THE EMBO JOURNAL

Manuscript EMBO-2010-74729

Direct dynamin-actin interactions regulate the actin cytoskeleton

Changkyu Gu, Suma Yaddanapudi, Astrid Weins, Teresia Osborn, Jochen Reiser, Martin Pollak, John Hartwig and Sanja Sever

Corresponding author: Sanja Sever, Massachusetts General Hospital and Harvard Medical School

Review timeline:

Submission date:	10 May 2010
Editorial Decision:	11 June 2010
Revision received:	30 July 2010
Editorial Decision:	25 August 2010
Revision received:	09 September 2010
Accepted:	10 September 2010

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 11 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see while all three referees consider the study as interesting in principle it becomes clear from their reports that the evidence presented is not yet sufficiently conclusive to fully justify the conclusions drawn. Given the perceived potentially controversial nature of the conclusions it will be particularly important that the evidence presented is strong and fully convincing. Given the interest expressed by the referees in principle we would thus be able to consider a revised version of this manuscript in which you need to address or respond to the criticisms raised by the referees in an adequate manner and to their satisfaction. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript as well as on the final assessment by the referees. Please do not hesitate to contact us at any time should you wish to discuss aspects of your revision with us.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer-Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript details experiments showing that dynamin, a large GTPase, interacts directly with the cytoskeletal protein actin. The authors identify a region on dynamin that binds to actin filaments, and they generate mutations in this region to show that mutant dynamin affects actin organization in vitro and in cells. Further studies show that actin promotes assembly of dynamin polymers that enhance actin polymerization, presumably by uncapping the barbed ends of actin.

This paper will be of interest to a wide audience, as it extends the current view of dynamin-mediated ffects on the actin cytoskeleton mediated through SH3-domain containing proteins while confirming the previous observations that dynamin is an important modulator of actin organization and dynamics.
There will be some resistance to this observation as many have dismissed the association between tubulin and Dyn as irrelevant based on charge, size, etc. Indeed, Sandy Schmid has suggested that the Dyn ring diameter is a perfect fit for a Mt (microtubule?), thus forming a spurious interaction. A similar off-target model will be made for these observations I am afraid. The fact that non-biological interactions (antibodies) with the dynamins increases polymerization/activity may also cause some concern.

Suggestion to improve the study are below.

Major:

The manuscript is generally well written, but it is huge and difficult to read. Possibly this was reviewed previously at other journals with demanding editors/reviewers, but the story in its current form is unwieldy. If one truly wants to understand all the data, it can take nearly a day to read. Each primary figure is complex and filled with data, which is a challenge, but the supplemental figs are putative. Examples are the 10 graphs/gels in Fig S1, the 40 IF images in S3, the 35 IF images in Fig S6, the 30 variables in Table 1, and so on. The story needs to be paired down substantially if anyone is going to read it or understand it.

The gelsolin data seems to muddy the whole story. The initial observation showed lots of Dyn coming down with polymerized actin. Subsequent figures showed a requirement of short filament induced by gelsolin, which seems inconsistent. Not sure why the gelsolin observation couldn't be just one part of one figure; instead, it dominates the story which is overly complex and distracting.

The expression of Dyn1 in Dyn2 KO epithelial cells that do not normally even express Dyn1 seems artificial and contrived. The Dyn2 actin binding is less than the Dyn1 in vitro, but doesn't that call into question the relevance of the observation in epithelial cells? This seems pretty important.

The EM of Dyn1 rings seem off both in size, diameter, and shape. How do these compare to those of Hinshaw that are smaller and more uniform?

Figure 1a/b: In this figure, purified dynamin is shown to bind to actin in ultracentrifugation assays. The authors should show the purity of the dynamin and actin, particularly by performing western blots to ensure that actin binding proteins such as cortactin are not present in the purified fraction. The authors use in vitro translation in Fig. S1 to show that truly pure Dyn can pellet with actin (a key piece of data), yet in this figure the actin band is not apparent so it is not clear that we are looking at a coomassie-stained gel that results from a co-sedimentation assay.

Figure S1C: It is clear that in vitro translated Dyn1d399-444 fails to pellet with actin to the same extent as wt Dyn1, yet it is not clear that a Dyn2 mutation in the same region has a substantial effect on actin-binding. There are at least two possibilities to explain the large amount of Dyn2 pelleting with actin: 1) Dyn2 contains one or more additional actin-binding sites, or 2) the pelleting assay is detecting Dyn2 oligomers that precipitate in the presence of actin (this is suggested by the slight band seen in the pellet in the absence of actin). If the latter is true, the authors will still need to explain how actin can influence Dyn2 oligomer formation in a Dyn2 mutant that lacks an actin-binding site.

Authors should either reference or provide the sequences of the shRNA used to knockdown Dyn2 (Fig 2, S3).

Fig. 3F shows a clear example of Dyn enhancing F-actin polymerization in the presence of lipids. Authors should show a control with actin + lipids, without dynamin.

On page 10 the authors write 'consistent with experiments in podocytes, dynΔPRD/KE failed to induce stress fiber formation', yet the image shown in Fig 3SO2 clearly shows an actin aster that appears very similar to those in cells expressing dynΔPRD or dynΔPRD/EK. It is not apparent that the constructs have an "opposite effect"? This should be clarified. Further, it is unclear how these data suggest that interactions with dynamin's PRD inhibit dynamin's ability to polymerize F-actin, as suggested by the authors in the same paragraph.

A major thesis put forth in the paper is that dynamin rings regulate actin polymerization/stabilization. The E/K 'gain of function' mutation in the dynamin actin binding domain enhances dynamin's effect on actin. The in-cell FLIP experiments suggest that dynamin E/K oligomerizes, but there is no direct evidence that the construct forms normal rings. Further, the distribution of the polymerized Dyn is confusing in the cells. While the color change is consistent with the manipulations performed, why does the polymerized Dyn not localize to sites of enriched actin such as lamellipodia? The distribution appears diffuse or even non-biological? Maybe a comet assay would be nice to see if the active Dyn can actually be localized to a cellular structure?

Fig. 6F: Show the Dyn band. Does it pellet with actin or in the supt with gelsolin?

Minor:

Authors should explain why they often used different amounts of Gsn:Actin ratios during the various biochemical assays.

Page 10: Authors state that 'interactions between dynamin's PRD and SH3-domain domain might inhibit dynamin's ability to promote polymerization of F-actin'. Dynamin does not have an SH3 domain. Do you mean to write SH3-domain containing proteins?

Referee #2 (Remarks to the Author):

In this manuscript, Gu et al. identify an F-actin binding site on the protein dynamin, which allows dynamin to bundle filaments in vitro and participate in stress fiber formation in vivo. They further show that short actin filaments trigger the formation of dynamin rings with concomitant GTP hydrolysis, while longer filaments do not. Finally they claim to show that oligomerized dynamin dissociates gelsolin from filament barbed ends, allowing filament elongation. The overall idea is that short, capped filaments generated by gelsolin at distinct sites in the cell incite dynamin to oligomerize and kick off the gelsolin, allowing filament growth.

This paper is an interesting study, using a battery of different techniques and comprising a very thorough study of the question of dynamin-actin interaction and function. However there are some major points (some quite major, like point 7 and point 9) that need to be addressed before this study can be considered for publication. There is also a certain sloppiness that is disconcerting to this reviewer; for example, almost all of the images lack scale bars and the few references this reviewer looked up were not correct (detailed below). The error detailed in point 7 is a little shocking. Finally

the paper is quite heavy and the authors might think about leaving out some data, and maybe shortening the discussion, which is excessively detailed.

Major Points
1. For all of the binding studies, the authors say that they are using a tetrameric form of dynamin, but did the authors check this and are they sure that they don't have dimers also? What form is the mutant dynK/A in since the authors show that it has impaired oligomerization?

2. How was the quantification done in Figure 2C and 2D for evaluating the loss of F-actin and focal adhesions?

3. Figure 3B and 3C evoke the use of gelsolin:actin complexes in the legend and in the panels, but no mention is made of why gelsolin is used for these experiments in the text and the discussion about regulating filament length with gelsolin comes much later.

4. The authors show evidence for dynamin associating with actin to form bundles and they claim that dynamin rings (GTPgammaS conditions) does likewise, but this reviewer sees a drastic difference in Fig 3A and a significant different in 3B. Why do the authors treat this result so lightly? If dynamin rings don't associate well with actin, this calls into question their model.

5. In Figure 3E, the little black arrows are mysterious. Are we looking at dynamin rings glued to the sides of filaments, or do the authors see the ring going around the filament like a belt? This reviewer can't tell. The sentence about the 17-20 nm spacing of filaments in the bundles is not well justified- what is the supposed spacing between the actin binding sites in the tetramer or the oligomer?

6. Figure 3F needs control panels showing what lipids look like with just actin or just dynamin.

7. The interpretation of Figure 4 and Figure S1K is incorrect. The authors' calculation of how many subunits their filaments are composed of at a given gelsolin concentration is erroneous. A gelsolin:actin ratio of 1/5 gives a filament that is 13.5 nm long and that corresponds to FIVE subunits, not 14 as written in the text. This is an important point, as with only 5 subunits, the authors' suggestion that dynamin rings change the twist of the filament to kick off gelsolin becomes very hard to swallow. The formula for length versus gelsolin is that the length in microns = 1/(370R) where R is the gelsolin/actin ratio. This comes from the fact that each monomer adds 2.7 nm to the filament (so a 1 micron filament has 370 monomers in it) and each gelsolin associates with one filament. The original reference for this is Hanson and Lowy 1963, but this reviewer found it more clearly stated in Janmey et al (J Biol Chem. 1986 Jun 25;261(18):8357-62.) At any rate (despite its title), the cited reference Yin et al 1981 says nothing about this issue, nor does Andrianantoandro et al. In fact, Andrianantoandro et al. is overall a very bad citation (top of page 16) since it says nothing about either how CytoD regulates filament length (this chemical is not even used in the article) nor does it say that a 0.4 micron long filament has 430 monomers. In fact in the legend of Figure 5 of Andrianantoandro et al., they give that 0.6 microns = 225 monomers, i.e. as stated above, each monomer adds 2.7 nm to the filament NOT 0.9 nm as used by the authors!!
This needs to be corrected in the text and Figure S1K, and the authors need to think about how this changes their story. On a five-subunit filament, how can gelsolin and a dynamin ring even bind together given that each needs to bind an actin subunit and that each has a certain radius? Unless dynamin/dynamin rings bind to gelsolin-did the authors check this?

What is the use of Figure S1J?

Why didn't the authors test G1:A2 or G1:A1 in Figure 4C? Does GTP hydrolysis keep going up even though now we are talking about filaments that are only a subunit or two long? Presumably G1:A1 would give no GTP hydrolysis since that corresponds to G-actin. Is there a minimal length where GTP hydrolysis (i.e. ring formation) is no longer triggered? This is important to test.

8. Page 22, the authors say that CytoD severs filaments. This reviewer has never heard that before. CytoD caps filaments, and so CytoD doesn't necessarily generate short actin filaments-it depends on the incubation time. When the filament is capped, it can only depolymerise from its pointed end and not polymerize at all, so over time, filaments will get shorter. However in the beginning, filaments are simply not growing (since pointed end dynamics are slow); conversely, at long incubation times,

all filaments could be depolymerized. The authors need to do a time dependence of their FRET study on CytoD cells and provide some quantification of filament length. In the Figure S5E, the difference between CytoD and Latrunculin is not convincing-quantification is needed or electron microscopy.

9. The section concerning the displacement of gelsolin from barbed ends by oligomeric dynamin is the weakest part of the paper. The experiments in Figure 6C and E were done with a G1:A1000 ratio-i.e. of a length that would give little or no natural GTPase activity by dynamin. Why were the experiments done this way and not with short filaments and no GTPgammaS? It is an interesting result that dynamin can uncap long filaments (this reviewer does not know of another uncapper), but what is the physiological relevance of this since the authors have taken great pains to show that this is NOT how it works in vivo?

Also the authors should see if dynamin can uncap filaments protected by capping protein.

Referee #3 (Remarks to the Author):

The manuscript 'Direct dynamin-actin interactions regulate actin cytoskeleton' by Gu et. al. deals with a long standing question in cell biology which is the effect of dynamin - and endocytosis in general - on actin remodeling.

The authors show binding of a dynamin 1 isoform to F-actin and the bundling effect on filaments. Point mutants in the putative F-actin binding domain affect bundling and show effects in vivo on actin organization. Furthermore, the authors show data, which suggest that dynamin 1 might work as an 'uncapping protein' for gelsolin. This would be the first protein that is able to remove Gelsolin from the barbed end of filaments.

The manuscript by Gu et al. is heavily loaded with data and experiments which were technically performed on a high level. Nevertheless it would need to be condensed to the important aspects relating to actin.

This manuscript is one of the examples were the referee is torn between 'high impact and high significance for the cytoskeleton field' and 'mixture of 'unspecific' dynamin activities and overinterpretation of data'. This is not meant in a negative way.

Direct binding (specific and in vivo) and an effect of dynamin on actin filament organization would be a great step in understanding the link of dynamin to actin. Coupled with dynamins GTPase and assembly activity would be a very intuitive way of regulation. And last, the uncapping of Gelsolin capped filaments by dynamin would be like finding the 'holy grail' in the gelsolin field, partly solving the long standing mystery of what removed gelsolin from filaments under physiological conditions (in vivo).
On the other hand, the manuscript breaks with a number of past findings, which I find very surprizing and hard to understand - not saying that old dogmas need to remain untouched. A large number of labs have looked into the link of dynamin to actin and to my knowledge have ended up with dynamin being indirectly linked to actin remodeling via the binding of actin regulators (such as cortactin, profilin, etc.). These labs have for sure searched very hard for a direkt binding of dynamin to actin and for effects on actin polymerization (it is the most straightforward explanation) with the (disappointing?) result that dynamin is not directly interacting with actin and is not controlling polymerization kinetics. How can this be reconciled? Second, the uncapping of Gelsolin and the mechanism is not obvious and convincing to me and I see no indication in the manuscript that this plays a role in vivo (see comments below). Uncapping by dynamin would be very interesting but is such an important finding that it needs to be investigated in more detail also in vivo. 'Uncapping activities' have become popular in the actin field, however the term has remained very fuzzy and has frequently been used wrongly by others. Last, the part of the manuscript dealing with FLIM is very lengthy (5 pages of results) and seems a bit artificially squeezed in. It really looks like the authors are taking the opportunity to introduce their FLIM system for dynamin, which belongs somewhere else and has limited relevance for the topic of the manuscript (seems like a manuscript in the manuscript).

Critique:

- the manuscript is not consistent in terms of using dynamin isoforms. The authors need to be specific in the text what they are showing in the experiments, just dynamin is missleading - is it dynamin 1 or 2 or which splice form?

- the manuscript is not consistent in using dynamin 1 for the in vitro/in vivo assays, in vitro dynamin 1 is used in most experiments, in vivo experiments are performed in dynamin 2 expressing cell lines? It is not clear to me how to interpret the results without knowing that dynamin 1 and 2 are equivalent in terms of actin regulation, and if not the interpretation by the authors is on shaky ground.

- if the putative actin binding domain is indeed an actin binding domain I find it surprizing that the authors have not tried to delete the entire domain, or transplanted the domain. Is it in the end a simple charge effect? Would synthesized peptides from the region have the same effects in the in vitro assays (bundling, uncapping)? It is well known that many positively charged peptides function like bundling proteins. Whether this then might have relevance for dynamin in vivo is not clear to me. The Sh-RNA/complementation experiments are an indication but not conclusive. We might look at very indirect effects - e.g. retraction fibers, toxic effects. We learn little of what the dynamin point mutations are doing to cell division, proliferation etc., which could explain the aberrant structures.

- I am a bit worried about the fact that expression of the dynamin point mutations have an effect on cells independently of the presence or absence of dynamin 2?! Arguing that the mutants have some sort of dominant negative effect on cells ...

- in Fig. 6C I wonder what the result would be in the absence of Gelsolin ? Is there an effect by GTP-gammaS? Same question for the Gelsolin release assay (6B)
why do the authors not consider pointed end nucleation as an alternative explanation for the gelsolin-dynamin effects?

- is the 'uncapping' gelsolin specific - how about capZ, capG?

- I find it very hard to follow the rational in explaining the mechanism of uncapping by dynamin. Dynamin has no affinity for the barbed end. Dynamin binds to the side of the filament - how should this cause uncapping? What is the effect of the mutant dynamins on 'uncapping' (e.g. K44A?)? Is GED, which inhibits oligomerization, having an effect on 'uncapping' by dynamin? The authors suggest a change in twist - is there any solid support for this?

- is there any indication for dynamin mediated uncapping in cells? Is the number of free barded ends different when overexpressed or when mutant forms are expressed?

- one point which is not addressed, but might be important - does dynamin interact with gelsolin itself??

- perhaps I missed it, but I do not recall any staining of F-actin structures (actin bundles!) in the manuscript using dynamin antibodies?!? Again, is the filament binding restricted to in vitro?

- it is not clear to me what I should see and learn from Fig. 3 ? Again here dynamin 2 is used, while dynamin 1 is used in the other experiments ... why?

1st Revision - authors' response 30 July 2010

We thank the reviewers for their thorough and thoughtful comments. In response to the reviewers comments, we have modified the manuscript in the following, major ways.

1. We show that dynamin cannot effect actin polymerization of actin seeds (Figure 5A).

2. We have added data for capping protein (CP). We now show that dynamin cannot induce actin elongation from filaments capped by CP, demonstrating a specific effect of dynamin on gelsolin-capped actin filaments (Figure 5B).

3. We show that dynamin can displace gelsolin from short actin filaments in the absence of GTPgS (Figure 5D).

4. We have co-localized dynamin with focal adhesions via EM (Figure 6B-D).

5. We show that expression of the dynamin mutant that binds better to actin also promotes the formation of barbed ends in cells (Figure 6E-H).

6. To simplify the manuscript, we have removed the FLIM data.

Below is our detailed response to the reviewers' comments

Referee #1 (Remarks to the Author):

This manuscript details experiments showing that dynamin, a large GTPase, interacts directly with the cytoskeletal protein actin. The authors identify a region on dynamin that binds to actin filaments, and they generate mutations in this region to show that mutant dynamin affects actin organization in vitro and in cells. Further studies show that actin promotes assembly of dynamin polymers that enhance actin polymerization, presumably by uncapping the barbed ends of actin. This paper will be of interest to a wide audience, as it extends the current view of dynamin-mediated ffects on the actin cytoskeleton mediated through SH3-domain containing proteins while confirming the previous observations that dynamin is an important modulator of actin organization and dynamics. There will be some resistance to this observation as many have dismissed the association between tubulin and Dyn as irrelevant based on charge, size, etc. Indeed, Sandy Schmid has suggested that the Dyn ring diameter is a perfect fit for a Mt (microtubule?), thus forming a spurious interaction. A similar off-target model will be made for these observations I am afraid. The fact that non-biological interactions (antibodies) with the dynamins increases polymerization/activity may also cause some concern.

Suggestion to improve the study are below.

Major:
(1) *The manuscript is generally well written, but it is huge and difficult to read. Possibly this was reviewed previously at other journals with demanding editors/reviewers, but the story in its current form is unwieldy. If one truly wants to understand all the data, it can take nearly a day to read. Each primary figure is complex and filled with data, which is a challenge, but the supplemental figs are putative. Examples are the 10 graphs/gels in Fig S1, the 40 IF images in S3, the 35 IF images in Fig S6, the 30 variables in Table 1, and so on. The story needs to be paired down substantially if anyone is going to read it or understand it.*

As suggested by the reviewer, the manuscript has been extensively rewritten in order to make it more concise. In line with this suggestion, we have removed supplemental data showing the actin cytoskeleton in cells expressing different dynamin mutants (original Fig S3F, S3G, S3H). In addition, we have removed all the FLIM data from the paper, but we describe it briefly as unpublished results.

(2) The gelsolin data seems to muddy the whole story. The initial observation showed lots of Dyn coming down with polymerized actin. Subsequent figures showed a requirement of short filament induced by gelsolin, which seems inconsistent. Not sure why the gelsolin observation couldn't be just one part of one figure; instead, it dominates the story which is overly complex and distracting.

We apologize if this was not clear in the original version of the manuscript, but there are several *distinct* ways by which dynamin can be induced to oligomerize as well as to pellet under high speed centrifugation. In Figure 1A, unoligomerized dynamin is brought into the pellet due to dynamin-actin interactions. Those interactions do not require dynamin oligomerization, nor can the long actin

filaments used in this assay promote dynamin oligomerization. When long actin filaments are used in the study (e.g. Figures 5C, 5G, 3E), dynamin oligomerization on filaments is induced by addition of GTPγS. Our study identifies dynamin oligomerization into rings in the presence of short actin filaments (Figures 4B, 4C). Thus, in experiments in which we use short filaments we do not use GTPγS (e.g. new Figure 5D). Thus, our experimental approaches are not inconsistent with each other, nor are the data.

As far as the prominence of gelsolin data are concerned, our results demonstrates an ability of dynamin to induce elongation from the gelsolin capped filaments but not those capped by capping protein (CP), demonstrating specificity for the role of dynamin in regulation of gelsolin-dependent actin polymerization. Thus, the data concerning gelsolin are an essential part of the manuscript since they provide an explanation for how dynamin can affect actin dynamics.

(3) The expression of Dyn1 in Dyn2 KO epithelial cells that do not normally even express Dyn1 seems artificial and contrived. The Dyn2 actin binding is less than the Dyn1 in vitro, but doesn't that call into question the relevance of the observation in epithelial cells? This seems pretty important.

Our data do not in fact suggest that dyn2 has a lower affinity for F-actin than dyn1. As shown previously (Warnock et al., 1997, Mol Biol Cell, 8:2553), dyn2 has a higher propensity to self assemble than dyn1. Thus, under experimental conditions used to measure actin-dependent dynamin pelleting (Figure 1A), dyn2 would be mostly present in the pellet regardless of the presence or absence of F-actin (our data not shown). This explains why other investigators that studied dynamin's role in regulation of actin could not examine whether dynamin 2 interacts with F-actin. Given the conservation of the actin binding domain between dyn1 and dyn2, and data shown in Fig S1F, it appears they have similar Kds for F-actin.

Furthermore, work by Sandy Schmid (Liu et al., 2008, Mol Biol Cell, 19:5347) examined in detail isoform and splice-variant specific functions of dyn2 versus dyn1. They showed that dyn1 expression can efficiently rescue actin driven processes such as macropinocytosis and cytokinesis in cells conditionally lacking dyn2. They also showed that expression of dyn1 could not efficiently rescue clathrin mediated endocytosis. Together, these experiments led them to suggest that dynamin isoforms do have distinct functions in cells, but that their roles in regulation of the actin cytoskeleton are overlapping. Indeed, we placed ABD mutations within dyn2 and showed that their expression leads to identical phenotypes to dyn1 mutants (Fig S5D).

(4) The EM of Dyn1 rings seem off both in size, diameter, and shape. How do these compare to those of Hinshaw that are smaller and more uniform?

As far as diameter is concerned, the most important parameter, they are identical to "Hinshaw rings," which also measure ~40 nm. In addition, being rings, our are similar in shape to Hinshaw rings. However, in contrast to Hinshaw rings, which seem empty in the middle, our rings have protein density in the middle. At present we do not know whether this protein density is due to dynamin or actin proteins.

(5) Figure 1a/b: In this figure, purified dynamin is shown to bind to actin in ultracentrifugation assays. The authors should show the purity of the dynamin and actin, particularly by performing western blots to ensure that actin binding proteins such as cortactin are not present in the purified fraction. The authors use in vitro translation in Fig. S1 to show that truly pure Dyn can pellet with actin (a key piece of data), yet in this figure the actin band is not apparent so it is not clear that we are looking at a coomassie-stained gel that results from a co-sedimentation assay.

As requested by the reviewer, we have used Western blot analysis of our dynamin and actin prep to show that they lack detectable amounts of cortactin (new Figure 1B). In addition, Figure S1 shows an autoradiograph of ^{35}S labeled total protein generated by in vitro transcription/translation, and we have included a Comassie stained gel showing the presence of actin in the pellet. We have included this explanation of the data in the current legend to Figure S1A.

(6) Figure S1C: It is clear that in vitro translated Dyn1d399-444 fails to pellet with actin to the same extent as wt Dyn1, yet it is not clear that a Dyn2 mutation in the same region has a substantial effect on actin-binding. There are at least two possibilities to explain the large amount of Dyn2 pelleting with actin: 1) Dyn2 contains one or more additional actin-binding sites, or 2) the pelleting assay is detecting Dyn2 oligomers that precipitate in the presence of actin (this is suggested by the

slight band seen in the pellet in the absence of actin). If the latter is true, the authors will still need to explain how actin can influence Dyn2 oligomer formation in a Dyn2 mutant that lacks an actin-binding site.

As pointed out by the reviewer, it does appear that there is significant binding of dyn2Δ399-444 to actin. Although we cannot rule out that this reflects a second binding site, we consider this somewhat unlikely. In any case, our data do show diminished binding of dyn2Δ399-444 to actin versus dyn2WT, which the reviewer acknowledges. Most importantly, knockdown of dyn2 causes an actin phenotype, and this phenotype is restored by dyn1WT but not dyn1Δ399-444. The simplest explanation of these results is that dyn2 is required for normal actin dynamics (since knockdown causes an actin phenotype), dyn1 can replace this function of dyn2 (since it restores the phenotype), and that this function is carried out by the ABD (since disruption of the dyn1ABD, which is identical to that of dyn2, prevents the rescue). In direct support of this interpretation, over-expression of dyn2K/E shows a strong dominant negative phenotype with a disrupted actin cytoskeleton (Figure S5D).

(7) Authors should either reference or provide the sequences of the shRNA used to knockdown Dyn2 (Fig 2, S3).

A detailed description of the experiments in Fig 2 including the shRNA sequences used to knock down dynamin are now provided in the Supplemental Material, and they were included in the original submission.

(8) Fig. 3F shows a clear example of Dyn enhancing F-actin polymerization in the presence of lipids. Authors should show a control with actin + lipids, without dynamin.

As requested we have included this control. However, we would like to clarify that Figure 3F does not show dynamin-driven F-actin polymerization, but crosslinking of F-actin into higher order structures such as bundles.

(9) On page 10 the authors write 'consistent with experiments in podocytes, dynΔPRD/KE failed to induce stress fiber formation', yet the image shown in Fig 3SO2 clearly shows an actin aster that appears very similar to those in cells expressing dynΔPRD or dynΔPRD/EK. It is not apparent that the constructs have an "opposite effect"? This should be clarified.

Looking at single cells can be misleading. In Figures 2C and 2D we show a quantitative analysis of F-actin and the number of focal adhesions in many cells under all experimental conditions. Those data clearly demonstrate that expression of dynΔPRD/KE failed to induce formation of stress fibers and focal adhesions in cells in which dynamin has been down regulated.

Further, it is unclear how these data suggest that interactions with dynamin's PRD inhibit dynamin's ability to polymerize F-actin, as suggested by the authors in the same paragraph.

We based this statement on the observation that overexpression of dynΔPRD in clone 9 cells appears to induce more stress fibers than expression of dynWT ((McNiven et al., 2000, JCB, 151:187) and Figure S4). However, we do not observe a similar phenomenon in the knockdown and rescue experiments (Figure 2C and 2D), so we have decided to remove the statement.

(10) A major thesis put forth in the paper is that dynamin rings regulate actin polymerization/stabilization. The E/K 'gain of function' mutation in the dynamin actin binding domain enhances dynamin's effect on actin. The in-cell FLIP experiments suggest that dynamin E/K oligomerizes, but there is no direct evidence that the construct forms normal rings. Further, the distribution of the polymerized Dyn is confusing in the cells. While the color change is consistent with the manipulations performed, why does the polymerized Dyn not localize to sites of enriched actin such as lamellipodia? The distribution appears diffuse or even non-biological? Maybe a comet assay would be nice to see if the active Dyn can actually be localized to a cellular structure?

Although we have removed the FLIM data from the paper, we would like to respond to this criticism of the data. The reviewer questions whether FLIM measures formation of rings and if so what is the cellular distribution of dynamin rings in Cos cells. First, in contrast to podocytes, Cos cells do not

exhibit a sophisticated organization of the actin cytoskeleton, and thus it is impossible to localize dynamin on any particular cellular structures in Cos cells. Second, FLIM measures higher order oligomerization of dynamin, which is demonstrated by in vitro data (former Figure 5D) using lipids (which promote dynamin oligomerization into spirals), or short actin filaments (which promote dynamin oligomerization into rings). In addition, in vitro dynE/K exhibits wild type oligomerization properties (Figures 1E and S1J). We see no reason to assume that this mutant will not form higher-order oligomers in the cell. Whether they are single perfect rings, or half a ring, or some other type of oligomers is not relevant for the interpretation of the data, though our EM analysis suggest that they are most likely rings (Figure 4F).

That said, we have performed EM analysis of dynamin on "unroofed" podocytes (cytoplasm was removed) demonstrating that dynamin concentrates at distinct sites in podocytes along the actin filaments (new Figures 6B-D). In addition, dynamin co-localized in part with paxillin, suggesting it localizes at the focal adhesions (new Figures 6A-D). Finally, in the new Figure 6H, we co-localized dynamin with EGF-induced free barbed ends, thus placing dynamin at the site of active actin polymerization.

(11) Fig. 6F: Show the Dyn band. Does it pellet with actin or in the supt with gelsolin?

We have added data showing the dynamin distribution (~30% in the sup and ~70% in the pellet). Importantly, the dynamin distribution was not altered in the presence of GTPgS or by displacement of gelsolin, demonstrating that the presence of dynamin in the pellet is due to F-actin binding.

Minor: *Authors should explain why they often used different amounts of Gsn:Actin ratios during the various biochemical assays.*

Our choice depends on whether we want to generate very short filaments, which promote dynamin oligomerization (G1:A5), or long filaments that cannot promote dynamin oligomerization. When long filaments are used, dynamin oligomerization is driven by addition of GTPγS. We tried to make that more clear in the current version of the manuscript.

(1) Page 10: Authors state that 'interactions between dynamin's PRD and SH3-domain domain might inhibit dynamin's ability to promote polymerization of F-actin'. Dynamin does not have an SH3 domain. Do you mean to write SH3-domain containing proteins?

Yes, we did, but we have removed this sentence completely.

Referee #2 (Remarks to the Author):

In this manuscript, Gu et al. identify an F-actin binding site on the protein dynamin, which allows dynamin to bundle filaments in vitro and participate in stress fiber formation in vivo. They further show that short actin filaments trigger the formation of dynamin rings with concomitant GTP hydrolysis, while longer filaments do not. Finally they claim to show that oligomerized dynamin dissociates gelsolin from filament barbed ends, allowing filament elongation. The overall idea is that short, capped filaments generated by gelsolin at distinct sites in the cell incite dynamin to oligomerize and kick off the gelsolin, allowing filament growth.

This paper is an interesting study, using a battery of different techniques and comprising a very thorough study of the question of dynamin-actin interaction and function. However there are some major points (some quite major, like point 7 and point 9) that need to be addressed before this study can be considered for publication. There is also a certain sloppiness that is disconcerting to this reviewer; for example, almost all of the images lack scale bars and the few references this reviewer looked up were not correct (detailed below). The error detailed in point 7 is a little shocking. Finally the paper is quite heavy and the authors might think about leaving out some data, and maybe shortening the discussion, which is excessively detailed.

Scale bars have been added to all images were this is important for the interpretation, and the references have been fixed. We have removed FLIM data to make the paper shorter and easier to understand. See below for detailed responses to other comments.

Major Points

1. For all of the binding studies, the authors say that they are using a tetrameric form of dynamin, but did the authors check this and are they sure that they don't have dimers also? What form is the mutant dynK/A in since the authors show that it has impaired oligomerization?

It has been shown by Sandy Schmid (Song et al, 2004, Mol Biol Cell, 15:2243) and subsequently by us (Sever et al., 2006, EMBO J, 25:4163) that mutations that block dynamin in the dimer form (e.g. dynI690K) impair its basal rate of GTP hydrolysis and even more importantly *completely* block dynamin's ability to be stimulated by lipids. These results argue that dynamin MUST form tetramers in order to be stimulated by lipids. Based on the fact that all the mutants including KA are stimulated by lipids (Figure 1E), we can conclude that they all form tetramers that can oligomerize into higher order structures.

2. How was the quantification done in Figure 2C and 2D for evaluating the loss of F-actin and focal adhesions?

In the current Materials and Methods, we have added a detailed description of the methodology used to quantify F-actin and focal adhesions. In addition, we would like to point out that the error bars on the original Figures 2C and 2D were done showing s.e.m. and not s.d. Thus, error bars were quite small. We have modified all the panels so that all error bars represent s.d., which in turn increased error bars in the current version of the manuscript.

3. Figure 3B and 3C evoke the use of gelsolin:actin complexes in the legend and in the panels, but no mention is made of why gelsolin is used for these experiments in the text and the discussion about regulating filament length with gelsolin comes much later.

The reason why we do not comment on the filament length in those experiments is because they are not relevant. The experiments use long actin filaments (G1:A1000), and gelsolin was used to generate long filaments with similar lengths. Not to cause confusion by over emphasizing the importance of gelsolin in these experiments, we have removed this labeling from the figure and added a description of the actin filaments in the current figure legend.

4. The authors show evidence for dynamin associating with actin to form bundles and they claim that dynamin rings (GTPgammaS conditions) does likewise, but this reviewer sees a drastic difference in Fig 3A and a significant different in 3B. Why do the authors treat this result so lightly? If dynamin rings don't associate well with actin, this calls into question their model.

The reviewer is correct in stating that when viewed by IF, it seems as if addition of GTPγS lowers dynamin's ability to crosslink actin filaments. In the following, we explain why these experiments do not challenge our model. First, in Figure 1A, we show that dynamin efficiently binds F-actin regardless of its oligomerization state. Thus, the amount of dynamin pelleting by F-actin in the presence of GTPγS is similar to that of dynamin alone (compare lanes 2, 4, 6 in Figure 1A), suggesting that dynamin's affinity for F-actin is independent of its oligomerization state. The same is true for dynamin-actin interactions detected in Figure 5G. These data are in agreement with EM analysis, which demonstrates that oligomerized dynamin binds F-actin (dynamin rings attached to actin filaments in Figure 3E). What the assays in Figure 3A and 3B measure is the ability of dynamin to *crosslink* actin filaments. Based on EM analysis, it is possible that dynamin rings form thinner bundles than the thicker, highly crosslinked bundles formed by unassembled dynamin, which could explain our inability to efficiently detect them by IF, or their inability to efficiently pellet at low speed centrifugation. We do not think that the difference in bundling activity between oligomerized and unoligomerized dynamin is very significant since our data show that dynamin is a *very weak crosslinker* when compared to α-actinin 4. To make this point more clear, we have included α-actinin 4 data in the main figure (new Figure 3A, panel 3), instead of showing it in the Supplement. Thus, we do not believe that the primary role of dynamin in the cell will be similar to that of known actin crosslinkers such as α-actinin 4 or myosin II. That said, it is possible that locally, dynamin might crosslink actin filaments, which in turn might have a biological role (e.g. formation of stress fibers at FAs).

5. In Figure 3E, the little black arrows are mysterious. Are we looking at dynamin rings glued to the sides of filaments, or do the authors see the ring going around the filament like a belt? This

reviewer can't tell. The sentence about the 17-20 nm spacing of filaments in the bundles is not well justified-what is the supposed spacing between the actin binding sites in the tetramer or the oligomer?

The reviewer is correct in both statements. Dynamin rings could be seen on the side of filaments, and dynamin rings could be seen going around the filament like a belt, but those are hard to point out. Dynamin rings are ~40 nm in diameter, and based on Zhang and Hinshaw (Nature Cell Bio, 2001, 3:922) one ring contains 13 monomers and the distances between GTPase domains of monomers in the ring is 9.4 nm. Based on our measurements, filament to filament spacing is 17-20 nm, which is less than the diameter of the whole ring. Together, these data suggest that dynamin rings act as a "donut" that aligns filaments, which we schematically show in current Figure 3E.

6. Figure 3F needs control panels showing what lipids look like with just actin or just dynamin.

As requested, we have included those control panels.

7. The interpretation of Figure 4 and Figure S1K is incorrect. The authors' calculation of how many subunits their filaments are composed of at a given gelsolin concentration is erroneous. A gelsolin:actin ratio of 1/5 gives a filament that is 13.5 nm long and that corresponds to FIVE subunits, not 14 as written in the text. This is an important point, as with only 5 subunits, the authors' suggestion that dynamin rings change the twist of the filament to kick off gelsolin becomes very hard to swallow. The formula for length versus gelsolin is that the length in microns = 1/(370R) where R is the gelsolin/actin ratio. This comes from the fact that each monomer adds 2.7 nm to the filament (so a 1 micron filament has 370 monomers in it) and each gelsolin associates with one filament. The original reference for this is Hanson and Lowy 1963, but this reviewer found it more clearly stated in Janmey et al (J Biol Chem. 1986 Jun 25;261(18):8357-62.) At any rate (despite its title), the cited reference Yin et al 1981 says nothing about this issue, nor does Andrianantoandro et al. In fact, Andrianantoandro et al. is overall a very bad citation (top of page 16) since it says nothing about either how CytoD regulates filament length (this chemical is not even used in the article) nor does it say that a 0.4 micron long filament has 430 monomers. In fact in the legend of Figure 5 of Andrianantoandro et al., they give that 0.6 microns = 225 monomers, i.e. as stated above, each monomer adds 2.7 nm to the filament NOT 0.9 nm as used by the authors!! This needs to be corrected in the text and Figure S1K, and the authors need to think about how this changes their story. On a five-subunit filament, how can gelsolin and a dynamin ring even bind together given that each needs to bind an actin subunit and that each has a certain radius? Unless dynamin/dynamin rings bind to gelsolin-did the authors check this?

We apologize for this confusion, but these references were not meant to define the length of actin filaments. We cited Yin et al because this study reports the ability of gelsolin to cap actin filaments in a calcium-dependent manner. Andrianantoandro et al. is cited because we use their experimental approach to generate short filaments by pushing F-actin through the needle. We now make it more clear why these papers are cited. We do not cite the proper references for calculating actin filament lengths because our impression is that this is such common knowledge that the original studies are generally not cited.

We did make a mistake, not in calculations of the length of the actin filaments in former Figure S1K, but in stating how many monomers that equals. In calculating the length of the filaments we were guided by the fact that as stated by the reviewer, each monomer comprises 2.7 nm within the filament. Thus, as stated in the original Figure S1K, a 1:5 ratio between gelsolin and actin gives a filament that is 13.2 nm long, a 1:10 ratio gives a filament that is 26.4 nm long etc. We have modified the original Figure S1K by removing any references to the filament length in the presence of different concentrations of gelsolin due to the following reason. We measured the length of the filaments using EM. Thus, for a 1:5 ratio of gelsolin to actin, the measured filament lengths using EM images shown in Figure 4F were 51±34 nm, which implies that the average length of the actin filament is ~ 19 monomers long, and also demonstrates a significant variation in filament lengths (based on the standard deviation of 34 nm). Thus, the filaments were not as short as theoretically predicted, nor were they highly uniform. The difference in length between the theoretical value for G1:A5 (13.2 nm) and that measured in the experiment (51 nm) is most likely due to the fact that not all of the gelsolin is active in our preparation. In addition, based on the GTPase assays, a filament length between 50-700 nm can promote dynamin oligomerization, a quite large spectrum. Thus, the effective filament lengths used in our experiments should be sufficient to

bind both dynamin and gelsolin. We are grateful to the reviewer to point this issue out to us, and we have modified the text to better explain our results.

We do agree with the reviewer that we have not defined the mechanism by which dynamin rings displace gelsolin. As stated more clearly in the current Discussion, this could be either due to direct gelsolin-dynamin interactions, or alterations in the filament twist. Since gelsolin does not affect dynamin's GTPase activity, and since dynamin-gelsolin interactions might occur only on F-actin and only when dynamin is oligomerized into rings, identification of those interactions may not be straightforward. Nor are experiments to test whether dynamin rings can alter filament twist, our current favorite model. We feel that identification of the exact mechanism by which dynamin displaces gelsolin goes beyond the scope of this manuscript.

What is the use of Figure S1J?

The point of former Figure S1J (current S6B) is to empirically demonstrate at what ratio of gelsolin to actin the actin filaments pellet (or not) at high speed centrifugation. This was prompted by our findings that the theoretical and actual filament lengths are not identical (as described above), so we wanted to define experimental conditions at which gelsolin capped actin filaments can pellet at high speed centrifugation. For example, in Figure 5G, we use a 1:300 ratio between gelsolin to actin, resulting in gelsolin-capped actin filaments that are sufficiently long to pellet under high speed centrifugation. This assay allowed us to test whether dynamin can release gelsolin from the actin filaments.

Why didn't the authors test G1:A2 or G1:A1 in Figure 4C? Does GTP hydrolysis keep going up even though now we are talking about filaments that are only a subunit or two long? Presumably G1:A1 would give no GTP hydrolysis since that corresponds to G-actin. Is there a minimal length where GTP hydrolysis (i.e. ring formation) is no longer triggered? This is important to test.

Because theoretical and experimental length of the actin filaments are not 100% identical, and because the filament lengths generated by the addition of gelsolin are not uniform, we do not think that proposed experiments can define exactly the filament length that supports dynamin oligomerization. As stated above, based on GTPase assays, any length between 50-700 nm can induce dynamin oligomerization. We believe that the use of latranculin A (which inhibits actin polymerization, Figure 4A) is a cleaner way to completely block actin polymerization, giving more straightforward results.

8. Page 22, the authors say that CytoD severs filaments. This reviewer has never heard that before. CytoD caps filaments, and so CytoD doesn't necessarily generate short actin filaments-it depends on the incubation time. When the filament is capped, it can only depolymerise from its pointed end and not polymerize at all, so over time, filaments will get shorter. However in the beginning, filaments are simply not growing (since pointed end dynamics are slow); conversely, at long incubation times, all filaments could be depolymerized. The authors need to do a time dependence of their FRET study on CytoD cells and provide some quantification of filament length. In the Figure S5E, the difference between CytoD and Latrunculin is not convincing-quantification is needed or electron microscopy.

As requested by the reviewers we have removed the FLIM experiments, so these comments do not pertain to the current version of the manuscript. However, to respond briefly to this comment, we stated that CytoD effects in cells include severing of the filaments based on a paper by Schliwa M (JCB, 1982, 92:79). Schliwa shows that effects of Cyto D on cells results from both a direct interaction of the drug with actin filaments as well as a secondary cellular response which leads to *breaking* of actin filaments.

9. The section concerning the displacement of gelsolin from barbed ends by oligomeric dynamin is the weakest part of the paper. The experiments in Figure 6C and E were done with a G1:A1000 ratio-i.e. of a length that would give little or no natural GTPase activity by dynamin. Why were the experiments done this way and not with short filaments and no GTPgammaS?

The experiments were intentionally performed with long filaments that cannot promote dynamin oligomerization, so that dynamin oligomerization could be induced by addition of GTPγS. Effects of GTPγS on dynamin are well known, and thus represent in our mind the cleanest way to

demonstrate that only dynamin oligomers but not unassembled dynamin can induce gelsolin release. As requested by the reviewer, we have also performed the same experiments using short actin filaments that can promote dynamin oligomerization. In the new Figure 5D we show that dynamin alone can induce elongation of short actin filaments, as predicted by our model.

It is an interesting result that dynamin can uncap long filaments (this reviewer does not know of another uncapper), but what is the physiological relevance of this since the authors have taken great pains to show that this is NOT how it works in vivo?

Based on the GTPase assays, dynamin oligomerization can be promoted by actin filaments that range in length from 50-700 nm. In addition, dynamin oligomerization is also regulated by PRD-SH3 interactions. Taken together, we suggest a model in which dynamin oligomerization (and actin polymerization) will be regulated by *both* PRD-SH3 interactions as well as the local concentration of short actin filaments. Thus, it is easy to envision that if filaments are long and thus unable to efficiently promote dynamin oligomerization, that can be achieved by PRD-SH3 interactions. Alternatively, a high, local concentration of shorter filaments might drive dynamin oligomerization *independently* of PRD-SH3 interactions.

Also the authors should see if dynamin can uncap filaments protected by capping protein.

As requested, we have performed experiments using mouse capping protein (CP). The new Figure 5B shows that dynamin cannot induce actin elongation from filaments capped by CP. Thus, our data show that dynamin specifically affects gelsolin-dependent actin polymerization.

Referee #3 (Remarks to the Author):

The manuscript 'Direct dynamin-actin interactions regulate actin cytoskeleton' by Gu et. al. deals with a long standing question in cell biology which is the effect of dynamin - and endocytosis in general - on actin remodeling.
The authors show binding of a dynamin 1 isoform to F-actin and the bundling effect on filaments. Point mutants in the putative F-actin binding domain affect bundling and show effects in vivo on actin organization. Furthermore, the authors show data, which suggest that dynamin 1 might work as an 'uncapping protein' for gelsolin. This would be the first protein that is able to remove Gelsolin from the barbed end of filaments.

The manuscript by Gu et al. is heavily loaded with data and experiments which were technically performed on a high level. Nevertheless it would need to be condensed to the important aspects relating to actin. This manuscript is one of the examples were the referee is torn between 'high impact and high significance for the cytoskeleton field' and 'mixture of 'unspecific' dynamin activities and overinterpretation of data'. This is not meant in a negative way.
Direct binding (specific and in vivo) and an effect of dynamin on actin filament organization would be a great step in understanding the link of dynamin to actin. Coupled with dynamins GTPase and assembly activity would be a very intuitive way of regulation. And last, the uncapping of Gelsolin capped filaments by dynamin would be like finding the 'holy grail' in the gelsolin field, partly solving the long standing mystery of what removed gelsolin from filaments under physiological conditions (in vivo).
On the other hand, the manuscript breaks with a number of past findings, which I find very surprizing and hard to understand - not saying that old dogmas need to remain untouched. A large number of labs have looked into the link of dynamin to actin and to my knowledge have ended up with dynamin being indirectly linked to actin remodeling via the binding of actin regulators (such as cortactin, profilin, etc.). These labs have for sure searched very hard for a direkt binding of dynamin to actin and for effects on actin polymerization (it is the most straightforward explanation) with the (disappointing?) result that dynamin is not directly interacting with actin and is not controlling polymerization kinetics. How can this be reconciled?

Naturally, it is difficult for us to guess why other laboratories have failed to discover the direct dynamin-actin interaction described in our paper. One possible explanation is that the pelleting assays are tricky due to the fact that dyn2 has a high propensity to spontaneously self assemble. In some instances, we assume that other investigators did see direct effects of dynamin on actin, but chose to ignore them. For example, in Mooren et al., JBC, 2009, Figure 1A shows that dynamin

induces pelleting of actin filaments in a cortactin-dependent manner. Even in the presence of a cortactin mutant (W525K) that cannot bind dynamin there is significant pelleting of actin filaments above the background level (compare columns 1 and 2 with column 4). Thus, we do not believe that our data are in contradiction with published studies. Our study provides a novel way of looking at the role of dynamin in regulation of the actin cytoskeleton, and it explains previously mysterious observations (e.g. role of GTP hydrolysis in regulation of actin cytoskeleton in vitro and in vivo, actin phenotypes in cells expressing dynΔPRD, etc…)

Second, the uncapping of Gelsolin and the mechanism is not obvious and convincing to me and I see no indication in the manuscript that this plays a role in vivo (see comments below). Uncapping by dynamin would be very interesting but is such an important finding that it needs to be investigated in more detail also in vivo. 'Uncapping activities' have become popular in the actin field, however the term has remained very fuzzy and has frequently been used wrongly by others.

We now provide evidence that the number of barbed ends is increased by the gain of function dynE/K mutant which binds more efficiently than WT to actin.

Last, the part of the manuscript dealing with FLIM is very lengthy (5 pages of results) and seems a bit artificially squeezed in. It really looks like the authors are taking the opportunity to introduce their FLIM system for dynamin, which belongs somewhere else and has limited relevance for the topic of the manuscript (seems like a manuscript in the manuscript).

As requested by this reviewer, we have removed the FLIM data.

Critique:

(1) The manuscript is not consistent in terms of using dynamin isoforms. The authors need to be specific in the text what they are showing in the experiments, just dynamin is missleading - is it dynamin 1 or 2 or which splice form?

We now state in the Materials and Methods that we used human dyn1 isoform 1. As requested, we specify in the text and in the figures which isoform we use in each experiment, though most experiments were performed using dyn1. We use dyn2 only to demonstrate that the reported effects are not isoform-specific.

-(2) The manuscript is not consistent in using dynamin 1 for the in vitro/in vivo assays, in vitro dynamin 1 is used in most experiments, in vivo experiments are performed in dynamin 2 expressing cell lines? It is not clear to me how to interpret the results without knowing that dynamin 1 and 2 are equivalent in terms of actin regulation, and if not the interpretation by the authors is on shaky ground.

Dyn2 is the ubiquitous form expressed in all cells, whereas the highly related dyn1 (79% identity; 88% similarity) is expressed in neurons. Work by Sandy Schmid (Liu et al., 2008, Mol Biol Cell, 19:5347) examined in detail isoform and splice-variant specific functions of dyn 2 versus dyn1. Importantly, they showed that dyn1 expression can efficiently rescue actin-driven processes such as macropinocytosis and cytokinesis in cells conditionally lacking dyn2. In agreement with this study, we performed several experiments to demonstrate that with respect to actin regulation in dyn1 and dyn2 are interchangeable: (1) Expression of dyn2 ABD mutants lead to identical phenotypes as dyn1 ABD mutants (current Fig S5D). (2) The ABD is conserved between all dynamin isoforms and splice variants, strongly suggesting that they regulate the actin cytoskeleton using a similar molecular mechanism. (3) Expression of dyn1 rescues the actin phenotype in dyn2 KO cells (Fig 2A). (4) Both dyn1 and dyn2 can crosslink actin filaments (Fig 3A and Fig 3F). The reason why we did not perform more experiments using dyn2 is because it has a much higher propensity to self assemble on its own, thus making actin binding assays impossible. As far as actin regulation is concerned, we could not detect any differences between isoforms in our study.

(3) If the putative actin binding domain is indeed an actin binding domain I find it surprizing that the authors have not tried to delete the entire domain, or transplanted the domain. Is it in the end a simple charge effect? Would synthesized peptides from the region have the same effects in the in vitro assays (bundling, uncapping)? It is well known that many positively charged peptides function

like bundling proteins. Whether this then might have relevance for dynamin in vivo is not clear to me. The Sh-RNA/complementation experiments are an indication but not conclusive. We might look at very indirect effects - e.g. retraction fibers, toxic effects. We learn little of what the dynamin point mutations are doing to cell division, proliferation etc., which could explain the aberrant structures.

In fact, one of the first mutants we generated was dyn lacking the entire ABD. It gave the same actin phenotypes as dynK/E and dynK/A in cells and in vitro. We do not show those experiments because the manuscript is already very long, and our point mutants (dynK/A and dynK/E) are obviously preferable to larger deletions since they are less likely to induce unpredicted changes in protein folding. The other experiments the reviewer proposes (transplantation of the domain etc.) are interesting but go beyond the scope of this study.

Second, the reviewer suggests that the inability of dynK/E to crosslink F-actin is due to loss of positive charge in the mutant, which the reviewer suggests would somehow imply a non-specific effect of dynamin on actin. We find it implausible that all of our observations can be explained by a non-specific interaction between dynamin and actin. If this were the case, one could possibly explain the binding, but not the oligomerization-dependent ability of dynamin to uncap gelsolin or the reciprocal effect of short actin filaments on dynamin's oligomerization status. Moreover, among all the in vivo effects, all of which are difficult to explain with a non-specific effect, it is particularly difficult to explain the gain of function phenotyope of the dynE/K mutant. Notably, podocytes are *terminally differentiated cells* and thus expression of dynamin mutants does not affect cell division or proliferation. These dynamin mutations did not even have major effects on endocytosis (Fig S2A), or signaling by other small GTPases (Fig S2C, S2D) strongly suggesting that the effects on the actin cytoskeleton are specific and direct.

(4) I am a bit worried about the fact that expression of the dynamin point mutations have an effect on cells independently of the presence or absence of dynamin 2?! Arguing that the mutants have some sort of dominant negative effect on cells .

It has been extensively shown that almost all dynamin mutants generated so far exhibit *dominant effects* on endogenous dynamin when overexpressed in cells (e.g. van der Bliek et al, JCB, 1993, 122:553; Damke et al., 2001, Mol Bio Cell, 12:2578). This is readily explained by the fact that dynamin is a tetramer and thus mutant monomers bind endogenous monomers to form heteroteramers, “poisoning” the endogenous enzyme.

(5) In Fig. 6C I wonder what the result would be in the absence of Gelsolin ? Is there an effect by GTP-gammaS? Same question for the Gelsolin release assay (6B)
why do the authors not consider pointed end nucleation as an alternative explanation for the gelsolin-dynamin effects?

In the new Figure 5A, we show that dynamin itself has no effect on actin polymerization using actin seeds with or without GTPγS. In addition, in the new Figure 5B we show that dynamin cannot induce actin polymerization from actin filaments capped by capping proteins (CP). This demonstrates that dynamin cannot induce pointed end nucleation.

(6) Is the 'uncapping' gelsolin specific - how about capZ, capG?

Yes, our new data demonstrates that dynamin specifically uncaps gelsolin, but not CP.

- I find it very hard to follow the rational in explaining the mechanism of uncapping by dynamin. Dynamin has no affinity for the barbed end. Dynamin binds to the side of the filament - how should this cause uncapping? What is the effect of the mutant dynamins on 'uncapping' (e.g. K44A?)?

Since in vitro “uncapping” experiments are performed with or without addition of nucleotides there is no need to examine effects of mutant dynamins (dynK44A cannot bind GTP thus its in vitro effect is identical to empty dynWT). In the Discussion we propose two possible mechanisms for uncapping; (1) by direct protein-protein interactions and (2) by altering the filament geometry (filament twist). A number of actin regulatory proteins are known to alter filament twist, thereby affecting interactions of other actin binding/regulatory proteins, most notably cofilin. We feel that elucidation of the molecular mechanism by which dynamin rings displace gelsolin goes beyond this manuscript.

Is GED, which inhibits oligomerization, having an effect on 'uncapping' by dynamin? The authors suggest a change in twist - is there any solid support for this?

Yes, experiments in Figure 5F address this question by demonstrating that addition of GED inhibits actin polymerization and thus there is less actin in the pellet.

(7) Is there any indication for dynamin mediated uncapping in cells? Is the number of free barded ends different when overexpressed or when mutant forms are expressed?

As requested by the reviewer, we have measured the number of free barbed ends in podocytes expressing different dynamin mutants upon EGF stimulation. In new Figure 6F, we show that direct dynamin-actin interactions promote the formation of free barbed ends.

(8) One point which is not addressed, but might be important - does dynamin interact with gelsolin itself??

This is a great question that is not easy to address. The straightforward way to ask this question is to look whether gelsolin can alter dynamin's GTPase activity as shown for other dynamin binding proteins. As shown in Figure 4C, this is not the case. Furthermore, while dynamin rings displace gelsolin from the actin filaments, dynamin itself stayed on the filaments (Figure 5G). Thus, dynamin distribution between sup and pellet was not altered by addition of GTPγS or release of gelsolin from the filaments. That said, it is formally possible that dynamin binds gelsolin only on actin filaments and when oligomerized, but that is not easy to test. We tried to state all questions raised by our study openly in the Discussion.

(9) Perhaps I missed it, but I do not recall any staining of F-actin structures (actin bundles!) in the manuscript using dynamin antibodies?!? Again, is the filament binding restricted to in vitro?

As requested, in the new Figure 6A, we first show that dynamin colocalizes with focal adhesions, as reported (Kruchten and McNiven, 2006, J. Cell Sci, 119:1683). In addition, electron micrographs of "unroofed cells" (cells in which the cytoplasm has been removed) show that dynamin antigenic sites concentrate at distinct locations in the cell and along the actin filaments. Co localization with paxillin suggests that those sites are focal adhesions (Figure 6B). Furthermore, in Figure 6F we show co-localization of dynamin with free barbed ends. Finally, dynamin has been localized to actin rich structures such as Listeria tails, lamellipodia, and podosomes (e.g. Lee and De Camilli, 2002, PNAS, 99:161; Bruzzaniti et al., 2005 Mol Bio Cell, 16:3301)

(10) It is not clear to me what I should see and learn from Fig. 3 ? Again here dynamin 2 is used, while dynamin 1 is used in the other experiments ... why?

In Figure 3 we used both dyn1 and dyn2 to demonstrate that crosslinking activity of dynamin is not dependent on the particular isoform. Thus, both dyn1 and dyn2 can crosslink actin filaments. Since we could not measure dyn2's Kd for F-actin (because this isoform pellets on its own), the data in Figure 3F demonstrates that dyn2 can bind F-actin. That said, we do want to point out that dynamin is a weak crosslinker, and thus its role in the cell will not be similar to that of α-actinin 4.

2nd Editorial Decision 25 August 2010

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referees 1 and 2 think that there are a few issues that need to be addressed (see below) before we can ultimately accept your manuscript. I would therefore like to ask you to deal with the issues raised. Please let us have a suitably amended manuscript as soon as possible.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The current form of the manuscript is much improved, more concise, and has provided additional data in cells. In particular, the authors now show that dynamin co-localizes with paxillin in cells and, importantly, that expression of the Dyn1K/E generates barbed ends in cells. Further, the manuscript now shows that dynamin can not remove CP from actin filaments, suggesting that dynamin-mediated uncapping of actin is selective for gelsolin. The mechanism of dynamin-mediated gelsolin release from actin filaments has still not been elucidated and it still is a bit peculiar that the story needs to even include gelsolin but overall the story represents a significant amount of careful experimentation.

Critique:

It seems to this reviewer that the best way to test the specificity of dyn1 to remove gelsolin but not CP from actin filaments would be to assay the two proteins under the same conditions. To what degree are the experimental assays in Fig 5b and 5d comparable? Are the actin seeds used in Fig 5a and 5b comparable in length to the short actin filaments in Fig.5d? Was the Dyn1-mediated enhancement of actin polymerization that is seen with preformed short actin filaments also seen with Gsn-capped actin seeds?

Could the increased actin pelleting in Fig. 5E and F result from Dyn1-mediated actin crosslinking, rather than gelsolin dissociation followed by actin polymerization? It is noteworthy that a substantial amount of Gsn is pulled to the pellet (Fig 5E, lane 4) after addition of Dyn1 to the reaction. This seems inconsistent with the interpretation that Dyn1 is removing gelsolin from the short actin filament. Does addition of the GED reduce actin crosslinking in experiments similar to those in Fig. 3?

Referee #2 (Remarks to the Author):

Remarks to editor and authors:

The manuscript by Gu et al. is considerably improved and the authors have responded adequately to most of my comments. There are some details that I would like to see addressed, listed below as they come up in order in the text.

Also the paper is still very heavy, and there is too much supplementary information. I suggest that all graphs that show no effect be left out and just mentioned in the text: Figure 1D and 1E, Figure 4A and 4D, combine Figure 5A and 5B to one graph, and those are just the ones that I noticed. I have not gone through the supplementary figures carefully, but there also perhaps things can be left out/condensed etc.

1. Bottom page 3, last 2 sentences: I think the authors mean "...significantly reduced formation of F-actin comets generated either by Listeria or on vesicles by overexpression etc."

2. Page 5, second to last line: word missing after "contain" (cortactin??).

3. Page 12 Figure 2F is not referred to in the text and it's just a quantification of 2E, so put in with 2E.

4. Page 13-14: There are several major mistakes on these two pages that need to be fixed. First, the

use of the word "crosslinked" as a synonym of "bundled" is incorrect (and this is also incorrect in the authors' rebuttal letter). These are two different arrangements of actin. The authors need to only use the word "bundle" or "crosslinked into bundles", never crosslinked. The actin in Fig 3A, panel 4 may well be crosslinked (can't tell by epifluorescence), but it's certainly not bundled.
Second, I have a problem with the cartoons shown in Fig 3 (the idea of putting in cartoons is excellent though). The cartoon in Fig 3D is a meshwork, not bundles. The authors should make loser bundles to better represent what we see in the EM. Mention that bundles are on average thinner and more tightly packed with GTPgammaS than without, and make this obvious in the cartoons.
Third, Fig 3F is still lacking the proper controls. Lipids plus dyn2 is lacking as is dyn2 plus actin (can't just refer to Fig 3A because that is dyn1). The authors can cut the first panel of just lipids in my opinion.

5. Page 16: This section is much improved. Very readable. Why not take it a step further and just leave out the predicted length? That would avoid the problem of having to say that the gelsolin is not as active as it should be. The authors have already measured the length for the G1:A5 ratio. They need to do the same for G1:A300 (the current estimate of 700 nm could be totally off!). Then the authors could rewrite this paragraph using the real, measured lengths.

6. Page 17: Cut Fig 4D (as suggested above), and just put G1:A5 data for K/A mutant in with other data in Fig 4C.

7. Page 23: Fig 6 brings up the question as to what the mutants K/E and E/K do in the assays shown in Fig 5C. Does K/E kick off gelsolin less than WT and, is E/K more active for uncapping than WT? This is really important, since it would bring the first part of the paper (identification and mutation of the actin binding site) together with the gelsolin data and with what the authors see in cells.

Referee #3 (Remarks to the Author):

The revised manuscript 'Direct dynamin-actin interactions regulate actin cytoskeleton' by Gu et. al. has improved significantly. To detangle it from the FLIM data and to focus on the main hypothesis has made the manuscript easier to read without loosing the impact. The main criticism and the concerns have been addressed in the revised version.

As it stands now the data are presented in a convincing way, although the manuscript will certainly raise some controversy in the field.

The main novel and interesting data are the a) mutual dependence of dynamin and actin in controlling oligomerization and actin polymerization, and b) the uncapping and extension of gelsolin capped filaments. Both findings contribute to long standing questions in how dynamin might crosstalk with actin and second, what physiological signals can lead to uncapping of gelsolin capped filaments and thereby can promote de novo actin polymerization. To my knowledge this is the first example of a protein that shows such activity. I am convinced that the findings presented here will gain significant attention and stimulate the further discussion on dynamin and actin interaction.

2nd Revision - authors' response 09 September 2010

Referee #1 (Remarks to the Author):

The current form of the manuscript is much improved, more concise, and has provided additional data in cells. In particular, the authors now show that dynamin co-localizes with paxillin in cells and, importantly, that expression of the Dyn1K/E generates barbed ends in cells. Further, the manuscript now shows that dynamin can not remove CP from actin filaments, suggesting that dynamin-mediated uncapping of actin is selective for gelsolin. The mechanism of dynamin-mediated gelsolin release from actin filaments has still not been elucidated and it still is a bit peculiar that the story needs to even include gelsolin but overall the story represents a significant amount of careful experimentation.

Critique:

It seems to this reviewer that the best way to test the specificity of dyn1 to remove gelsolin but not CP from actin filaments would be to assay the two proteins under the same conditions. To what degree are the experimental assays in Fig 5b and 5d comparable? Are the actin seeds used in Fig 5a and 5b comparable in length to the short actin filaments in Fig.5d? Was the Dyn1-mediated enhancement of actin polymerization that is seen with preformed short actin filaments also seen with Gsn-capped actin seeds?

We have performed the suggested experiment and the data are shown in the current Figure 5C. Thus, under identical conditions, dynamin can displace Gelsolin but not CP.

Could the increased actin pelleting in Fig. 5E and F result from Dyn1-mediated actin crosslinking, rather than gelsolin dissociation followed by actin polymerization? It is noteworthy that a substantial amount of Gsn is pulled to the pellet (Fig 5E, lane 4) after addition of Dyn1 to the reaction. This seems inconsistent with the interpretation that Dyn1 is removing gelsolin from the short actin filament. Does addition of the GED reduce actin crosslinking in experiments similar to those in Fig. 3?

We have already addressed this question, by demonstrating that dynamin cannot induce pelleting of short actin filaments generated by CP (Fig S6D). If pelleting was due to crosslinking and not polymerization then dynamin should have had identical effect on short filaments generated by CP, but it does not. The lower band that the reviewer refers to is proteolyzed dynamin (highly common product during purification that uses HAP column). In fact, lane 7 shows the presence of the lower band. Nevertheless, in order to prove that gelsolin was not brought into the pellet with F-actin, we performed a Western blot of the samples shown in Figure 5D. The data are included in this letter (Figure 1), but we did not add them into the manuscript since we feel that the CP experiments adequately addresses the reviewer's concern.

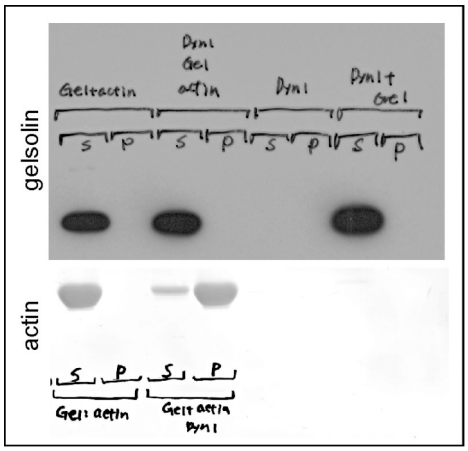

Figure 1. Experiments were performed as shown in current Figure 5D. Gelsolin was detected using Western blot, and actin was visualized using Ponceau solution after protein transfer. Notice that while addition of dynamin shifts actin into the pellet faction (lane 4), gelsolin stays in the supernatant.

Referee #2 (Remarks to the Author):

Remarks to editor and authors:
The manuscript by Gu et al. is considerably improved and the authors have responded adequately to most of my comments. There are some details that I would like to see addressed, listed below as they come up in order in the text. Also the paper is still very heavy, and there is too much supplementary information. I suggest that all graphs that show no effect be left out and just mentioned in the text: Figure 1D and 1E, Figure 4A and 4D, combine Figure 5A and 5B to one graph, and those are just the ones that I noticed. I have not gone through the supplementary figures carefully, but there also perhaps things can be left out/condensed etc.

As suggested by the reviewer we have removed Figures 1D, 1E, 4A and 4D. We have combined Figure 5A and 5B into one graph. We have not changed our supplementary figures since after carefully consideration we think that they all contain essential data.

1. Bottom page 3, last 2 sentences: I think the authors mean "...significantly reduced formation of F-actin comets generated either by Listeria or on vesicles by overexpression etc."

Changed as suggested.

2. Page 5, second to last line: word missing after "contain" (cortactin??).

Added.

3. Page 12 Figure 2F is not referred to in the text and it's just a quantification of 2E, so put in with 2E.

We do refer to Figure 2F in the text.

4. Page 13-14: There are several major mistakes on these two pages that need to be fixed. First, the use of the word "crosslinked" as a synonym of "bundled" is incorrect (and this is also incorrect in the authors' rebuttal letter). These are two different arrangements of actin. The authors need to only use the word "bundle" or "crosslinked into bundles", never crosslinked. The actin in Fig 3A, panel 4 may well be crosslinked (can't tell by epifluorescence), but it's certainly not bundled.

Changed as suggested.

Second, I have a problem with the cartoons shown in Fig 3 (the idea of putting in cartoons is excellent though). The cartoon in Fig 3D is a meshwork, not bundles. The authors should make loser bundles to better represent what we see in the EM. Mention that bundles are on average thinner and more tightly packed with GTPgammaS than without, and make this obvious in the cartoons.

We altered the cartoon based on the reviewers suggestion.

Third, Fig 3F is still lacking the proper controls. Lipids plus dyn2 is lacking as is dyn2 plus actin (can't just refer to Fig 3A because that is dyn1). The authors can cut the first panel of just lipids in my opinion.

We have added all the requested controls.

5. Page 16: This section is much improved. Very readable. Why not take it a step further and just leave out the predicted length? That would avoid the problem of having to say that the gelsolin is not as active as it should be. The authors have already measured the length for the G1:A5 ratio. They need to do the same for G1:A300 (the current estimate of 700 nm could be totally off!). Then the authors could rewrite this paragraph using the real, measured lengths.

We have measured the length of the actin filaments and added this into the text.
Of note, 700 nm refers to the length of the filaments generated by severing and then addition of Cyto D and not to G1:A300.

6. Page 17: Cut Fig 4D (as suggested above), and just put G1:A5 data for K/A mutant in with other data in Fig 4C.

Modified as suggested.

7. Page 23: Fig 6 brings up the question as to what the mutants K/E and E/K do in the assays shown in Fig 5C. Does K/E kick off gelsolin less than WT and, is E/K more active for uncapping than WT? This is really important, since it would bring the first part of the paper (identification and mutation of the actin binding site) together with the gelsolin data and with what the authors see in cells.

We have performed the requested experiment. The current Figure 5B shows that ‘gain of function’ dynE/K stimulates actin polymerization better than dynWT whereas ‘loss of function’ dynK/E is significantly impaired in stimulating actin polymerization from gelsolin capped actin filaments.

Referee #3 (Remarks to the Author):

The revised manuscript 'Direct dynamin-actin interactions regulate actin cytoskeleton' by Gu et. al. has improved significantly. To detangle it from the FLIM data and to focus on the main hypothesis has made the manuscript easier to read without loosing the impact. The main criticism and the concerns have been addressed in the revised version.

As it stands now the data are presented in a convincing way, although the manuscript will certainly raise some controversy in the field.
The main novel and interesting data are the a) mutual dependence of dynamin and actin in controlling oligomerization and actin polymerization, and b) the uncapping and extension of gelsolin capped filaments. Both findings contribute to long standing questions in how dynamin might crosstalk with actin and second, what physiological signals can lead to uncapping of gelsolin capped filaments and thereby can promote de novo actin polymerization. To my knowledge this is the first example of a protein that shows such activity. I am convinced that the findings presented here will gain significant attention and stimulate the further discussion on dynamin and actin interaction.

