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Dynamin GTPase regulation is altered by PH domain mutations found in centronuclear myopathy patients

Jon A. Kenniston and Mark A. Lemmon

Corresponding author: Mark A. Lemmon, University of Pennsylvania

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1st Editorial Decision 04 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. As you will see all three referees consider the study as interesting in principle and referees 2 and 3 are very positive already at this stage of analysis. Still, referee 1 feel strongly that the mechanism of the activation of GTPase activity in the PH domain mutants needs to be analysed in more depth - in particular in respect to the conclusiveness of the evidence presented for a model of direct allosteric coupling between the PH domain and the GTPase reaction. Taking into account all issues raised we have come to the conclusion that we would be happy to consider a revised version of the manuscript in which the referees' concerns - in particular those raised by referee 1 - need to be addressed or responded to in an adequate manner. 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. Please do not hesitate to contact us at any time should you wish to discuss certain aspects of the revision with us.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the 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,

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Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript from Kenniston et al. describes a comprehensive study with a number of surprising findings concerning increased GTPase activities of PH domain mutants. Furthermore, the new low resolution structural data of a dimeric dynamin construct obtained by SAXS measurements are very exciting for the dynamin field. My main worries concern the fact that the mechanism of GTPase activation in the PH domain mutants is not sufficiently addressed. Consequently, I am not yet convinced that a direct coupling of the GTPase reaction to the PH domain is important for the function of dynamin.

Chappie et al. (2010) demonstrated that the GTPase activity of dynamin is stimulated by association of the G domains. Furthermore, the GTPase reaction of dynamin is dependent on the protein concentration, e.g. higher protein concentrations lead to increased specific GTPase rates. A structural model of the dynamin oligomer by Gao et al. (2010) suggested that the GTPase activity of dynamin when assembled on a bud neck is activated between neighboring dynamin rings.
The results presented here by Kenniston et al. indicate that under the reaction conditions (presence of GTP, 37° C) the mutations in the PH domain promote oligomerization of dynamin to different degrees concomitant with an increased GTP hydrolysis rate. There are several possibilities why the GTPase reaction is promoted in vitro (and only three are listed here).

a) There is a direct coupling of the PH domain via the GED with the G domain which directly influences the GTPase activity in the G domain, as discussed by the authors. The twofold increased maximal GTPase rate of the A617T mutant compared to wildtype might speak in favor for such a scenario although there could be plenty of other explanations for this increase. The main reason which speaks against such a mechanism is the structural model of the dynamin dimer (Fig. 7), where the G domain and PH domain are separated by the MD / GED stalk which is built of a stable four helical bundle. It appears highly unlikely, that conformational changes from the PH domain are relayed through the whole stalk to the G domain, and GED mutations known to interfere with GTPase activity are far away from the PH domain on the opposite side of the stalk, close to the G domain.

b) The PH domains might themselves be involved in promoting oligomerization by interacting with each other (possibly with low affinity) in the oligomer, as suggested by the structural model of the dynamin dimer (Fig. 7). This low affinity PH domain interactions might be inhibited in solution by an interaction of the PH domain with the neighboring middle domain / GED and the described mutations would interfere with that inhibition. In such a scenario, the PH domain mutations would result in increased oligomerization rates of dynamin in solution, concomitant with increased GTPase activity in vitro. However, the increased GTPase rates in vitro would have little to do with the regulation of the GTPase reaction on a bud neck.

c) It might also be that the PH domain mutations induce a local destabilization of the PH domains leading to non-specific aggregation of two PH domains within the dynamin oligomer (as for example suggested from experiments seen in Fig. 4b) which would also lead to increased GTPase rates in vitro (partial aggregation is often observed for protein variants with destabilizing mutations). The stability measurements of the mutated PH domains in Fig. 5 speak in favor for such a scenario since the most destabilizing mutations lead to the highest GTPase rates. In this scenario, the increased GTPase rates of the PH domain mutants in the in vitro assay might have nothing to do with the normal function of the PH domain in dynamin.

The main problem of the paper is that the in vitro GTPase reaction of dynamin is a poor readout for the function of dynamin at the neck of a budding vesicle. The GTPase reaction of dynamin and dynamin-related proteins includes a number of different reaction steps, and it is unclear from the current manuscript which step is accelerated by the mutations in the PH domain and how relevant

this is for dynamin function. In the recent manuscript from Gao et al. (2010), oligomerization-compromised mutants in the dynamin-related MxA protein also showed increased GTPase rates; however, the underlying mechanisms of increased GTPase activities appear to be completely different for the PH domain mutants presented here.

If this paper was published in EMBO Journal, the authors would need to experimentally address the detailed mechanism how the GTPase reaction is stimulated in the PH domain mutants and show that the increased GTPase rates in the in vitro assays are not an artifact, as e.g. in scenario (c). The new findings should result in a conclusive model how the PH domain influences oligomerization and/or GTPase activity.

Specific comments:
Fig. 2: Fitting of Michaelis-Menten parameters to the dynamin reaction is not correct since the underlying reaction model is completely different. The dynamin reaction does not only involve GTP binding and turnover (as in a typical Michalis-Menten reaction) but also assembly of the G domains (most likely from different dynamin tetramers) which is also promoted by GTP binding / hydrolysis. Consequently, the observed KMs reported here are not equal Kd for the nucleotide. Not the affinity to the nucleotide but the percentage of assembled dynamin molecules at a given protein concentration might differ between wildtype and PH domain mutants which would also result in the observed differences in GTP saturation.
To dissect nucleotide binding affinity and dynamin assembly, GTPase reactions for wildtype and PH domain mutations with saturating GTP concentrations and varying protein concentrations should be carried out. In this way, dissociation constants for G domain dimerization can be derived. Nucleotide binding affinities of the PH domain mutants should be determined with other methods such as calorimetry or fluorescence measurements.

Fig. 2d - Since basal GTP hydrolysis for the PH mutants is most likely dependent on dynamin assembly, the reported value is kobs rather than kcat.

Fig. 4: Interesting finding which might argue for uncontrolled (unspecific?) oligomerization of the S619L mutant in the presence of GTP and at 37° C. In fact, since the mutant runs in the void volume it looks like a large soluble aggregate which, however, still maintains functional G domains.

Fig. 5: Could be removed to the Supplement and discussed in detail there.

Fig. 6: I am not an expert for SAXS measurements and cannot judge the relevance of the observed differences in Fig. 6c. However, the resulting envelope and suggested structural model look reasonable and interesting. The proposed model argues against a direct coupling of the PH domain with the GTPase reaction (see above).

Fig. 7: It might be helpful to include the MxA stalk in Fig. 7A.

Fig. 6 and Fig. 7: It has been proposed that the PH domain mutations also induce conformational changes in the background of a dimeric dynamin mutant. How do the GTPase reactions of dimeric dynamin with PH domain mutations compare to the dimeric dynamin variant and wildtype dynamin? Do they also show accelerated GTPase rates? Do these mutants display protein-concentration dependent GTPase activity?

Since the GTPase reaction of dynamin is such an indirect readout for dynamin function (see above), it would be very informative to characterize the effect of a few representative PH domain mutants on the cellular uptake of cargo (e.g. the data not shown for the S619L mutant). It would also be interesting to see whether the PH domain mutants described here can be recruited to budding vesicles or whether they have lipid binding defects in vivo.

Discussion
The discussion should also include the novel findings on the mechanism of GTPase activation in dynamin (e.g. dimerization-dependent GTP hydrolysis, etc.) including a final model how the PH domain mutations affect GTP hydrolysis (see above). The comparison of dynamin to the various other PH domain containing proteins is somehow misleading since in most of these cases, there is a

direct contact between the PH domain and the second domain whose activity is controlled, which is different for dynamin (at least, this difference should be mentioned).

Referee #2 (Remarks to the Author):

In this manuscript Kenniston and Lemmon analyze the large GTPase dynamin to suggest new aspects of its regulatory mechanism at a molecular level. These insights are generated from analysis of disease-related dynamin mutations linked to centronuclear myopathy (CNM). This study is particularly important because in most cases of CNM and similar disorders we do not understand in depth the links connecting the genetic defects with the clinical manifestations of the diseases. The main conclusion from this study is that the PH domain allosterically couples lipid binding to dynamin GTPase activation and that mutations in the PH domain C-terminal α-helix cause conformational changes that mimic or facilitate effects of lipid binding. These conclusions are based on extensive analysis using a range of biochemical and biophysical methods including measurements of relevant parameters of GTPase activity, lipid binding, oligomerization, protein stability using urea chemical melts and conformational changes using SAXS. Specific comments are below:

1) The idea that lipid binding causes conformational change should be supported more directly. Would binding of the lipid head-group or a short-chain PIP2 cause an increase in self-association or cause a conformational change?

2) The authors should make a reference and further comment on their observation in the light of recent and important findings described in Nature (Article) 2010 465:435-440. Although the paper was published after submission of their manuscript, the importance and relevance of this article has to be taken into account.

3) The data are presented in a large number of Figures distributed between the main text and supplementary material. The distribution of the data between "main" and "supplemental" could be organized better. For example, the finding that mutations in the PH domain C-terminal α-helix, as expected, do not affect lipid binding (a negative observation) is in the main Figure 1 and could be supplemental, while the key observations that these mutations affect GTPase activity are distributed between Figures 2 and 3. The original 3D model of dynamin based on the EM ("Head", "Salk" and PH-domain "Leg") should be presented together with already included schematic diagram of dynamin. Efforts in this direction would be beneficial for the potential, wider interest in this work.

4) It should be noted that mutations in a range of proteins (not just dynamin) are found in disorders linked to aberrant trafficking.

Referee #3 (Remarks to the Author):

This is a clearly written and scholarly paper on an exceptionally insightful study of the dynamin PH domain and CNM disease mutations. A compelling case is made that the dynamin PH domain allosterically regulates dynamin's GTPase activity. The SAXS analysis pushes the limits of the technique in drawing conclusions from very small differences in the P(r) distributions, but nevertheless I found the analysis convincing given the care taken to repeat the data collection under various conditions, and the corroborating evidence from hydrodynamics. After going through the whole study with a critical eye, I have no revisions to request, and recommend rapid publication of this superb manuscript.

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1st Revision - authors' response 29 June 2010

We have considered all of the points raised by Reviewers 1 and 2, and have found them very constructive and useful in revising the manuscript. Both brought up several interesting and valid points that we have addressed in revisions. In particular, to address the comments of Reviewer 1, we have significantly altered our focus and discussions of how changes in the PH domain seen in human disease lead to altered GTPase regulation. Since GTPase activity (and its regulation) is undoubtedly key to dynamin's function, our findings that the characteristics and sensitivity of GTPase regulation are altered in CNM mutants provide an important foundation for delineating the molecular basis of this disease. Also, in response to Reviewer 1's comments, we have generalized our consideration of mechanistic models to include the possibility that CNM mutations activate dynamin by enhancing oligomerization – although it seems highly likely that specific oligomers are involved. The only request that we are unable to address is definition of the detailed mechanism for stimulation of the GTPase domain in the CNM mutants. To do this will require high-resolution structural studies that have not yet been achieved even for wild-type dynamin. Indeed, the state-of-the-art here is reflected in structures of fragments of dynamin (or related proteins) that were published in *Nature* just last month – from the laboratories of Daumke, Schmid, and Dyda. A major challenge is to understand the interplay between the different domains within dynamin, which is not addressed experimentally in those studies – although we would like to think that the low-resolution SAXS studies of near full-length dynamin presented in our manuscript represent a valuable advance in that direction.

In revising our manuscript to address the Reviewer's comments, we have also changed the title so as to better portray the focus on PH domain mutations found in human centronuclear myopathy (CNM): "*Dynamin GTPase regulation is altered by PH domain mutations found in centronuclear myopathy patients*".

Our responses to the Reviewers' comments (and resulting actions in revision) are summarized below.

Reviewer 1:

This Reviewer commented that our manuscript '*describes a comprehensive study with a number of surprising findings concerning increased GTPase activities of PH domain mutants*', and that '*the new low resolution structural data of a dimeric dynamin construct obtained by SAXS measurements are very exciting for the dynamin field.*' We are grateful to the Reviewer for these comments.

The Reviewer goes on to comment that his/her '*main worries concern the fact that the mechanism of GTPase activation in the PH domain mutants is not sufficiently addressed. Consequently, I am not yet convinced that a direct coupling of the GTPase reaction to the PH domain is important for the function of dynamin.*'

As we have been digesting the results of the very recent *Nature* papers on the MxA stalk and dynamin GTPase from the Daumke and Schmid/Dyda labs respectively, we now agree with the Reviewer that we had overstated the case for direct coupling between the GTPase and the PH domain mutations in the original manuscript. A key change in the way that we now present the various possibilities has been to include the possibility that mutations in the PH domain (or its ligation) exert indirect effects on GTPase activity by modulating dynamin oligomerization – which seems to be the overarching comment of this Reviewer (appropriately).

We hope the Reviewer will agree that there is little doubt that the CNM-derived mutations studied here do activate dynamin as a GTPase in our studies. We would argue that this is an important finding – since it has generally been assumed that PH domain mutations linked to disease exert their effects by altering phosphoinositide/lipid binding affinity. As shown in Figure 1C of the manuscript, none of the PH domain-located CNM mutations affect lipid binding by dynamin: a surprising finding. We surmised that these mutations must therefore exert their

effects otherwise – and showed that they increase basal GTPase activity and indeed promote dynamin oligomerization.

The Reviewer lists three possibilities for how the GTPase reaction is promoted *in vitro*.

a) The first invokes direct (allosteric) coupling via the GED domain – which had been a significant focus (perhaps too much so) of our discussions in the original paper. We agree 100% with the Reviewer that other possibilities are equally likely at this stage, and accept that we had overstated the case for this type of model. We have revised the manuscript substantially in order to broaden the mechanistic view. Indeed, it seems clear that answers about specific mechanistic characteristics responsible for GTPase activation by the PH domain mutations will require high-resolution structural views of dynamin and its inter- (and intra) molecular interactions. We are working hard to generate crystallographic views of intact (or nearly intact) dynamin – as are many other groups – but our efforts have not yet borne fruit.

The Reviewer suggests here that the *'main reason which speaks against such a* (direct coupling) *mechanism is the structural model of the dynamin dimer (Fig. 7), where the G domain and PH domain are separated by the MD / GED stalk which is built of a stable four helical bundle. It appears highly unlikely, that conformational changes from the PH domain are relayed through the whole stalk to the G domain, and GED mutations known to interfere with GTPase activity are far away from the PH domain on the opposite side of the stalk…'.* We have considered this issue a great deal – and thought similarly. However, an analogy with receptor tyrosine kinases has persuaded us not to dismiss the possibility of allosteric coupling – albeit in the context of dynamin oligomers. Arguments about relaying 'conformational information' through the stalk in dynamin are similar to those that concern communication through the single transmembrane domain in receptor tyrosine kinases (RTKs). For most RTKs (such as the EGF receptor), this has led to acceptance of ligand-induced receptor dimerization as the only reasonable mechanism for transmembrane signaling – and this is analogous to arguments for dynamin oligomerization. However, the insulin receptor stands out as a counter example. Extracellular insulin binding activates the intracellular kinase domain without altering the oligomerization state of the receptor. Rather, insulin appears to change the relationship between the two molecules within a receptor dimer (or possibly higher order oligomer). A shift in the relative positions of the two dynamin molecules in the dimer shown in Fig. 7 may be promoted by PH domain ligation or mutation in a manner analogous to insulin binding to insulin receptor.

There is evidence for such conformational coupling between the GTPase and PH domains of dynamin within oligomers. Using dynamin with a fluorescently labeled PH domain, Ramachandran and Schmid (*EMBO J.* **27,** 37; 2008) found that adding a GTP analogue to pre-assembled dynamin promotes a conformational rearrangement that brings adjacent PH domains into closer proximity (*without dissociating from the membrane*). Driving this reaction from the other side – if mutations promote closer proximity of PH domains – would conversely alter GTPase activity. Thus, in the context of dynamin oligomers, allosteric changes promoted by PH domain ligation (or mutation) may well be important for allowing optimal interactions between G domains in neighboring dynamin rings (and thus enhanced GTPase activity).

In revisions, we have suggested this as one of several possibilities (remaining open minded, pending a crystal structure). It is clear that effects on phospholipid-binding cannot explain the influence of the CNM mutations found in the dynamin PH domain (a major result of our manuscript), and to be precise about how they do so would require advances in understanding of dynamin regulation that significantly surpass those published in *Nature* a few weeks ago.

b) *The PH domains might themselves be involved in promoting oligomerization by interacting with each other (possibly with low affinity) in the oligomer, as suggested by the structural model of the dynamin dimer (Fig. 7). This low affinity PH domain interactions might be inhibited in solution by an interaction of the PH domain with the neighboring middle domain / GED and the described mutations would interfere with that inhibition. In such a scenario, the PH domain mutations would result in increased oligomerization rates of dynamin in solution, concomitant*

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with increased GTPase activity in vitro. However, the increased GTPase rates in vitro would have little to do with the regulation of the GTPase reaction on a bud neck.

The scenario painted here by the Reviewer is not dissimilar to that suggested above. Again, specifics along the lines sketched by the Reviewer really require high-resolution structural views (which we do not yet have, despite several years of effort). PH domain mutations could indeed disrupt autoinhibitory interactions that normally hold oligomerization at bay. Or, they could directly promote oligomerization as gain-of-function mutations.

We are not sure, though, why the Reviewer would dismiss the possibility that the increased GTPase rates *in vitro* are relevant for regulation of the GTPase reaction on a bud neck (or – importantly – elsewhere in the cell). From the perspective of dynamin's role in human pathologies, we would argue that this is an important result. Most studies of dynamin mutants with defective GTPase activity have employed artificial mutations (K44A, T65A/F, etc) – which impact both *in vitro* GTPase and endocytosis in overexpression-based assays. Here, we have mutations identified in patients with centronuclear myopathy (CNM): mutations that clearly alter dynamin activity in a way that leads to disease. In an effort to understand this, we asked what characteristics of dynamin activity are altered by the CNM mutations. We showed that phospholipid binding is not affected by any of the CNM mutations in the C-terminal part of the PH domain. Unexpectedly, we found that GTPase activity is enhanced by all of these mutations (and oligomerization by some), and is sensitized to enhancement by lipid binding. We would argue that these altered characteristics are highly likely to be relevant for CNM. Indeed, they are the key molecular properties changed by all the mutations (in a way that correlates with phenotype) – and are manifest with *in vitro* assays that have formed the basis of what the endocytosis community considers dynamin's active role in vesicle scission. Moreover, the V625del and S619L mutations have been shown (by us and others) to impair transferrin uptake (implying important effects at the bud neck, at least). We therefore respectfully disagree with the Reviewer that the increased GTPase rates observed for CNM mutants *in vitro* have little to do with regulation of the GTPase reaction.

It may well be that GTPase regulation *at the bud neck* is not the key issue. Indeed, it remains unclear exactly what cellular processes are defective in CNM patients, and what dynamin even does in the diseased cells. For instance, McNiven and colleagues have suggested that dynamin is active in centrosome cohesion, an intriguing possibility given that central nuclei are the hallmark phenotype of CNM disease. Even with respect to endocytosis, dynamin's role is not well defined. A growing body of work from the Schmid laboratory concludes that basal tetrameric dynamin also contributes to endocytosis – *before the bud neck is even formed*. Specifically, these studies have demonstrated that differences in basal GTPase activities (even small 2-fold increases such as the two-fold increase in basal rate observed for the A618T mutant studied here) can significantly change overall endocytosis rates, particularly by changing the rate of coated pit <u>formation</u>. Our goal has been to understand what molecular properties are affected by the CNM mutations – with a view to exploiting the results both for understanding dynamin and understanding CNM. Elevated basal (and lipid-stimulated) GTPase rates are a common and notable characteristic of all CNM mutants studied here – so it seems inappropriate to dismiss this as a likely causal factor without evidence to support another possibility.

c) *It might also be that the PH domain mutations induce a local destabilization of the PH domains leading to non-specific aggregation of two PH domains within the dynamin oligomer (as for example suggested from experiments seen in Fig. 4b) which would also lead to increased GTPase rates in vitro (partial aggregation is often observed for protein variants with destabilizing mutations). The stability measurements of the mutated PH domains in Fig. 5 speak in favor for such a scenario since the most destabilizing mutations lead to the highest GTPase rates. In this scenario, the increased GTPase rates of the PH domain mutants in the in vitro assay might have nothing to do with the normal function of the PH domain in dynamin.*

We think that the Reviewer may well be correct that at least some of the PH domain mutations

alter the nature of dynamin self-association in oligomers. However, it seems highly unlikely that this is a consequence of non-specific aggregation. Partial aggregation is indeed often observed for protein variants with destabilizing mutations as the reviewer points out. However, this is a *very* stable PH domain. As we tried to make clear in the manuscript, the reduced stability arising from the CNM mutations occurs within the context of a particularly stable protein domain, and therefore all of the mutants are still stable and well folded proteins even when expressed as isolated domains in bacteria. In fact, we specifically show in the manuscript that even one of the most destabilizing CNM mutations (S619L) *still leaves the PH domain ~10 times more stable than the PLC-δ_1 PH domain*. The *PLC-δ_1* PH domain is a stable and well-used protein that has become a standard in the field. It does not self-associate, crystallizes readily, and is frequently used as a GFP fusion in cells to decorate PtdIns(4,5)P_2-containing membranes. The fact that one of the most destabilized CNM-mutated dynamin PH domain is still an order of magnitude more stable than this already-stable domain argues against the Reviewer's assertion. The 'lipid sensitized' A618T CNM variant is even more striking in this regard. The A618T-mutated dynamin PH domain is about *2,000 times more stable* than the PLC-δ_1 PH domain. Moreover, none of the C-terminal CNM mutations impair or enhance lipid binding, demonstrating that the PH domain retains structural integrity.

Given these considerations, we hope that the Reviewer will agree that GTPase activation by non-specific aggregation arising from local destabilization is very unlikely. Instead, it seems more likely (in an oligomerization model) that native-like intermolecular interactions are enhanced; either between PH domains or between native-like PH domains (that interact with lipid) and/or native oligomers of the middle/GED stalk. The oligomerization must be sufficient to promote the specific dimerization of the GTPase domains as reported in the recent structure by Chappie *et al.* (2010). Indeed, the CNM mutations in the C-terminal part of the PH domain appear to mimic the effects of phosphoinositide binding in this regard (which is unlikely to promote non-specific dynamin aggregation).

Reviewer 1 had additional general comments:

i). The main problem of the paper is that the in vitro GTPase reaction of dynamin is a poor readout for the function of dynamin at the neck of a budding vesicle. The GTPase reaction of dynamin and dynamin-related proteins includes a number of different reaction steps, and it is unclear from the current manuscript which step is accelerated by the mutations in the PH domain and how relevant this is for dynamin function.

A major concern of the Reviewer – mentioned in several of the above comments – is that the *in vitro* assay might have nothing to do with the normal function of the PH domain in dynamin. Specifically here, he/she comments that it is *'a poor readout for the function of dynamin at the neck of a budding vesicle'*.

As mentioned above, our goal was to understand how the CNM mutations studied here (in the PH domain) affect the properties of dynamin as a molecule – since this will certainly underlie the mechanism of the molecular lesion that leads to disease in CNM patients. We went into the project expecting to detect effects on phosphoinositide binding, and there are essentially none – and this seemed a surprising result. Since dynamin is a GTPase – and it is well established that GTP hydrolysis plays an important role in its function – we next studied the GTPase characteristics of the mutants. We hope the Reviewer will agree that our data show that regulation of dynamin's GTPase activity is altered by CNM mutations – and all seem to have the same effect (to a greater or lesser degree). Much of what we currently know about dynamin function is predicated on mutations in the GTPase and GED domain, and assessment of their effects on endocytosis in overexpression studies. Examples include the K44A, and T65A/F artificial mutations.

Schmid, Zimmerberg, and colleagues carefully analyzed the behavior of dynamin in different GTP-binding states as reported in two 2008 *Cell* papers. They proposed that 'dynamin transmits GTP's energy to periodic assembling of a limited-curvature scaffold that brings lipids

to an unstable intermediate'. In the Pucadyil and Schmid paper (*Cell* 25, 135), the quantitative *in vitro* studies suggest that 'the primary response generated upon GTP hydrolysis in preassembled dynamin is its dissociation from the membrane without concomitant fission'. Dynamin-catalyzed membrane fission requires both its ability to bind-and-hydrolyze GTP and its ability to self-assemble. Self-assembly stimulates the GTPase activity of dynamin, which in turn causes its release from the membrane. These authors therefore argued that the presence of GTP *in vivo* should maintain dynamin as a self-limited assembly on the membrane, reflecting a balance between spontaneous assembly and GTP hydrolysis-dependent disassembly. Nice data were presented to support this.

All that we are suggesting here is that CNM mutants disrupt this balance, perhaps by altering the balance towards GTP hydrolysis-dependent disassembly so that self-limited assemblies may be smaller. According to our current understanding of dynamin, this should certainly affect dynamin function at the neck of a budding vesicle.

It is also important to note that there is a convincing and growing body of work (some mentioned above) that highlights the importance of dynamin in other cellular activities, including the proposed role of non-assembled dynamin in endocytosis, and dynamin's activity in centrosome cohesion (which may be of particular relevance for CNM). Before its assembly, dynamin's basal GTPase activity is suggested to regulate the rate of coated pit formation. We see substantial changes in basal dynamin GTPase rates in CNM mutations, in addition to a sensitized response to lipid in some instances. It is difficult to imagine, given the current state of the field, that this will not affect dynamin function in cells.

There are certainly caveats – as in all cases – with the use of *in vitro* assays. However, given the central role played by GTP hydrolysis (and its regulation) in dynamin function, we believe that our discovery that CNM mutations alter the characteristics of dynamin's GTPase (and its stimulation upon membrane binding) is an important step forward in understanding both dynamin and its role in disease.

We and others have taken steps towards investigating the effects of the CNM mutations on dynamin function in cells. Overexpressing the 'severe' GTPase dysregulation mutations found in CNM (V625del and S619L) causes dominant inhibition (not acceleration) of transferrin endocytosis in cultured cells. This is consistent with the *in vitro* biophysical studies of Zimmerberg, Schmid, and colleagues mentioned above. Enhanced GTPase activity would likely reduce the size of self-limited dynamin assemblies – which would impair membrane fission. As expected, the 'milder' A618T does not cause overt dominant inhibition (new Figure S7).

The next phase of this study will certainly be to decipher how the CNM mutations influence cell behavior and ultimately cause CNM disease. Reagents (MEFs lacking both dynamin 1 and 2) have only very recently become available for such a study – and may well not have the correct cellular context. We have initiated such studies, but view them as a separate project that will require significant development and refinement before the point of publication can be reached. We would also need to analyze centrosome cohesion, and ask whether CNM mutants influence this process either directly or indirectly following improper endocytosis/trafficking events.

ii). In the recent manuscript from Gao et al. (2010), oligomerization-compromised mutants in the dynamin-related MxA protein also showed increased GTPase rates; however, the underlying mechanisms of increased GTPase activities appear to be completely different for the PH domain mutants presented here.

The excellent MxA paper from the Daumke laboratory, published in *Nature*, analyzed the effects on *in vitro* MxA GTPase activity of mutations designed based on an interface observed in a crystal structure of a ~220 residue GED/middle domain fragment. It is intriguing that the M527D, L617D and ΔL4 mutations enhanced GTPase activity despite seeming to reduce self-assembly in other assays – but this was only seen at very elevated protein concentrations that are well above those used in our assays. Studies of nucleotide-binding affinities led the authors to suggest that nucleotide release is normally influenced by oligomerization via the stalk region. This does indeed appear to be a completely different mechanism from what is observed with PH

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domain mutations in CNM. However, this is not entirely unexpected, since MxA has no PH domain, and the MxA mutations studied by Daumke and colleagues also impaired binding to liposomes – which is not the case for the CNM mutations. Since regulation of the GTPase activity of dynamin family members by oligomerization is so central to their function, it seems reasonable to anticipate that several different domains will contribute – and in different precise ways. It would be most interesting and informative to compare the MxA mutations studied by Daumke and colleagues with mutations in the dynamin GED.

iii). If this paper was published in EMBO Journal, the authors would need to experimentally address the detailed mechanism how the GTPase reaction is stimulated in the PH domain mutants and show that the increased GTPase rates in the in vitro assays are not an artifact, as e.g. in scenario (c). The new findings should result in a conclusive model how the PH domain influences oligomerization and/or GTPase activity.

If we are interpreting this comment correctly, the only way that we can imagine satisfying this request is to determine a crystal structure of intact dynamin in different oligomeric states – and/or of mutated dynamin in different nucleotide-bound states as done for hGBP1. This would be a wonderful advance. The current state-of-the-art (as published in the May 27th issue of *Nature*) for dynamin is limited to studies of a minimal GTPase-GED fusion protein (for dynamin) or a 220aa GED/middle domain fragment (for MxA). We are actively pursuing crystal structures of near-intact dynamin variants – and so far have managed to obtain what we believe are useful solution scattering data that suggest domain rearrangements upon oligomerization. The Reviewer commented that these results are '*very exciting for the dynamin field*'.

We have shown that specific CNM-derived mutations in the C-terminal part of the PH domain lead to enhanced GTPase activity and oligomerization (to which it is coupled). The mutations do not affect lipid binding, but do appear to mimic its effects. There are several scenarios in which enhancing dynamin's GTPase activity could impair its function (such as altering the balance between assembly and disassembly, as outlined above). Thus, our data provide a model for how CNM mutations may exert their pathological effects. It is not clear that the effects of GED domain mutations on MxA GTPase activity mentioned above by the Reviewer, for example, are any better defined in terms of detailed mechanism than the CNM mutations studied here.

We hope that – in light of the discussion above – the Reviewer will agree that our work does represent a significant advance that might be published in the *EMBO Journal* in the absence of additional high-resolution structural and mechanistic data, which we are quite some considerable time away from completing.

Reviewer 1 also had several helpful specific comments, for which we are most grateful, and which we address below (and with revisions):

Fig. 2: *Fitting of Michaelis-Menten parameters to the dynamin reaction is not correct since the underlying reaction model is completely different. The dynamin reaction does not only involve GTP binding and turnover (as in a typical Michalis-Menten reaction) but also assembly of the G domains (most likely from different dynamin tetramers) which is also promoted by GTP binding / hydrolysis. Consequently, the observed KMs reported here are not equal Kd for the nucleotide. Not the affinity to the nucleotide but the percentage of assembled dynamin molecules at a given protein concentration might differ between wildtype and PH domain mutants which would also result in the observed differences in GTP saturation.*
To dissect nucleotide binding affinity and dynamin assembly, GTPase reactions for wildtype and PH domain mutations with saturating GTP concentrations and varying protein concentrations should be carried out. In this way, dissociation constants for G domain dimerization can be derived. Nucleotide binding affinities of the PH domain mutants should be determined with other methods such as calorimetry or fluorescence measurements.

We appreciate the Reviewer pointing this out – and he/she is quite correct to do so. We have revised the text, figures, and tables to make it clear that we cannot extract classical Michaelis-Menten parameters from our analysis. We fit a plot of initial hydrolysis rates against [GTP] to a saturation kinetics model, and thus obtain estimates of what we now term k_{obs} and K_M^{app}. This takes into account the fact that GTP binding also promotes assembly. We have also corrected our statement that $K_M^{app} = K_d$ for GTP binding. The lower K_M^{app} values measured for the CNM mutants might indeed reflect enhanced assembly (or reduced disassembly).

Wild-type dynamin is well known to have significant basal activity ($k_{obs} = 1.1$ min^{-1}) under conditions where its self-assembly does not exceed tetramerization. The key result with the CNM mutants is that dynamin activity is elevated above wild-type levels in the absence of stimulation by lipid binding. This may reflect increases in basal activity and/or in self-assembly. The latter is certainly suggested for the S619L mutant in Figure 4B of the manuscript, but we have no evidence to suggest that A618T self-assembly is enhanced.

It is clear, though, that self-assembly is not the only issue. Importantly, even when fully assembled on PtdIns(4,5)P_2-containing membranes, the A618T mutant, for example, shows GTP hydrolysis rates that are significantly higher than wild-type dynamin (Figure 3C/D of manuscript). This suggests that the nature of the oligomer is altered in some way – such that assembly-induced GTPase activity is higher in mutated than in wild-type dynamin. It is not clear how to obtain more detailed mechanistic or structural insight into the reasons for this (which are likely to be physiologically important) without high-resolution structural studies of dynamin oligomers.

Fig. 2d: *Since basal GTP hydrolysis for the PH mutants is most likely dependent on dynamin assembly, the reported value is kobs rather than kcat.*

We have made this change – as described above – and thank the Reviewer for pointing this out.

Fig. 4: *Interesting finding which might argue for uncontrolled (unspecific?) oligomerization of the S619L mutant in the presence of GTP and at 37˚C. In fact, since the mutant runs in the void volume it looks like a large soluble aggregate which, however, still maintains functional G domains.*

We agree with the Reviewer that this result indicates enhanced oligomerization properties of the S619L mutant at 37˚C, as described in the text. It is certainly a large oligomer (>700 kDa), and its formation is promoted by the S619L mutation despite the fact that the isolated PH domain harboring this mutation remains a stable, well-folded, monomeric protein (more stable than the PLC-δ_1 PH domain as described in Figure S1).

It seems less likely that this is a large soluble aggregate than a native-like oligomer. Indeed, the protein retains wild-type lipid binding, and is actually more active upon stimulation with 40 μM lipid than wild-type dynamin. S619L shares this property with the A618T mutant, which Figure 4 shows does not oligomerize detectably under the same conditions.

In considering the possibility of specific effects, it is interesting to note that mutations in the C-terminal part of the dynamin PH domain – the 'motif' that attracted our attention – appear to be linked exclusively to CNM. Other mutations in the PH domain cause CMT, which has no clinical overlap with CNM. The clustering of CNM mutations to this one motif in the dynamin PH domain suggests some specificity in the defects caused.

Fig. 5: *Could be removed to the Supplement and discussed in detail there.*

As we have discussed above, we think it important to note that the dynamin PH domain is really very stable – even for a PH domain – and Figure 5 allows this point to be made clearly. Even dynamin PH domain variants that harbor CNM mutations are significantly more stable that many well-studied (and well-behaved) PH domains. Yet, the mutations clearly have an effect on

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stability that we hypothesize reflects altered ability to participate in intra- and/or inter-molecular interactions that are important for dynamin regulation.

Fig. 6: *I am not an expert for SAXS measurements and cannot judge the relevance of the observed differences in Fig. 6c. However, the resulting envelope and suggested structural model look reasonable and interesting. The proposed model argues against a direct coupling of the PH domain with the GTPase reaction (see above).*

We very much agree with the Reviewer that the SAXS-derived model argues against direct PH-GTPase ***interactions.*** However, it does not speak to the issue of ***coupling***. In the context of dynamin tetramers or higher-order oligomers, alterations in PH•PH and/or PH•M/GED interactions (be they intra- or intermolecular) could alter the arrangement of G domains and modulate GTPase activity. This is discussed in both the results and discussion, and has been edited for clarity. Indeed, the scheme depicted in Figure 7 illustrates how the relative positions of the G domains in oligomers reconstructed from EM studies are different from those seen in the free dynamin dimer. The two M/GED regions of the dimer 'T' are drawn closer together, and their relationship with the adjacent PH domain appears to be altered. This transition is effected upon dynamin binding (via its PH domain) to PtdIns(4,5)P_2-containing membranes, and we suggest that it may be promoted by CNM mutations found in the PH domain.

Fig. 7: *It might be helpful to include the MxA stalk in Fig. 7A.*

We have modified Figure 7A to include the MxA stalk structure recently reported by Daumke and colleagues as suggested – and thank the Reviewer for the suggestion.

Fig. 6 and Fig. 7: *It has been proposed that the PH domain mutations also induce conformational changes in the background of a dimeric dynamin mutant. How do the GTPase reactions of dimeric dynamin with PH domain mutations compare to the dimeric dynamin variant and wildtype dynamin? Do they also show accelerated GTPase rates? Do these mutants display protein-concentration dependent GTPase activity?*

We have analyzed the GTPase activity of dimeric dynamin variants with PH domain mutations, and they do indeed show accelerated GTPase rates. In fact, the behavior of the A618T and S619L dimeric variants (at very low protein concentrations – 50 µg/ml or 0.5 µM – with high salt) mirrors that seen for the equivalent variants of intact dynamin with respect to both basal activity and self-association in experiments similar to those shown in Figure 4.

Since the GTPase reaction of dynamin is such an indirect readout for dynamin function (see above), it would be very informative to characterize the effect of a few representative PH domain mutants on the cellular uptake of cargo (e.g. the data not shown for the S619L mutant). It would also be interesting to see whether the PH domain mutants described here can be recruited to budding vesicles or whether they have lipid binding defects in vivo.

As mentioned in the text, Bitoun et al. (*Hum. Mutat.* **30,** 1419; 2009) have reported that the V625del mutation inhibits clathrin-mediated endocytosis when overexpressed in COS7 cells. We have also observed similar effects for the S619L mutant, and now show representative images in the new Supplementary Figure S7. Consistent with its milder effects on GTPase activity, the A618T mutation does not cause dynamin to exert a dominant negative effect. Thus, it seems clear that the changes in dynamin behavior caused by the CNM mutations do impair its function in endocytosis (in a way that correlates with 'severity' *in vitro* – although it remains unclear how this is related to the origin of CNM, as outlined above).

For the purposes of understanding CNM, a much more thorough analysis will be required, including investigation not only of endocytosis, but also centrosome cohesion, and perhaps indirect effects of endocytosis on centrosome cohesion. These latter activities are not well defined, and will certainly need to be done in an appropriate null background – which has not

been possible until very recently. Thus, such careful analyses are really beyond the scope of the current manuscript.

We have not investigated lipid binding in cells, and do not expect this to be a productive line of enquiry. First, none of the CNM mutations studied here impair phosphoinositide binding *in vitro*, so there is no reason to expect that they will do so in a cellular context. Second, we have already reported (Bethoney et al., *PNAS* 2009) that even severely lipid-binding-deficient mutations in the dynamin PH domain still localize to the bud neck (largely because dynamin is recruited via PRD interactions, not lipid binding).

Discussion: *The discussion should also include the novel findings on the mechanism of GTPase activation in dynamin (e.g. dimerization-dependent GTP hydrolysis, etc.) including a final model how the PH domain mutations affect GTP hydrolysis (see above). The comparison of dynamin to the various other PH domain containing proteins is somehow misleading since in most of these cases, there is a direct contact between the PH domain and the second domain whose activity is controlled, which is different for dynamin (at least, this difference should be mentioned).*

As the Reviewer suggests, we have significantly expanded our discussion of the dimerization-dependent GTP hydrolysis as revealed by the recent papers from the Schmid/Dyda and Daumke laboratories. We hope that the discussion of what our findings suggest for the role of the PH domain in dynamin GTPase activation – and specifically the effects of the CNM mutations – is now acceptable. Naturally, this remains a model – which will be falsified or otherwise only when high-resolution structures of dynamin tetramers and assemblies are determined.

Since the Reviewer considered that it might be misleading, we have substantially altered discussion of the comparison of dynamin with other PH domain containing proteins. The revised discussion now focuses only on the fact that the C-terminal alpha helix of these PH domains is often involved in regulatory protein-protein interactions, many of which indirectly or directly regulate the activity of associated GTPases.

Reviewer 2:

This Reviewer commented that '*This study is particularly important because in most case of CNM and similar disorders we do not understand in depth the links connecting the genetic defects with the clinical manifestations of the diseases. The main conclusion from this study is that the PH domain allosterically couples lipid binding to dynamin GTPase activation and that mutations in the PH domain C-terminal α-helix cause conformational changes that mimic or facilitate effects of lipid binding. These conclusions are based on extensive analysis using a range of biochemical and biophysical methods including measurements of relevant parameters of GTPase activity, lipid binding, oligomerization, protein stability using urea chemical melts and conformational changes using SAXS.*'

We thank the Reviewer for his/her very positive comment about the importance of our manuscript, and for appreciating the value of our study for understanding the origin of molecular defects in CNM.

The Reviewer also had several constructive and interesting suggestions and comments, for which we are grateful, and which we address below:

1. *The idea that lipid binding causes conformational change should be supported more directly. Would binding of the lipid head-group or a short-chain PIP2 cause an increase in self-association or cause a conformational change?*

This is an interesting idea, on which we expended quite a bit of effort. The difficulty is that the dynamin PH domain has very low affinity for monomeric inositol phosphate head groups or short-chain phosphoinositides. Indeed, K_D for dynamin (or dynamin-PH) binding to Ins(1,4,5)P_3

is in the 1 – 4 millimolar range. One can only see appreciable lipid binding by dynamin when the phosphoinositide is presented as a membrane surface such that multivalent interactions of dynamin oligomers can occur with multiple lipids in the same membrane. Indeed, significant membrane binding by the isolated PH domain can only be seen when it is oligomerized artificially (using GST, cysteine cross-linking or such) or by native dynamin tetramers/higher-ordered oligomers.

Given this, it is perhaps not surprising that experiments in which very high concentrations of Ins(1,4,5)P_3 headgroup to our GTPase reactions did not result in observable activation of dynamin – so were abandoned. We suggest that the PH-domain mediated activation that results from lipid binding (suggested by our manuscript) follows from reorientation of the PH domains within an oligomer when dynamin assembles on a lipid surface/tubule. Indeed, coupling between the GTPase and PH domains of this sort has been reported by Ramachandran and Schmid (*EMBO J.* **27,** 37; 2008), who observed a conformational change in the PH domain of pre-oligomerized dynamin upon addition of a GTP analogue. This was achieved using a form of dynamin with a fluorescent label attached to the PH domain, and indicated that upon GTP binding to self-assembled dynamin, a conformational rearrangement occurs that brings adjacent PH domains into closer proximity. Driving this reaction from the other side – if mutations (or lipid binding) promote(s) closer proximity of PH domains – GTPase activity and/or GTP binding should be affected.

2. *The authors should make a reference and further comment on their observation in the light of recent and important findings described in Nature (Article) 2010 465:435-440. Although the paper was published after submission of their manuscript, the importance and relevance of this article has to be taken into account.*

We strongly agree, and have amended the discussion to reflect these important findings in discussing possible models for coupling of the PH and GTPase domains.

3. *The data are presented in a large number of Figures distributed between the main text and supplementary material. The distribution of the data between "main" and "supplemental" could be organized better. For example, the finding that mutations in the PH domain C-terminal α-helix, as expected, do not affect lipid binding (a negative observation) is in the main Figure 1 and could be supplemental, while the key observations that these mutations affect GTPase activity are distributed between Figures 2 and 3. The original 3D model of dynamin based on the EM ("Head", "Salk" and PH-domain "Leg") should be presented together with already included schematic diagram of dynamin. Efforts in this direction would be beneficial for the potential, wider interest in this work.*

We thank the Reviewer for these suggestions.
In Figure 1, we would prefer to keep the (negative) data on lipid binding in the main manuscript. We believe that it is important to do so because it is generally assumed (incorrectly) that all dynamin PH domain mutations found in CNM and CMT patients cause aberrant lipid binding – and that this is the molecular lesion. These negative data are therefore rather important for the paper (and consideration of mechanisms of CNM pathology).
We have rearranged Figure 1, though, to accommodate the Reviewer's suggestion that a schematic based on the original 3D model of dynamin from the Hinshaw lab EM studies might be included. This is indeed useful for the ability of the reader to consider mechanistic possibilities while going through the paper, and we are grateful for the suggestion.
Regarding the GTPase data in Figures 2 and 3, we wished to keep data on basal activity (Figure 2) and lipid-stimulated activity (Figure 3) in separate figures, so as not to have too unwieldy a figure – and to guide the flow of the text. We have smoothed the text to help with this, and hope that the break between these figures now seems more natural.

4. It should be noted that mutations in a range of proteins (not just dynamin) are found in disorders linked to aberrant trafficking.

We have noted this important point in the revised manuscript (page 19), with reference to key reviews.

Reviewer 3:

We thank this Reviewer very much for his/her very positive comments. Reviewer 3 suggested no revisions.

2nd Editorial Decision 06 July 2010

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed the criticisms in a satisfactory manner, and that the paper will therefore be publishable in The EMBO Journal.

Before this will happen, however, I was wondering whether you would like to consider addressing the minor issues suggested by referee 1 (see below). Please let us have a suitably amended manuscript as soon as possible. I will then formally accept the manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The manuscript is clearly improved and I am satisfied with the changes and responses to the points which I raised. I completely agree with referees 2 and 3 that the manuscript is a significant advance in our understanding of dynamin's function and mechanism and of the molecular basis of CNM and suggest that it should be published without delay. I have two minor points which the authors still should address before publication.

p7 main text. The CNM mutations studied here thus appear to represent gain-of-function mutations. I would remove that sentence since the authors show in Supp. S7 that these mutants, despite having increased GTPase rates, are dominant negative for endocytic uptake, e.g. the mutations interfere with the function of dynamin in endocytosis. This again shows that the in vitro GTPase activity is not a clear readout for the function of dynamin (e.g increased GTPase rates are not equal to an increased function).

p7 rebuttal letter. Fig. 6 and 7: It has been proposed that the PH domain mutations also induce conformational changes in the background of a dimeric dynamin mutant ...
The authors provide new GTPase data for some CNM mutations in a dimeric dynamin background, but they fail to include them in the manuscript. Since this is a very interesting and important point for the paper and the arguments, the data should be included as small panel in Fig. 6 and also in the main text.

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2nd Revision - authors' response 13 July 2010

We thank Reviewer 1 for his/her comments on our revised manuscript – and indeed for making numerous helpful suggestions in the last round of review that helped improve the manuscript.

The Reviewer raised two further minor points that he/she suggested that we might address before publication:

1. "*p7 main text. The CNM mutations studied here thus appear to represent gain-of-function mutations. I would remove that sentence since the authors show in Supp. S7 that these mutants, despite having increased GTPase rates, are dominant negative for endocytic uptake, e.g. the mutations interfere with the function of dynamin in endocytosis. This again shows that the in vitro GTPase activity is not a clear readout for the function of dynamin (e.g increased GTPase rates are not equal to an increased function).*"

We have removed this sentence as suggested. However, since doing so results in a rather abrupt end to that paragraph, we have added an alternative sentence that focuses on increased GTPase rates rather than function – and therefore does not equate GTPase activity with function. The new sentence reads:
"...our data show that, rather than affecting the phospholipid-binding properties of dynamin, CNM mutations in the C-terminal region of the PH domain enhance dynamin's GTPase activity."

We believe that this addresses the Reviewer's concern.

2. "*p7 rebuttal letter. Fig. 6 and 7: It has been proposed that the PH domain mutations also induce conformational changes in the background of a dimeric dynamin mutant ... The authors provide new GTPase data for some CNM mutations in a dimeric dynamin background, but they fail to include them in the manuscript. Since this is a very interesting and important point for the paper and the arguments, the data should be included as small panel in Fig. 6 and also in the main text.*"

We have now included the new GTPase data that the Reviewer discusses in the manuscript. Since Figure 6 is already on the verge of becoming unwieldy, we would very much prefer not to add to it – particularly for this experiment (which we view largely as a control). We have therefore added a new Supplementary Figure (new Figure S4) to present these data – with appropriate description in the figure legend. We have added a statement in the main text (on page 14) as suggested, which reads:

"Importantly, the effects of these mutations on basal GTPase activity described above are retained in the dimer context (Figure S4)."

We agree that this is an important point, and thank the Reviewer for his/her suggestion to include it explicitly.

We thank the Reviewer for these two suggestions, and trust that our revisions address them fully.

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