Direct dynamin–actin interactions regulate the actin cytoskeleton

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The large GTPase dynamin assembles into higher order structures that are thought to promote endocytosis. Dynamin also regulates the actin cytoskeleton through an unknown, GTPase-dependent mechanism. Here, we identify a highly conserved site in dynamin that binds directly to actin filaments and aligns them into bundles. Point mutations in the actin-binding domain cause aberrant membrane ruffling and defective actin stress fibre formation in cells. Short actin filaments promote dynamin assembly into higher order structures, which in turn efficiently release the actin-capping protein (CP) gelsolin from barbed actin ends in vitro, allowing for elongation of actin filaments. Together, our results support a model in which assembled dynamin, generated through interactions with short actin filaments, promotes actin polymerization via displacement of actin-CPs.

The EMBO Journal (2010) 29, 3593–3606. doi:10.1038/emboj.2010.249; Published online 8 October 2010
Subject Categories: cell & tissue architecture
Keywords: actin; cytoskeleton; dynamin

Introduction

Dynamic cellular properties such as migration and division depend on the actin cytoskeleton, a dense meshwork of protein polymers that undergoes rapid cycles of assembly and disassembly and that is controlled by a large number of actin-associated proteins (reviewed in Pollard and Cooper, 2009). One of the proteins implicated in regulation of cell motility is the GTPase dynamin (reviewed in Schafer, 2004), best known for its essential role in clathrin-coated vesicle formation during endocytosis (reviewed in Mettlen et al., 2009). In addition to its role in endocytosis, dynamin co-localizes with actin filaments, often in locations where membranes undergo remodelling such as cortical ruffles and podosomes. Dynamin binds numerous actin-regulating or -binding proteins such as profilin, Nck and cortactin (reviewed in Schafer, 2004). These interactions are mediated through dynamin’s C-terminal proline, arginine-rich (PRD) domain and the Src homology 3 (SH3) domains of actin-binding proteins. It is through these interactions that dynamin is thought to link the plasma membrane to actin and thereby assemble actin filaments on membranes that are undergoing remodelling (Orth and McNiven, 2003). According to this model, dynamin’s regulation of the actin cytoskeleton is tightly connected to its membrane localization and its ability to bind actin-binding proteins.

Several lines of evidence suggest an alternative model in which dynamin has a more direct role in regulation of the actin cytoskeleton. Thus, expression of a dynamin mutant that cannot bind GTP, dynK44A, but can still bind SH3-domain containing proteins, significantly reduced formation of F-actin comet tails generated either by Listeria or on vesicles by overexpression of type I phosphatidylinositol 5-phosphate kinase (Lee and De Camilli, 2002; Orth et al., 2002). Interestingly, dynamin was not only concentrated at the actin-vesicle-membrane interface, but also extended along the length of the comet tails. Thus, in addition to nucleotide-independent, PRD-dependent interactions with actin-binding proteins (McNiven et al., 2000), these data raised the possibility of dynamin GTPase-dependent regulation of actin. Supporting this connection are studies showing a role for dynamin’s GTPase activity in podosome structure and function (Bruzzone et al., 2005). Podosomes are highly dynamic actin-containing adhesion structures found in osteoclasts, macrophages and Rous sarcoma virus-transformed fibroblasts. While expression of dynK44A decreased osteoclast resorption and migration, overexpression of dynWT increased these processes (Bruzzone et al., 2005). Recent work by Mooren et al. (2009) attempted to explain the role of dynamin’s GTPase cycle in regulation of the actin cytoskeleton. They showed that in the presence of dynamin, GTP led to remodelling of actin filaments in vitro via the actin-binding protein cortactin. This suggested that a GTP hydrolysis-induced conformational change within dynamin is transduced to cortactin, which in turn alters orientation of the actin filaments. However, as interactions between dynamin’s PRD and cortactin’s SH3 domain do not require GTP binding or hydrolysis by dynamin, it is uncertain how such a conformational change within dynamin might be transmitted to cortactin.

In this study, we identify a direct interaction between dynamin and actin, which is mediated by a conserved domain in dynamin. Expression of dynamin mutants with impaired affinity for F-actin in podocytes decreased the number of actin stress fibres and disrupted the cortical actin cytoskeleton. In contrast, expression of a dynamin mutant with increased affinity for F-actin enhanced stress fibre formation. Importantly, actin binding was closely linked to the assembly status of dynamin. Thus, short actin filaments stimulated dynamin self-assembly via direct dynamin–
actin interactions in vitro. Importantly, assembled dynamin displaced the capping protein (CP) gelsolin (Gsn) from barbed ends, which in turn promoted elongation of actin filaments. The effect was Gsn specific as dynamin could not displace another mammalian CP. Together, the data suggest that short actin filaments, generated by actin severing and CPs, lead to dynamin self-assembly. Dynamin oligomers in turn promote de-capping of barbed ends, allowing for actin filament elongation. The experiments reveal an intricate functional interplay between the actin cytoskeleton and the assembly status of dynamin.

**Results**

**Dynamin binds to filamentous actin**

To revisit the question of how dynamin regulates the actin cytoskeleton, we tested whether dynamin binds to F-actin using a co-sedimentation assay. We used the neuronal isoform, dynamin 1 (dyn1), which has a lower propensity to spontaneously oligomerize (Warnock et al., 1997). At physiological ionic strength, dyn1 was soluble and thus found in the supernatant after ultracentrifugation (Figure 1A, lanes 13, 15 and 17). However, in the presence of F-actin, dynamin was found in the pellet (Figure 1A, compare lanes 2 and 14; Supplementary Figure S1A) independently of GTP or GTPγS (Figure 1A, compare lanes 2, 4 and 6). Recombinant dyn1 and isolated actin did not contain cortactin, an actin-binding protein known to interact with dynamin (Supplementary Figure S1B), suggesting that interactions between dynamin and actin are direct. The binding constant ($K_d$) of dynamin for actin was $\approx 0.4 \mu M$ (Figure 1B, black circles); which is comparable with the $K_d$s of known actin-binding proteins such as $\alpha$-actinin 4 or cortactin (van Rossum et al., 2003; Weins et al., 2005). Based on a Scatchard analysis (Supplementary Figure S1C; saturation-binding curve), one dynamin tetramer bound 4–6 actin subunits of the filament.

We next mapped the actin-binding domain (ABD) within dynamin. Importantly, dynamin lacking its PRD domain (dynΔPRD) bound F-actin as efficiently as dyn1WT with a $K_d$ of $\approx 0.3 \mu M$ (Figure 1B; Supplementary Figure S1D). Thus, dynamin–actin interactions are not mediated through SH3 containing actin regulators. Dynamin’s GTPase effector domain (GED) did not bind F-actin (data not shown), suggesting that the actin-binding site was situated within the GTPase, middle or PH domains. Using *in vitro* transcription/translation (IVT), we generated several dynamin fragments and thereby mapped the actin-binding site to a region between amino acids 399 and 444 (Supplementary Figures S1E–G). As predicted for an ABD (Van Troys et al., 1996), this region contains several positively charged amino acids, and these are conserved from yeast to mammals (Figure 1C).

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**Figure 1** Direct dynamin–actin interactions are mediated by dynamin’s middle domain. (A) Dyn1 co-sediments with F-actin. Representative Coomassie blue-stained gel of supernatants (S) and pellets (P) after centrifugation at 150 000 g in the presence and absence of 100 μM nucleotides as indicated in the figure; 1 μM dynamin was incubated with 5 μM rabbit skeletal muscle actin. (B) Actin-binding analysis of dynamin (wild-type, APRD and actin-binding site mutants) to F-actin. Increasing concentrations of dyn1 were added to 2.5 μM F-actin. After centrifugation at 150 000 g, proteins were separated on SDS–PAGE and bands were analysed using densitometry. (C) Top, schematic diagram of dynamin domains and fragments tested in the actin co-sedimentation assay using IVT proteins. The presence or absence of an actin-binding domain (ABD) is indicated. Bottom, amino-acid sequence alignment of dyn2 (splice variants a and b), dyn1 (splice variants a and b), *Drosophila* dynamin (Shi), *Caenorhabditis elegans* dynamin (Cce), and yeast dynamin (Vps1). Dyn1 is a dynamin family member involved in mitochondrial morphogenesis. (D) Dynamin mutants with altered affinity for actin exhibit wild-type GTPase activities. Kinetic parameters were determined performing the GTPase assays using 0.2 μM dynamin and when indicated 80 μM lipids.
Interestingly, this region is alternatively spliced within different mammalian dynamin isoforms (called variant a and b).

Site-directed mutagenesis was performed on conserved, charged residues within the ABD of dyn1b to generate putative ‘loss-of-function’ mutants, dyn1K/E and dyn1K/A, and a putative ‘gain-of-function’ mutant, dyn1E/K (Figure 1C). As predicted, the affinities of dyn1K/E and dyn1K/A for actin were reduced (Kd of 1.7 and 2.8 μM, respectively), whereas the affinity of dyn1E/K for actin was increased (Kd = 0.03 μM) (Figure 1B; Supplementary Figures S1H and I). All three mutant proteins exhibited wild-type basal rates of GTP hydrolysis (Figure 1D), indicating proper folding. In addition, all three mutants supported lipid-stimulated GTP hydrolysis (Figure 1D), with dyn1K/A being partially impaired because of its reduced self-assembly (Supplementary Figure S1J, lane 3). In agreement with their normal GTPase activities, dyn1K/E and dyn1K/R supported endocytosis of transferrin (Supplementary Figures S2A and B). Expression of these mutant proteins did not change the overall level (Supplementary Figure S2C), or GTP-bound forms of known Rho-family GTPases (Supplementary Figure S2D) in cells. Together, these data suggest that the actin-binding mutants of dynamin are properly folded and that they do not detectably affect clathrin-mediated endocytosis or signalling by Cdc42, Rac1 or RhoA.

**Direct dynamin–actin interactions regulate the actin cytoskeleton**

To explore the function of direct dynamin–actin interactions in cells, we used cultured podocytes. Podocytes are specialized cells of the glomerulus containing a highly organized actin cytoskeleton that is necessary for their function as part of the glomerular filtration apparatus in the kidney (Saleem et al., 2008). Changes in podocyte morphology and attachment to the glomerular basement membrane, which are driven by reorganization of the actin cytoskeleton, are directly linked to chronic kidney diseases (Oh et al., 2004). The actin cytoskeleton in conditionally immortalized mouse podocytes supports podocyte adhesion, motility and morphogenesis, and like many other cell types can be roughly divided into three domains: (1) a lamellipodial actin network, (2) filopodial actin bundles and (3) contractile actin stress fibres (Figure 2A, panel 1).

Downregulation of the ubiquitously expressed dynamin 2 (dyn2) using lentivirus-based short hairpin RNAs was monitored using RT-PCR (Supplementary Figure S3A) and western blot analysis (Supplementary Figure S3B). Three different shRNAs downregulated dyn2 protein levels by 70–90% (Supplementary Figure S3B), resulting in similar alterations in cell morphology with cells becoming smaller, less round and eventually detaching from the coverslip (Supplementary Figure S3C). There was a dramatic loss of F-actin and the number of focal adhesions (FAs) in dynamin-deficient cells (Figure 2A, panel 2 and 2C, 2D for quantification). These data are in agreement with the global alteration in the actin cytoskeleton observed after dyn2 was downregulated in U2OS cells (Mooreen et al., 2009). The data are also in agreement with our previous finding that downregulation of dynamin by Catl processing resulted in loss of stress fibres (Sever et al., 2007).

We next examined whether expression of dyn1WT or our novel ABD mutants of dynamin could rescue the dyn2-depletion phenotype. Of note, >70% loss of dyn2 resulted in significant loss of cells from the coverslips (Supplementary Figure S3C and data not shown). Thus, all rescue experiments were performed under viral infection conditions in which downregulation of dynamin was ~70% (Supplementary Figures S3D and E). Expression of the neuronal isoform of dynamin, dyn1WT, restored normal actin organization in dyn2-deficient cells (Figure 2A, panel 3; Supplementary Figure S3F). Quantification of the data showed that dyn1WT restored levels of F-actin (Figure 2C) and FAs (Figure 2D) to wild-type levels. In contrast, expression of ‘loss-of-function’ dyn1K/E failed to rescue the depletion of dyn2 (Figure 2A, panel 4; Supplementary Figure S3F). Whereas dyn1K/E induced formation of FAs and stress fibres above levels seen in cells lacking dynamin (Figure 2C and D), it mostly induced formation of focal complexes and some disorganized actin cables within the cell body. Expression of ‘gain-of-function’ dyn1E/K rescued formation of FAs within the cell body (Figure 2A, panel 5, 2D; Supplementary Figure S3F), as well as formation of stress fibres (Figure 2C). While expression of dyn1E/K clearly restored formation of stress fibres within the cell body (Figure 2A), it seems that these structures are also somewhat disorganized. As ABD mutants exhibit wild-type GTPase activity (Figure 1D) and do not affect clathrin-mediated endocytosis or signalling by Cdc42, Rac1 and RhoA (Supplementary Figure S2), our data indicate that direct dynamin–actin interactions are essential for actin polymerization, which drives formation of stress fibres as well as cortical actin cytoskeleton in podocytes.

**Interactions between dynamin and SH3-domain containing proteins are not essential for stress fibre formation in podocytes**

Until now, the role of dynamin in regulation of the actin cytoskeleton has been viewed through its interactions with actin-binding/regulatory proteins such as cortactin. As these interactions are mediated by dynamin’s PRD domain, we examined effects of ABD mutants in the context of dynamin mutants that lack the PRD, dyn1APRD. Unexpectedly, expression of dyn1APRD complemented dyn2 knockdown to a similar extent as dyn1WT, as seen by the restoration of stress fibres and FAs within podocytes (Figure 2B, panel 1, 2C, 2D; Supplementary Figure S3F). In contrast, expression of the double mutant, dyn1APRD/KE, which is impaired in actin binding, failed to restore stress fibres and FAs (Figure 2B, panel 2), whereas dyn1APRD/KE behaved like dyn1WT (Figure 2B, panel 3). Together, these results further support the conclusion that the effects of dynamin on the actin cytoskeleton occurred through direct dynamin–actin interactions.

The importance of direct dynamin–actin interactions in actin regulation is not unique to podocytes. Thus, it has been shown that expression of dyn1APRD can induce stress fibre formation in rat hepatocytes cultured clone 9 cells (Supplementary Figure S4A, panel 1; McNiven et al., 2000). Consistent with our experiments in podocytes, dyn1APRD/KE failed to induce stress fibre formation in clone 9 cells, whereas dyn1APRD/K retained this activity (Supplementary Figure S4A). In fact, expression of dyn1K/E induced stress fibre formation, whereas expression of dyn1K/E had the opposite effect (Supplementary Figure S4B). Together, these data suggest that in clone 9 cells, interactions between dynamin’s PRD and SH3-domain containing proteins are
not essential for formation of stress fibres, but that direct dynamin–actin interactions are essential.

**Direct dynamin–actin interactions regulate actin polymerization**

To further examine the role of dynamin–actin interactions in regulation of actin cytoskeleton, we overexpressed ABD mutants in podocytes. If dynamin–actin interactions are essential for actin polymerization, then overexpression of ABD mutants might be predicted to function as dominant negative or dominant positive with respect to actin polymerization. As shown previously (Sever et al, 2007), expression of dyn1K44A, a mutant that cannot bind GTP, dramatically altered organization of the actin cytoskeleton in podocytes, whereas expression of dyn1WT had no effect (Supplementary Figures S5A–C). Expression of ‘loss-of-function’ dyn1K/E resulted in elongated podocytes whose cytoplasm collapsed into a narrow space around the nucleus (Supplementary Figure S5A), which correlated with a statistically significant loss of stress fibres and FAs (Supplementary Figures S5B and C). DynK/E was also generated in the context of dyn2, and the cells exhibited the same phenotypes as dyn1 K/E (compare Supplementary Figure S5A, row 4 and Supplementary Figure S5D, row 3), showing that the effects were not isoform specific. In contrast, expression of the ‘gain-of-function’ dyn1E/K exhibited a statistically significant increase in the number of FAs and stress fibres (Supplementary Figures SSB and C). Further supporting a role for dynamin–actin

**Figure 2** Direct dynamin–actin interactions are essential for organization of the actin cytoskeleton in podocytes. (A, B) Podocytes were first infected with adenoviruses expressing different dyn1 constructs as indicated; 18 h post-infection, cells were infected with lentivirus expressing shRNA construct S4, and dyn2 was downregulated for 3 days, after which cells were examined via immunofluorescence. Focal adhesions and F-actin were visualized with anti-paxillin antibodies and rhodamin phalloidin, respectively. In (A), white asterisk marks cells that were not infected with adenoviruses expressing dyn1 constructs. (C, D) Expression of dyn1 WT, dyn1K/E, dyn1APRD/EK, but not dyn1K/E or dyn1APRD/KE promotes formation of stress fibres and focal adhesions in podocytes. Bar graphs depicting total F-actin (C) and the number of focal adhesions (D). Data represent measurements of >50 cells and are plotted as ± s.d. (n = 3). (E) Effects of expression of dynamin mutants on the partitioning of actin into Triton-X 100 soluble and insoluble fractions. (Top) Flow chart of experiment, (bottom) western blot analysis of actin distribution among low-speed pellet (LSP), high-speed pellet (HSP), and high-speed supernatant (HSS) fractions in three separate experiments. (F) Bar graphs depicting the partitioning of actin in (E). The actin distribution in each fraction was expressed as a percentage of the total. Values shown are the mean ± s.d. (n = 3) shown in (E). (G) Fractionation of α-actinin 4 by differential centrifugation in podocyte lysates expressing different dynamin mutants. The result from three separate experiments are shown.
interactions in these phenotypes, both ABD mutants were able to interact with cortactin, an SH3-domain containing protein, to the same extent as the wild-type enzyme (Supplementary Figures SSE and F). Together, these data further show a role for direct dynamin–actin interactions in regulation of the actin cytoskeleton.

As a complementary means to examine dynamin-mediated regulation of actin polymerization, we performed subcellular fractionation using differential centrifugation (Watts and Howard, 1992). In this assay, higher order bundles and networks of F-actin are isolated in the low-speed fraction in the presence of Triton-X 100 (low-speed pellet, LSP in Figure 2E). The soluble fraction is recovered and re-centrifuged at high speed to separate non-crosslinked F-actin (high-speed pellet, HSP) from soluble G-actin monomers (high-speed supernatant, HSS). Wild-type mouse podocytes contained almost identical amounts of actin in the LSP (bundles) and HSP (F-actin) fractions and almost no actin in HSS (G-actin; Figure 2E, Endo; see Figure 2F for quantification), and this distribution was maintained in cells over-expressing dyn1WT (Figure 2E). Expression of dyn1K44A shifted F-actin into the LSP fraction (Figure 2E) consistent with formation of hyper-bundled cortical actin (Supplementary Figure S5A). In contrast, expression of ‘loss-of-function’ dyn1K/E and dyn1K/A resulted in a significant increase in the amount of G-actin in HSS, while decreasing the levels of crosslinked F-actin in LSP and HSP (Figure 2E). These data are in agreement with the overall loss of stress fibres observed by immunofluorescence microscopy (Supplementary Figures S5A and B). Cells expressing the ‘gain-of-function’ dyn1K/S exhibited a dramatic increase in F-actin pelleting into the LSP, and a concomitant decrease of F-actin in the HSP fraction (Figure 2E), consistent with increased stress fibre formation (Supplementary Figures S5A and B). A known podocyte actin bundling protein, α-actinin 4, co-fractionated with actin in cells expressing the different dynamin mutants (Figure 2G); thus, similar to the effects on actin, dyn1K44A and dyn1K/S expression shifted α-actinin 4 into the LSP, whereas dyn1K/E expression resulted in an increase of α-actinin 4 into the HSS fraction (Figure 2G, lanes 7–9). Together, these data show that dynamin–actin interactions have a major function in regulating actin polymerization in podocytes. Our data suggest that dynamin–actin interactions regulate both the formation of stress fibres and the cortical actin cytoskeleton in podocytes.

As the coordinated interplay between the cortical actin cytoskeleton and stress fibres underlies cell motility, we examined whether the dynamin–actin interaction influences podocyte motility. Podocyte motility was examined using a scratch wound-healing assay (Asanuma et al, 2006). As predicted from actin cytoskeleton organization, overexpression of dyn1WT had no effect on podocyte motility, in contrast to diminished podocyte motility in cells expressing dyn1K44A (Supplementary Figures S5G and H for quantification). Importantly, motility was also impaired in cells expressing any of the three ABD mutants of dynamin (Supplementary Figure S5G). Thus, both ‘loss-of-function’ and ‘gain-of-function’ ABD dynamin mutants act as dominant negatives with respect to podocyte motility. The loss of motility in cells expressing dyn1E/S can be explained by the increase in the number of FAs within the cell body (Supplementary Figure S5C). Together, these data show that direct dynamin–actin interactions control global organization of the actin cytoskeleton by regulating actin polymerization. To our knowledge, dynamin is the only GTPase known to directly regulate global organization of the actin cytoskeleton in cells.

**Dynamin bundles filamentous actin**

We next investigated the molecular mechanism by which dynamin–actin interactions influence the organization of the actin cytoskeleton. First, we examined whether dynamin alters F-actin organization. Figure 3A shows that dyn1 bundles F-actin in vitro, as visualized by fluorescence microscopy of actin after FITC-phalloidin staining (Figure 3A, panel 2). Importantly, dyn1E/S also bundled F-actin (panel 5), whereas dyn1K/E did not (panel 4), indicating that bundling is mediated by direct dynamin–actin interactions mediated by the dynamin ABD. Although dynamin can bundle F-actin, it does so less effectively than α-actinin 4, a known actin bundling protein (Figure 3A, panel 3). Given dynamin’s ability to oligomerize into higher order structures such as rings, we tested whether dynamin rings also bind and bundle F-actin. Dynamin was induced to oligomerize into rings by GTPγS, a non-hydrolysable GTP analogue. Although bundles were detected, the presence of GTPγS diminished the extent of bundling (Figure 3A, compare panels 2 and 6). Co-sedimentation at low speed was used to independently measure the ability of dynamin to crosslink F-actin into bundles. In the absence of dyn1, <10% of F-actin sedimented during centrifugation (Figure 3B, lane 2, and 3C for quantification). Addition of 1 μM dyn1 increased the amount of F-actin found in the pellet to ~35% of the total (Figure 3B, lane 8), but this was slightly reduced in the presence of GTPγS (Figure 3B, lane 10). Therefore, unassembled dynamin tetramers and, to a lesser extent, dynamin rings can bundle actin filaments.

To visualize actin bundles generated by dynamin, negative staining and electron microscopy were performed. In agreement with the light microscopy, the addition of dyn1 to F-actin resulted in bundles that could be visualized in the electron microscope (Figure 3D). White ‘knobs’ attached to actin filaments are recombinant dynamin. As observed by the IF, the EM analysis also showed that dynamin bundled F-actin in the presence of GTPγS (Figure 3E). In Figure 3E, black arrows point to the dynamin rings that associate with actin bundles in the presence of GTPγS. The filament-to-filament spacing in the presence of GTPγS was 17–20 nm and quite regular, showing that in contrast to unassembled dynamin, dynamin rings generate bundles with defined spacing (see model in Figure 3E). Therefore, both tetrameric and oligomeric dynamin can interact with and crosslink F-actin filaments into bundles and modify actin structure.

Dynamin is known to bind phospho-lipids. Thus, we tested whether dynamin might recruit actin filaments to membranes. Actin filaments and PIP2-containing lipid vesicles were visualized with rhodamine phalloidin and fluorescein phosphatidylethanolamine (PE), respectively (Schafer et al, 2002). As seen before (Schafer et al, 2002), addition of dyn2 induced aggregation of lipid vesicles and resulted in association of actin filaments with lipid aggregates (Figure 3F). In fact, addition of lipid vesicles increased dynamin’s ability to bundle actin filaments into disordered and entangled networks of thin filament bundles. Together, these data show that dynamin can crosslink actin filaments into bundles even when associated with the membranes, and thus suggest that...
it can directly influence the organization of the actin filaments in the vicinity of the membrane.

**Short actin filaments promote dynamin oligomerization**

To fully explore the functional connection between actin and dynamin, we addressed whether actin also modulates the properties of dynamin. The GTPase activity of dynamin is tightly connected to its oligomerization cycle. In its basal state at high ionic strength, dynamin exhibits a dimer–tetramer equilibrium (Muhlberg et al., 1997). At low ionic strength, or in the presence of lipid vesicles (reviewed in Praefcke and McMahon, 2004), dynamin tetramers self-assemble into higher order structures such as rings and/or spirals. Dynamin self-assembly increases its GTPase activity because of activation of an internal GTPase-activating protein domain (Sever et al., 1999). GTP hydrolysis in turn drives dynamin disassembly (Warnock et al., 1996).

GTPase assays were performed in the presence and absence of actin. Addition of G-actin, G-actin treated with latrunculin A (to ensure that actin remains monomeric at the salt conditions of the assay) or F-actin did not significantly alter the basal rate of dynamin GTP hydrolysis (data not shown). To determine whether actin filament length might affect the GTPase activity of dyn1, F-actin (2 mM final G-actin concentration) was sheared by five passages through a 27-gauge needle, followed immediately by the addition of different concentrations of cytochalasin D (cytoD), an inhibitor of actin polymerization. This shearing procedure
generates short filaments that are $\sim 0.7 \mu m$ long (Supplementary Figure S6A), which are efficiently capped by 0.5 mM cytoD. As uncapped actin filaments can anneal through an end-to-end mechanism (Andrianantoandro et al., 2001), lower concentrations of cytoD are expected to generate actin filaments longer than 0.7 $\mu m$. As shown in Figure 4A, the shortest actin filaments generated by the addition of 0.5 mM cytoD stimulated dynamin’s GTPase activity by $\sim 3.5$-fold, whereas a lower concentration of cytoD or cytoD alone had no effect. These data suggest that short actin filaments can promote dynamin oligomerization into higher order structures, which in turn increases dynamin’s GTPase activity.

In cells, short actin filaments are generated as a result of F-actin cleavage and capping by severing proteins such as Gsn (Barkalow et al., 1996; Pollard and Borisy, 2003). Thus, we next tested whether dynamin’s GTPase activity can be stimulated by the addition of short filaments generated in the presence of Gsn, a calcium-activated protein, and calcium (Yin et al., 1981). Under our experimental conditions, Gsn caps actin barbed ends, thus inhibiting actin polymerization. By varying the ratio of Gsn to actin (G:A), actin filaments of different lengths can be generated (Supplementary Figure S6B). Strikingly, Gsn-treated F-actin complexes increased dynamin’s basal rate of GTP hydrolysis up to eight-fold, with the highest stimulation achieved by the shortest filaments ($k_{cat} \sim 8 \text{ min}^{-1}$; Figure 4B, black diamonds). EM images revealed that actin filaments were $51 \pm 34 \text{ nm}$ long for a G1:A5 ratio (which is equivalent to $\sim 19$ monomers), whereas they were $3.2 \pm 1.7 \mu m$ long for a G1:A300 ratio (which poorly stimulated dynamin). Together, these data show that filament lengths between $51 \pm 34$ and $700 \text{ nm}$ have the best ability to stimulate dynamin oligomerization into higher order structures.

The observed increase in GTPase activity was dependent on dynamin–actin interactions, as Gsn–F-actin complexes could neither activate the dyn1K/A mutant (Figure 4B, red...
circles), nor could addition of only Gsn (Figure 4B, black squares). As dynamin’s GTPase activity is dependent on its oligomerization state, these findings suggest that actin filaments can promote dynamin oligomerization into rings in a length-dependent manner, with the shortest filaments being most potent. As an alternative approach to investigate the ability of short actin filaments to promote dynamin oligomerization into rings, GTP hydrolysis was monitored continuously in a coupled assay (Ingerman and Nunnari, 2005). Addition of Gsn–F-actin complexes to this assay overcame the kinetic lag that reflects dynamin assembly into rings (Figure 4C, grey circles).

To directly visualize dynamin’s oligomerization status in the presence of Gsn–F-actin complexes, samples were negatively stained with uranyl acetate and observed in the electron microscope. In agreement with biochemical experiments, Gsn–F-actin complexes induced limited assembly into ring-like structures ~40 nm in diameter (Figure 4D), similar to rings that self-assemble at low ion strength (Hinshaw and Schmid, 1995). Oligomerization into single rings is consistent with the maximum eight-fold increase in the rate of dynamin’s GTP hydrolysis, as assembly into spirals causes up to a 100-fold increase (Warnock et al., 1996). Together, these data suggest that short actin filaments can have a direct and profound effect on the oligomerization status of dynamin.

**Dynamin rings induce elongation of Gsn-capped actin filaments**

The results presented so far show that direct binding of dynamin to actin promotes actin stress fibre formation in cells. Moreover, we found that short actin filaments in turn promote dynamin assembly. To understand how assembled dynamin might modulate actin dynamics, we first tested whether dynamin can promote actin polymerization. A solution-based actin polymerization assay using pyrene-labelled actin was performed (Fujiiwara et al., 2009). In this assay, pyrene fluorescence increases when monomeric actin is assembled into filaments. We tested whether addition of dynamin stimulates actin polymerization from actin seeds by reducing the lag phase, as shown for other actin regulatory proteins such are formins (Moseley et al., 2006). As shown in Figure 5A, addition of dynamin with or without GTPγS had no effect on the rate of actin polymerization. Given the ability of short actin filaments to promote dynamin oligomerization, we next tested whether dynamin rings can induce elongation of actin filaments that are capped by CP. CP is a ubiquitously expressed heterodimeric actin-binding protein that is essential for normal actin dynamics in cells. As filaments in this experiment are relatively long, dynamin oligomerization is promoted by addition of GTPγS. Addition of dynamin in the presence or absence of GTPγS failed to induce actin polymerization from the CP-capped filaments (Figure 5A). In striking contrast, dynamin induced rapid actin polymerization of filaments that were capped by Gsn (Figure 5B). Interestingly, the actin polymerization was achieved only in the presence of GTPγS, suggesting that only assembled dynamin can promote actin elongation. Importantly, ‘loss-of-function’ dyn1K/R mutant was significantly impaired in stimulating actin polymerization (blue circles), whereas ‘gain-of-function’ dyn1E/K mutant stimulated actin polymerization better than dyn1WT (green circles). Together, these data suggest that dynamin oligomerization driven by direct dynamin–actin interactions is essential for observed actin polymerization. If this conclusion is correct, it is predicted that Gsn:actin ratios that stimulate dynamin oligomerization (based on the GTPase assays presented in Figure 4B) should promote dynamin oligomerization in the absence of GTPγS. Indeed, dynamin stimulated actin polymerization at Gsn–actin ratios of 1:5 (Figure 5C, red circles), but not those capped by CP (red triangle). Together, these data show that dynamin rings can promote elongation of actin filaments capped by Gsn, but not those capped by CP.

As an alternative means to measure dynamin-dependent actin polymerization, we performed sedimentation assays. At low salt conditions, dynamin is induced to oligomerize into single rings, which pellet during high-speed centrifugation (Warnock et al., 1996), but at physiological salt concentration, dyn1 stays in the supernatant (Figure 5D, lane 7). Short actin filaments generated at a Gsn:actin ratio of 1:10 did not pellet in this assay (Figure 5D, lane 1). However, when dyn1 was mixed with Gsn–F-actin filaments, the majority of both actin and dynamin were recovered in the pellet (Figure 5D, lane 4). In contrast, dyn1 did not bring short actin filaments capped by CP into the pellet (Supplementary Figure S6D), in agreement with its inability to promote actin elongation of CP-capped filaments (Figure 5C). Together, these data argue that sedimentation of actin filaments in this assay was due to their elongation, which was promoted by actin-dependent dynamin oligomerization.

The sedimentation assay allowed us to further explore the role of dynamin rings in inducing elongation of capped actin filaments. Thus, we performed co-sedimentation assays under conditions that impaired the oligomerization of dyn1. Dynamin’s GED is directly involved in dynamin oligomerization through GED–GED contacts (Sever et al., 1999; Zhang and Hinshaw, 2001). Hence, recombinant GED competes for these GED–GED interactions and impairs dynamin oligomerization (Sever et al., 1999). As shown in Figure 5E, GED counteracted the ability of dyn1 to draw Gsn–F-actin complexes into the pellet, leaving more actin and dynamin in the supernatant (compare lanes 5 and 7). Importantly, GED had no significant effect on the fractionation of Gsn–F-actin in the absence of dyn1 (Figure 5E, compare lanes 1, 2 and 3, 4). Thus, pelleting of Gsn–F-actin in this assay was dependent on dynamin oligomerization and because of elongation of actin filaments. Together, these data show that dynamin oligomers can induce actin elongation from filaments capped by Gsn.

**Dynamin rings displace Gsn from the barbed ends of actin filaments**

Next, we attempted to elucidate the mechanism by which dynamin induces elongation of Gsn-capped actin filaments. Thus, we first investigated whether dynamin itself binds barbed ends by using actin annealing assays (Andrianantoandro et al., 2001). Short actin filaments generated by shearing F-actin can re-associate through end-to-end annealing, which is impaired by saturating concentrations of CPs (Andrianantoandro et al., 2001). G-actin was allowed to polymerize for 1 h, resulting in actin filaments that were on average ~7.4 μm long (Supplementary Figure S6A, panel 1). Shearing of F-actin by passage through a 27-gauge needle generated short actin filaments that were ~0.75 μm long (panel 2). After annealing for 2 h, the sheared filaments grew
to a length of \(3.1\,\text{m (panel 3). Addition of dyn1 with or without GTP\(\gamma\)S resulted in actin filaments that were \(3.9\,\text{m}\) long and that appeared crosslinked into bundles (panels 4 and 5). Thus, addition of dynamin did not impair annealing of short actin filaments, suggesting that dynamin, even when oligomerized into rings, did not bind naked barbed ends.

The differential effects of dynamin rings on filaments capped by Gsn and CP, both of which bind to barbed ends, suggested that dynamin might increase actin polymerization by removing Gsn from the barbed ends. To test this, actin was assembled in the presence of Gsn at a ratio of G1:A300. Under these conditions, \(490\%\) of the Gsn pellets with F-actin after high-speed centrifugation (Figure 5F, lane 2). Addition of dyn1 to Gsn–F-actin promoted release of Gsn from F-actin in a GTP\(\gamma\)S-dependent manner (Figure 5F, lane 5), whereas F-actin stayed in the pellet (lane 6). These data show that,
when oligomerized into rings, dynamin can efficiently displace Gsn from the barbed ends of F-actin.

As an alternative means to examine actin elongation that is dependent on displacement of Gsn from the barbed ends, we performed an actin re-polymerization assay (see model in Figure 5G; Barkalow et al, 1996; Pryune et al, 2002). As shown in Figure 5H, dyn1 induced actin re-polymerization, but only in the presence of GTPγS (compare black diamonds and red circles with red squares). These data show that dynamin did not inhibit actin depolymerization from the pointed end, and that only dynamin rings can promote actin re-polymerization at the barbed ends capped by Gsn.

To our knowledge, dynamin is the first protein that has been shown to induce actin elongation from Gsn-capped actin filaments by displacing Gsn from the barbed ends.

**Dynamin–actin interactions promote formation of free barbed ends in cells**

Given the role of dynamin–actin interactions in formation of FAs and stress fibres in cells (Figure 2), together with the biochemical analysis, our data suggested that dynamin rings might regulate actin polymerization at FAs by generating free barbed ends. Thus, we next examined localization of endogenous dynamin in podocytes. As shown before (Kruchten and McNiven, 2006), endogenous dynamin could be detected at FAs, marked by paxillin staining (Figure 6A). To examine whether endogenous dynamin associates with actin filaments, we performed electron microscopy on detergent permeabilized podocytes (Figure 6B; Schliwa et al., 1981). This method visualizes the cortex of cells from which the cytoplasm has been released. Dynamin antigenic sites were visualized in the electron microscope by treatment with monoclonal anti-dynamin antibody followed by secondary antibody labelled with 10 nm gold particles (white arrow). Paxillin antigenic sites were visualized by polyclonal anti-paxillin antibody followed by secondary antibody labelled with 5 nm gold particles (black arrow). Scale bars, 200 nm.

**Figure 6** Dynamin–actin interactions at focal adhesions drive actin polymerization by generating free barbed ends. (A) Dynamin localizes to focal adhesions in podocytes. Podocytes were stained using anti-dynamin antibody, rhodamin phalloidin for F-actin or anti-paxillin antibody. Dynamin co-localizes with paxillin at focal adhesions and focal complexes along the membrane. (B–D) Electron micrographs of the podocyte cytoskeleton. Podocytes contain a thick actin network. Dynamin antigenic sites were visualized in the electron microscope by treatment with monoclonal anti-dynamin antibody followed by secondary antibody labelled with 5 nm gold particles (black arrow). Scale bars, 200 nm. (E, F) Dynamin–actin interactions promote formation of free barbed ends in podocytes. Podocytes expressing the indicated dynamin mutants were stimulated with 5 nM EGF to induce de novo actin polymerization. After 5 min, cells were permeabilized in the presence of 0.45 μM biotin-labelled G-actin for 45 s, fixed and stained using rhodamin-conjugated anti-biotin antibody (red). In (E), total F-actin was labelled using FITC phalloidin (green). In (F), cells were stained using anti-dynamin antibody (green). Scale bars, 20 μm. (G, H) Quantification of barbed ends per cell (G) and length of the newly synthesized actin filaments (H). Data represent measurements of >20 cells (except for dyn1K/E where only 10 cells were examined) and are plotted as ± s.d. *P<0.05, **P<0.01. (I) Working model for the role of dynamin oligomerization in regulation of actin cytoskeleton. Short actin filaments are generated by gelsolin-driven cleavage of the actin filaments. Their high local concentration promotes dynamin oligomerization into rings, which in turn displace gelsolin from the barbed end and allow filament extension.
detected using monoclonal anti-dynamin antibody followed by 10-nm gold-conjugated secondary antibody (white arrow in Figure 6C), whereas paxillin antigenic sites were detected using anti-paxillin polyclonal antibody followed by 5-nm gold-conjugated secondary antibody (black arrow in Figure 6C). Dynamin antigenic sites were concentrated along actin filaments at distinct locations. Groups of large gold particles co-localized with small gold particles (Figure 6B–D), suggesting that in some instances, dynamin clustered around FAs.

Next, we examined whether dynamin–actin interactions underlie the formation of free barbed ends in cells. Formation of barbed ends in cells expressing different dynamin mutants was examined upon EGF stimulation (Figure 6E and F; Symons and Mitchison, 1991; Baillie et al., 1999). In this assay, permeabilized cells were allowed to incorporate biotin-labelled G-actin at barbed ends in situ for 45 s (red in Figure 6E and F). Thus, newly polymerized actin incorporated at barbed ends will be labelled red (compare biotin-actin filaments at distinct locations. Groups of large gold particles co-localized with small gold particles (Figure 6B–D), suggesting that in some instances, dynamin clustered around FAs.

The data presented in this paper suggest a novel, dynamin-dependent mechanism that promotes actin polymerization (Figure 6I). In this model, proteins such as Gsn first fragment and cap F-actin filaments; the resulting short filaments bind and promote dynamin assembly into rings; dynamin rings in turn displace CPs; finally, the uncapped barbed ends undergo polymerization. The model is based on four key observations. First, we discovered a direct, functional interaction between dynamin and actin. Mutations in a previously unrecognized ABD of dynamin reduced actin-binding affinity stimulated formation of stress fibres in cells. Therefore, direct binding of dynamin to actin is crucial for maintenance of the actin cytoskeleton. Second, actin directly regulates dynamin self-assembly. Thus, using GTPase assays and electron microscopy, we found that the interaction of short actin filaments with dynamin promotes dynamin self-assembly. Third, assembled dynamin dissociates the actin-CP Gsn from barbed ends in vitro, thereby allowing filament elongation. Fourth, direct dynamin–actin interaction promotes formation of free barbed ends in podocytes. Together, the data establish a feedback loop in which short actin filaments promote formation of dynamin rings, which in turn displace barbed end CPs, thereby allowing actin filament elongation.

A role for dynamin rings in regulating Gsn-dependent actin polymerization

Many cellular processes in which dynamin has been implicated are driven by actin polymerization (e.g. cell motility, endocytosis, cytokinesis). They all depend on rapid bursts of actin filament assembly at specific subcellular locations. Actin polymerization can be achieved either by de novo filament formation, or by severing of pre-existing filaments and elongation of the resulting barbed ends. The extent of filament elongation in vivo is limited by the presence of high-affinity barbed end CPs (for review see Chesarone and Goode, 2009). Our study identifies a novel role for dynamin rings in regulating actin polymerization by displacing barbed end CPs such as Gsn. As dynamin rings displaced Gsn, but not CP, our study suggests specificity in dynamin’s interaction with different CPs.

Dynamin’s novel role in regulating actin polymerization through Gsn is consistent with the previous studies, which implicate Gsn in the same cellular processes that are regulated by dynamin. Thus, Gsn has been implicated in podosomal assembly (Chellaiah et al., 2000), reorganization of cortical actin filament networks that underlie protruding lamellae (Saflieko-Mroczka and Bell, 2001) and the length of Listeria actin tails (Laine et al., 1998). Indeed, immunofluorescence micrographs revealed that both dynamin and Gsn concentrate just behind motile bacteria, at the junction between the actin filament rocket tail and the bacterium (Laine et al., 1998; Lee and De Camilli, 2002; Orth et al., 2002). Finally, it has been shown that Gsn also has a function in stress-fibre-dependent cell contraction (Arora et al., 1999). While Gsn has been implicated in actin polymerization, the mechanism by which Gsn, once associated with the barbed ends, can be displaced has not been identified (Kuhn and Pollard, 2007). Our study suggests that dynamin rings regulate actin polymerization at the FAs by displacing the CP Gsn. We are currently exploring in detail how dynamin rings promote the de novo formation of FAs.

We can envision two different mechanisms by which dynamin rings displace gelosolin from the barbed ends. One possibility is that dynamin binds and displaces Gsn. However, Gsn did not affect the GTPase activity of dynamin, suggesting that these two proteins might not form direct protein–protein interactions. Nevertheless, oligomerization-dependent interaction of dynamin with Gsn on actin filaments cannot be ruled out. A second possibility is that dynamin affects Gsn indirectly. We show that dynamin rings bind and align F-actin filaments into bundles with defined filament–filament spacing (Figure 3E). Thus, it is possible that dynamin rings alter filament geometry/twist and thereby affect the binding properties of other actin-binding/regulatory proteins including Gsn (Stokes and...
DeRosier, 1987). We favour the second model for the following reasons. Gsn binds to filament ends and changes the twist of actin filaments, which in turn displaces phalloidin (Cooper, 1987). This observation implies that locking F-actin into an unfavourable conformation could disfavour Gsn binding. In contrast, CP is not known to alter actin filament twist, and CP was not displaced by dynamin rings. These data are consistent with the idea that dynamin rings stabilize terminal actin subunits in a conformation that disfavours Gsn binding. By analogy with cofilin, another protein that binds and changes actin geometry (McGough et al., 1997), we speculate that dynamin rings promote the formation of a distinct population of conformationally unique actin filaments, which binds or releases specific sets of actin-binding/regulatory proteins.

**Role of dynamin–actin interactions in regulation of dynamin oligomerization**

Our study identifies unexpected regulation of dynamin’s oligomerization status by actin filaments. Interestingly, while long actin filaments had no effect on dynamin oligomerization, shorter actin filaments (50–700 nm) promoted dynamin oligomerization into single rings. In addition, we have developed a novel, cell-based assay that measures dynamin oligomerization into higher order structures using fluorescence lifetime imaging microscopy. This assay confirms that dynamin oligomerization is promoted by short actin filaments in live cells (Gu et al., manuscript in preparation). As dynamin oligomerization is highly cooperative with respect to dynamin concentration (Warnock et al., 1996), our data suggest that local concentration of short actin filaments promotes actin-dependent dynamin oligomerization at distinct cellular locations. Supporting our hypothesis, we found that dynamin antigenic sites are concentrated at distinct locations on actin filaments, some of which are most likely FAzs (Figure 6C and D).

The mechanism by which short actin filaments promote dynamin oligomerization is not readily apparent and is topic of future studies. It is plausible that short filaments exhibit lower steric hindrance, thus allowing more efficient oligomerization of bound dynamin. Regardless of the mechanism, the preference for short filaments is expected to provide temporal and spatial regulation of dynamin assembly.

Importantly, a number of SH3-domain containing proteins that bind/regulate actin such as SNX9 and cortactin have been shown to regulate dynamin oligomerization (Yarar et al., 2007; Mooren et al., 2009). Thus, it seems reasonable to conclude that dynamin oligomerization is regulated not only by the length of actin filaments, but also by PRD–SH3 interactions. In summary, our study provides a direct link between the oligomerization cycle of dynamin and the regulation of the actin cytoskeleton. It further suggests that dynamin’s effect on the actin cytoskeleton will ultimately depend on actin binding, its intrinsic oligomerization cycle and other actin regulators that modulate dynamin’s assembly state.

**Materials and methods**

**Cells, antibodies, reagents and standard techniques**

Mouse podocyte cell lines were grown as described previously (Mundel et al., 1997). Antibodies: anti-dyn1/2 hudy 1 (Upstate Technology, Lake Placid, NY); mouse anti-dyn1 VAM-SVO41 (StressGene, Victoria, Canada); anti-dyn2 (Santa Cruz Biotechnology, Delaware Avenue, CA); rabbit polyclonal anti-paxillin (StressGene, Victoria, Canada); mouse monoclonal anti-Gsn (Sigma-Aldrich, Saint Louis, MO); anti-cortactin (Millipore, Billerica, MA). Subcellular fractionation was performed as described (Damke et al., 1994). GTPase assays were performed as described (Leonard et al., 2005). PC/PIP vesicles and actin bundling experiments were performed as described (Schafer et al., 2002). Adenoviral infections of cultured podocytes were performed as described (Schafer et al., 2002). Internalization of rhodamine transferrin was performed using HeLa cells, 18 h post-infection with 20 µg/ml-1 of rhodamine-Tfn in PBS containing 1 mM CaCl2, 1 mM MgCl2, 5 mM glucose and 0.2% BSA for 10 min at 37°C. The wound-healing assays were performed as described (Asanuma et al., 2006). Rho type GTPase activation assays were performed using commercial Rho activation kit (Cell Biolabs, San Diego, CA).

**Plasmid constructs**

Plasmid construction for adenovirus production and in vitro transcription and translation was accomplished by Gateway Technology System vector (Invitrogen). Donor vector was generated by cloning full-length human dyn1 or dyn2 cDNA into the pDONR201 vector using site-specific recombination reaction of BP clonase II enzyme. Actin-binding defective dyn mutant constructs were generated using appropriate site-directed mutagenesis. Resulting fragments were cloned using BstXI and ClaI into the pDONR201-containing gene for dyn. IVT expression vector (pCTT4 (Invitrogen) was generated using pDONR201 vector and the site-specific recombination reaction of LR clonase II. Adenoviral vectors were generated by recombining pDONR201 with pAd/CMV/V5-DEST. C-terminally His-tagged dynamin was generated by subcloning dyn1 cDNA into the pGE-60 vector (Qiagen) using Ncol and BamHI enzymes. All subsequent mutants were generated by subcloning BstXI and ClaI fragments from pDONR201 into pQE-60 (Qiagen). Vectors expressing ECFP and EYFP N-terminally tagged dynamin were generated using pECFP and pEYFP vectors (BD Biosciences Clontech). To generate the dyn1 APRD mutant, first, 970-bp PCR products corresponding to nt 1414–2250 of human dynamin1 coding DNA sequence were amplified using specific primers, incorporating three stop codons and Narl restriction site at COOH terminus of PCR products. Subsequently, ClaI/Narl digested PCR fragments were subcloned into the corresponding region of full-length dynamin1 cDNA in pDONR201 vector via T/A cloning using pGEM-F-easy vector (Promega). To construct double mutants, dyn1 APRD/EK and –KE, dyn1 EK and –KE mutant constructs were restricted by BstXI and ClaI enzymes and then the resulting DNA fragments were subcloned into the corresponding regions of dyn1 APRD cDNA in pDONR201 vector. Adenoviral vectors were generated by recombining pDONR201 with pAd/CMV/V5-DEST, using LR clonase II and production of adenoviruses was accomplished followed by manufacturer’s manual (Invitrogen). All constructs were verified by DNA sequencing.

**Dynamin expression and purification**

Non-tagged human dyn1 (isoform 1) was expressed using baculovirus expression system in Trn5 insect cells, and was purified as described (Damke et al., 2001). Baculoviruses expressing C-terminally His-tagged rat dyn2 were generous gift from D. Doerling and were generated as described (Damke et al., 2001). Dynamin oligomerization into single rings was induced by incubation of Trn5 infected insect cells with 1 mM GTPγS and 200 µM ATP in presence of 40 µM dynamin1 and dynamin2. Dynamin oligomerization was confirmed by gel filtration chromatography.

**Dynamin–actin interactions regulate the actincytoskeleton**

Non-tagged human dyn1 (isoform 1) was expressed using baculovirus expression system in Trn5 insect cells, and was purified as described (Damke et al., 2001). Dynamin oligomerization into single rings was induced by incubation of Trn5 infected insect cells with 1 mM GTPγS and 200 µM ATP in presence of 40 µM dynamin1 and dynamin2. Dynamin oligomerization was confirmed by gel filtration chromatography.
References


References


Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

We are indebted to Fujwara Ikuko and John A Hammer III for providing recombinant CP as well as pyrrole-labelled actin. This work was supported by the National Institute of Health (R01 DK64787 to SS) and the NephCure Foundation.
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