by the spatiotemporal regulation of membrane composition and grossly to correspond to the curvature of the underlying membrane. As CCPs are metastable structures, the endocytic machinery needs to maintain a delicate balance between the generation and stabilization of curved membrane nanodomains and their destabilization as the structure moves forward toward fission (Bocourt et al., 2012). A salient feature of the pathway appears the fact that the curvature of each BAR domain protein sequentially added appears to follow an increasing curvature sequence en route to dynamin-mediated fission (Figure 2) (Mim and Unger, 2012; Qualmann et al., 2011). The F-BAR domains of FCHO proteins, which participate in CCP nucleation (Henne et al., 2010), display a shallow curvature and can accommodate a corresponding membrane with a diameter of 110–130 nm (Henne et al., 2007), much larger than the diameter of a final clathrin-coated vesicle. Furthermore, these proteins lack an SH3 domain and the ability to associate themselves. Importantly, the sequential recruitment of BAR domain proteins to the maturing CCP. In this scenario, dynamin is finally added to the “tip” of the asymmetric BAR protein scaffold close to the coated vesicle, a model that is supported by super-resolution microscopy analysis of CCPs formed from plasma membrane sheets in vitro (Wu et al., 2010).

Apart from differential curvatures, the resulting BAR-dynamin protein assembly also displays asymmetry with respect to the number of amphipathic helices inserted into the underlying membrane. FCHO proteins lack bona fide amphipathic helices (Henne et al., 2007), indicating that their primary function in CME may be the stabilization of shallowly curved membrane domains. Amphiphysin and endophilin dimers, by contrast, harbor two or four amphipathic helices (Bocourt et al., 2012; Mim et al., 2012; Mim and Unger, 2012), respectively, thereby poising the vesicle neck for fission by dynamin at the interface with the vesicle coat. Insertion of hydrophobic residues within the PH domain loops of dynamin (Liu et al., 2011; Ramachandran et al., 2009) and from other endocytic proteins such as epsin (Bocourt et al., 2012) may further aid the local membrane destabilization at the boundary between the coated vesicle and its neck. Thus, we anticipate that membrane fission will occur preferentially at the coat-to-neck boundary, in agreement with recent data from electron microscopy (Sundborger et al., 2011).

Another prediction derived from this model is that the location of the fission reaction is controlled by the sequential addition of BAR domain proteins, thereby determining the volume enclosed by the budding endocytic vesicle. Lack of individual endocytic proteins, thus, would not only decrease the efficiency of endocytosis, but would also result in the generation of variably sized vesicles. Indeed, variations in the size of synaptic vesicles formed by CME have been observed at synapses from a variety of endocytic protein mutants (Dittman and Ryan, 2009; Ferguson et al., 2007; Saheki and De Camilli, 2012).

A burning open question relates to the temporal regulation of fission. The above considerations suggest that BAR domain proteins indeed may fulfill dual roles in stabilizing curved membrane domains, thereby aiding constriction via the assembled BAR scaffold and promoting fission by amphipathic helix insertion, e.g., in the case of endophilin. Further mechanisms, for example phosphorylation events that could modulate SH3-PRD interactions with dynamin, may impose additional layers of regulation upon membrane fission in vivo. Future studies will need to address these possibilities in detail. Furthermore, principles similar to those described here for dynamin will likely apply to other fission reactions mediated by dynamin family members such as the fission of mitochondria (Frohlich et al., 2013; Mears et al., 2011) or chloroplasts (Watanabe et al., 2013b), though the dimensions of the organelles involved and the timescale of these reactions differ substantially from endocytic membrane fission.

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