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Regulation of Cargo-Selective Endocytosis by Dynamin 2 GTPase-Activating Protein Girdin

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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 10 March 2014

Thank you for the submission of your manuscript entitled "Regulation of Cargo-Selective Endocytosis by Dynamin 2 GTPase-Activating Protein Girdin". We have now received the reports from the two referees that were asked to evaluate your study, which I copy below.

As you can see from their comments, both referees are rather positive, in particular referee #2, and support the publication of your manuscript. In general, they are convinced that the evidence presented is conclusive, but referee #1 still points out to some technical shortcomings, procedural confusion and clarifications that will need to be addressed. These concerns are explicitly mentioned in the referee report and I will thus not repeat them here. However, I would like to draw your attention to points 7 and 8 of referee #1. Regarding point 8, we believe that the identification (or not) of Girdin in previous RNAi screens is, in any case, not within the scope of this manuscript as none of the conclusions presented here would be affected by this fact. Similarly, while we agree with the referee that the mass-spectrometric characterization of CCVs upon Girdin knock-down (point 7) would certainly increase the interest and potential reach of your study, we also believe that it would be out of the scope of this manuscript for you to embark on such experimental work. Naturally, if you already have data at hand that could address these concerns, it would be only in your best interest to include them, although this information will not be determinant for the acceptance of your manuscript.

Given these positive evaluations, I would like to invite you to submit a revised version of the manuscript. It is 'The EMBO Journal' policy to allow a single round of revision only, which should

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be submitted within the next three months. In this regard, do not hesitate to contact me if you have any question, need any further input or anticipate any problems along the revision process. Should you foresee a problem in meeting the three-month deadline, please let us know in advance and we may be able to grant an extension.

I would also like to point out that as a matter of policy, competing manuscripts published during this period will not be taken into consideration in our assessment of the novelty presented by your study ("scooping" protection). However, we request that you contact me as soon as possible upon publication of any related work in order to discuss how to proceed.

When preparing your letter of response to the referees' comments, 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://emboj.msubmit.net/html/emboj_author_instructions.html#a2.12

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

REFEREE REPORTS

Referee #1:

Weng et al., "Regulation of Cargo-selective endocytosis by Dynamin 2 GTPase-activating protein Girdin."

In this study the authors report the association of Girdin with Dynamin 2 and go on to investigate the functional significance of that interaction providing evidence that Girdin acts as a GAP for Dynamin 2 and is required for the efficient endocytosis of specific cargo proteins, notably the Transferrin receptor (TfnR) and E-cadherin.
Dynamin 2 is an important regulator of calthrin-mediated endocytosis being required for the scission (pinching off) of coated vesicles at the plasma membrane. Additionally Dynamin 2 has been implicated in regulation of vesicle and tubule scission at other sites in the cell including endosomes. Given the importance of Dynamin 2 in regulating CME, this study will be of wide interest.
The authors present a series of experiments that together show that Gridin associates with Dynamin 2 and is important for the CME of cargo proteins such as the TfnR and E-cadherin but not other cargoes such the EGFR and b1-integrin.
For the most part the experiments are reasonably well controlled and the data appears fairly solid. I do have some concerns with aspects of this study however and feel that the authors may have over interpreted some of the data. Set out below are the main concerns I have.

1. Does Girdin directly bind to Dynamin 2? The data presented in the manuscript does not unequivocally answer this question as it relies mostly on the use of transient transfection and immunoprecipitation experiments. Where data describing direct binding experiments is presented (e.g. Fig 1G) the findings do not appear to agree with other experimental data, i.e. the binding of the Girdin NT domain to Dynamin 2 GTPase domain does not appear to occur in the experiment shown in Fig 1F - although the anti-Flag blot of the GFP IP in Figure 1F is so dominated by non-specific bands that it is hard to know what is specific in that experiment. It is strange that the anti-Flag blot of the IP is so dirty and the yet the blot of the lysates in the same figure is so clean, why should this be so?
Some additional corroborating data (e.g. using the yeast two hybrid system) to clearly demonstrate the direct binding of Gridin to Dynamin 2 would be helpful to resolve the apparent contradictions in the data in Figure 1.

2. The authors show that knockdown of Gridin selectively inhibits CME uptake of TfnR and E-cadherin but not EGFR or b1-integrin (Figure 2). It must be said that the effect on TfnR uptake is modest. I also have some concerns regarding the time points for the uptake experiment in Figure 2. Whilst uptake of the TfnR is rapid and therefore should be studied at early time points, the kinetics of E-cadherin uptake are not necessarily the same and it would be useful therefore for the authors to investigate E-cadherin uptake at later time points than 10 mins to show that a Girdin knockdown

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does not simply inhibit the kinetics of E-cadherin uptake.

3. Was the EM-based analysis of coated pit morphology (Fig 3G and H) performed 'blind', i.e. what steps were taken to ensure that images were not selected to show what the authors wanted to see?

4. The authors show that TfnR and E-cadherin uptake occurs at "central" sites whilst EGFR and b1-integrin uptake occurs at "peripheral" sites (Figure 4A). Does the localisation of Girdin correlate with the sites where TfnR and E-cadherin uptake occurs? This would be expected given the apparent requirement for Girdin in TfnR and E-cadherin uptake.

5. What happens to Girdin localisation after a knockdown of clathrin or Dynamin 2?

6. The authors show that Girdin can co-IP with the EGFR and, to a lesser extent, b1-integrin (Figure 4B) but apparently not with E-cadherin or the TfnR. This seems to be at odds with the proposed role for Girdin in mediating the uptake of TfnR and E-cadherin - why the discrepancy? How can the authors explain this? Does a knockdown of clathrin or Dyamin 2 affect the ability of Girdin to co-IP with these cargo proteins? Overall the data pertaining to the role of Gridin in cargo selection seems like it has been over interpreted.

7. Studies of clathrin-coated vesicles (CCVs) using mass spectrometry have been reported fairly recently and have shown how loss of specific proteins can affect the cargo composition of CCVs (see PMID: 17116749 and 22472443). Could the authors perform a similar analysis for Girdin?, i.e. isolate CCVs after Girdin knockdown and then analyse for cargo proteins using mass spectrometry - this would help strengthen the conclusion that Girdin regulates cargo selective endocytosis.

8. Has the requirement for Girdin in endocytosis been reported through the use of genome-wide siRNA screens, e.g. PMID: 20190736 and 23263279? If not, can the authors provide an explanation as to why?

Referee #2:

This manuscript by Weng et al. addresses fundamental issues in cell biology relating to clathrin mediated endocytosis. The authors examine the molecular determinants of cargo specificity and, more importantly, how that process is mediated. The manuscript centers around a recently discovered protein, girdin (girders of actin filaments), which associated with dynamin2 and enhances its GTPase activity. Evidence that girdin is a cargo-specific regulator of endocytosis comes from experiments in which knock down of girdin prevents transferrin and E-cadherin uptake, but not EGFR or integrin endocytosis. Mechanistically, this occurs because EGFR and integrin competitively bind dynamin. This prevents girdin form mediating its endocytic function. The authors suggest that girdin function may be in nucleation of clathrin coated pits via an interaction with the adaptor protein AP2. The final set of experiments that tries to tie everything together uses polarized cells in which girdin is knocked down. The consequence is that TfnR and E-cadherin localization is perturbed, but integrin beta1 is unaffected.

The premise of the manuscript is important, the experiments are clearly described, and the data are of high quality.

One strength of the manuscript is in mapping the domains of girdin that bind to dynamin and its GAP activity. In addition, the role of girdin in cargo selective uptake is clear, as is the mechanism by which this occurs. Less clear is the spatial regulation of clathrin coated pit (CCP) formation. While it is clear that this occurs from multiple experiments, the reason why is simply not clear at this point. This sentiment is echoed by the authors, as they admit "the mechanism by which girdin spatially controls CCP formation in the center of the cells but not the periphery is unknown". I think the manuscript would be strengthened if less emphasis were placed on those findings, so as not to distract from girdins role as a GAP and in cargo selective uptake.

Minor problems

1. Figure 5G. The co-immunoprecipitation of girdin with adaptin-a is not strong data. The concern is that IgG also pulled down the same proteins. Could this experiment be designed such that it is more

convincing?

1st Revision - authors' response 29 May 2014

Response to the reviewers:

We are grateful to reviewers for the critical comments and useful suggestions that have helped us improve our paper. As indicated in our responses, we have taken all of the comments and suggestions into account in the revised version of our paper.

Response to Reviewer #1:

1. Does Girdin directly bind to Dynamin 2? The data presented in the manuscript does not unequivocally answer this question as it relies mostly on the use of transient transfection and immunoprecipitation experiments. Where data describing direct binding experiments is presented (e.g. Fig 1G) the findings do not appear to agree with other experimental data, i.e. the binding of the Girdin NT domain to Dynamin 2 GTPase domain does not appear to occur in the experiment shown in Fig 1F - although the anti-Flag blot of the GFP IP in Figure 1F is so dominated by non-specific bands that it is hard to know what is specific in that experiment. It is strange that the anti-Flag blot of the IP is so dirty and the yet the blot of the lysates in the same figure is so clean, why should this be so?
Some additional corroborating data (e.g. using the yeast two hybrid system) to clearly demonstrate the direct binding of Gridin to Dynamin 2 would be helpful to resolve the apparent contradictions in the data in Figure 1.

We appreciate the reviewer's comment. We agreed that the results from original Fig. 1F contained many non-specific bands. This was due to the presence of IgG heavy and light chains derived from the anti-GFP antibody, which often happens in IP experiments. We point out, however, if we look at the result carefully, you find a band for co-immunoprecipitated dynamin 2 GTPase domain in the cells transfected with GFP-NT plasmid but not in the cells transfected with control GFP plasmid, which we labeled with a red asterisk (the second lane from the left in the original Fig. 1F, see below in the next page).

To overcome this problem and make the bands more visible, in the revised experiment, we transfected Flag-tagged dynamin 2 fragments along with GST or GST-girdin-NT plasmids into COS7 cells, and incubated the cell lysate with glutathione beads to pulldown GST-fusion protein and its interacting proteins (Fig. 1F in the revised manuscript). The result showed that girdin interacts with dynamin 2 GTPase and GED domain, which is consistent with our previous finding.

To show the direct interaction between girdin and dynamin 2, we performed in vitro GST-pulldown experiment by using purified GST/GST-NT and dynamin 2-His6 (Fig. 1G in the original and revised manuscript). In addition, as shown in Fig. 1H, we showed that the direct interaction is regulated by GTP-loading to dynamin 2, which strengthened our conclusion that girdin directly binds to dynamin 2.

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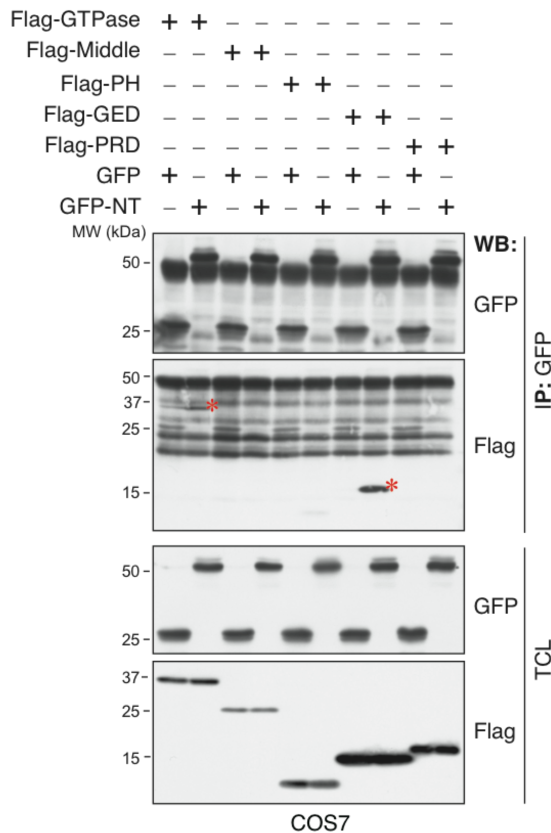


(Original Fig. 1F)

<u>2. The authors show that knockdown of Gridin selectively inhibits CME uptake of TfnR and E-cadherin but not EGFR or b1-integrin (Figure 2). It must be said that the effect on TfnR uptake is modest. I also have some concerns regarding the time points for the uptake experiment in Figure 2. Whilst uptake of the TfnR is rapid and therefore should be studied at early time points, the kinetics of E-cadherin uptake are not necessarily the same and it would be useful therefore for the authors to investigate E-cadherin uptake at later time points than 10 mins to show that a Girdin knockdown does not simply inhibit the kinetics of E-cadherin uptake.</u>

Following the reviewer's suggestion, we described in the revised manuscript that the effect on TfnR is rather modest compared with E-cadherin (page 7, line 9). Also, to address the reviewer's concern, we performed additional experiments to observe the effect of girdin knockdown on E-cadherin endocytosis over a longer time frame (10, 20 and 30 minutes) as shown in Fig. 2D in the revised manuscript. The result showed that girdin knockdown inhibited E-cadherin endocytosis even at 30 minutes, which indicates the significance of girdin function on the dynamics of E-cadherin trafficking and excludes the possibility that girdin knockdown simply inhibited the kinetics of E-cadherin uptake.

<u>3. Was the EM-based analysis of coated pit morphology (Fig 3G and H) performed 'blind', i.e. what steps were taken to ensure that images were not selected to show what the authors wanted to see?</u>

All of the electron microscopic pictures were taken blindly by an experienced technician in our laboratory, who was not familiar with and shielded from the projects in the study. She took the images of almost all of clathrin-coated vesicles available in each field without arbitrarily excluding any of them. We then independently analyzed all of the images she took to get our quantitative data. Therefore, we believe that our analysis is reliable without any arbitrary interference. In the revised manuscript, we incorporated these information in the Method section of Expanded Materials and Methods.

<u>4. The authors show that TfnR and E-cadherin uptake occurs at "central" sites whilst EGFR and b1-integrin uptake occurs at "peripheral" sites (Figure 4A). Does the localisation of Girdin correlate</u>

with the sites where TfnR and E-cadherin uptake occurs? This would be expected given the apparent requirement for Girdin in TfnR and E-cadherin uptake.

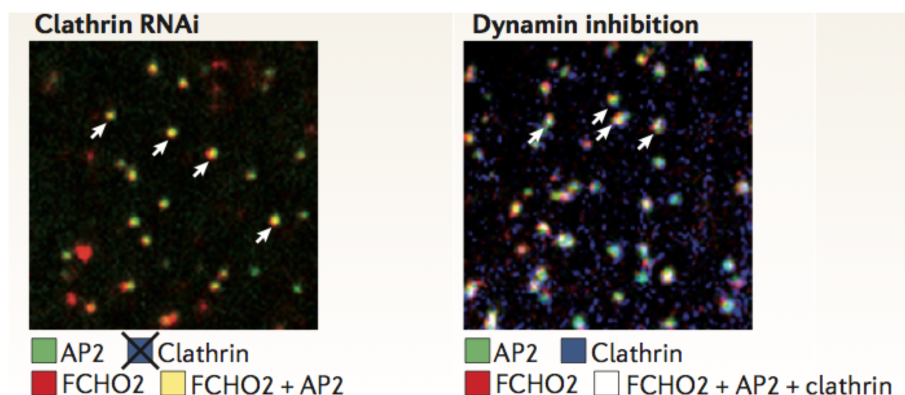
We appreciate the reviewer's comment that gets to the point in our paper. Following the reviewer's suggestion, we examined the localization of girdin and endocytic sites for the four cargoes which we have focused on in this study by TIRF microscope. As shown in Expanded Fig. E2, A-D, girdin partially colocalized with endocytic sites of all of the cargoes, which seemed not to contradict our biochemical experiments and hypothesis by the following reasons. First, out biochemical experiments have shown that girdin physically interacted with EGFR and integrin β1 in peripheral clathrin-coated pits (CCPs) (Fig. 4, B, D-G), which competitively inhibits the interaction between girdin and dynamin 2 to prevent girdin from executing its GAP function. Second, girdin also located in the center CCPs with Tf and E-cadherin, where girdin interacted with dynamin 2 to regulate Tf and E-cadherin endocytosis. Together with our previous biochemical data, the data suggested that colocalization per se is not sufficient to determine the selectivity for the cargoes. We incorporated these findings into the revised manuscript (page 9, lines 22-25).

In the revised manuscript, we also incorporated additional TIRF image that showed the colocalization of endogenous girdin with CCPs (Expanded Fig. E3D).

5. What happens to Girdin localisation after a knockdown of clathrin or Dynamin 2?

Following the reviewer's suggestion, we compared the localization of endogenous girdin in control and clathrin- or dynamin 2-depleted cells (Expanded Fig. E4B in the revised manuscript). We found that the colocalization between girdin and AP-2 was not affected by the knockdown of clathrin or dynamin 2.

It is of note that previous studies have shown that the knockdown of clathrin or dynamin inhibits clathrin-mediated endocytosis (CME) without affecting the recruitment of AP-2 to the CCPs, which indicates that different CCPs components are recruited there and regulated at distinct stages (McMahon and Boucrot, Nat. Rev. Mol. Cell Biol. 12: 517-533, 2011) as indicated below. Consistent with this notion was our observation that girdin localization was not affected by clathrin and dynamin 2 knockdown (Expanded Fig. E4B), which further confirmed the role of girdin in the early stage of CME. We incorporated these findings into the revised manuscript (page 11, lines 14-21).



6. The authors show that Girdin can co-IP with the EGFR and, to a lesser extent, b1-integrin (Figure 4B) but apparently not with E-cadherin or the TfnR. This seems to be at odds with the proposed role for Girdin in mediating the uptake of TfnR and E-cadherin - why the discrepancy? How can the authors explain this? Does a knockdown of clathrin or Dyamin 2 affect the ability of Girdin to co-IP with these cargo proteins? Overall the data pertaining to the role of Gridin in cargo selection seems like it has been over interpreted.

We appreciate the reviewer's comment and apologize for the confusion on our hypothesis. We would like to argue, however, that selective interaction of girdin for cargoes is a critical determinant of whether girdin is able to regulate dynamin activity to pinch off the membrane; i.e., in TfR/E-cadherin-positive vesicles, girdin, which is "not" interacting with TfR nor E-cadherin, is vital for the regulation of dynamin function. In integrin/EGFR-positive vesicles, girdin interacts with these cargoes, which makes girdin unable to regulate dynamin function (Fig. 7). We also noted that the

precise mechanisms for this hypothesis remain to be proven, which we believe is a subject of further studies. In the revised manuscript, taking into account the reviewer's criticism that the role of girdin in cargo selection has been over interpreted, we described the limitations of our hypothesis as follows:

"However, the generality of our hypothesis in other cargo-selective endocytosis remains to be proven, which should await further studies." (page 14, lanes 7-8).

The reviewer also suggested us to perform additional knockdown experiments. Following the reviewer's comment, we compared the interaction between girdin and the cargo proteins in control and clathrin or dynamin 2 knockdown cells by IP experiments (Expanded Fig. E2E in the revised manuscript). The data showed no apparent effects of clathrin/dynamin knockdown on the interaction between girdin and either integrin β1 or EGFR, which we believe is reasonable considering the following:

1) Girdin could regulate endocytosis at two stages: a) to control CCPs formation at early nucleation or cargo selection stages (Fig. 5, C-G), and b) to regulate dynamin GTPase activity at scission stage (Fig. 3D).
2) Cargoes are recruited to CCPs at cargo selection stage, in which girdin is beforehand located in the CCPs (Fig. 5F and Expanded Fig. E4A).

All these data considered, we argue that it is reasonable that the knockdown of clathrin (that is involved in the coating stage) or dynamin 2 (that is involved in the scission stage) has no effect on the interaction between girdin and cargoes. We incorporated these findings into the revised manuscript (page 10, lines 10-13).

7. Studies of clathrin-coated vesicles (CCVs) using mass spectrometry have been reported fairly recently and have shown how loss of specific proteins can affect the cargo composition of CCVs (see PMID: 17116749 and 22472443). Could the authors perform a similar analysis for Girdin?, i.e. isolate CCVs after Girdin knockdown and then analyse for cargo proteins using mass spectrometry - this would help strengthen the conclusion that Girdin regulates cargo selective endocytosis.

We appreciate the reviewer's suggestive comment. We carefully looked into these two papers. The method used in these papers can be applied to analyze the difference in the constituents of CCPs between control and girdin knockdown cells, which would be helpful to address the mechanism how girdin regulates heterogeneous CCPs formation. Unfortunately, however, those technologies and tools are not readily accessible to us, and, as the Editor suggested, it seems to be out of the scope of this manuscript for us to embark on the experiments at this time. In the revised manuscript, we cited and referred to these two papers in the Discussion section (page 15, lines 22-26).

8. Has the requirement for Girdin in endocytosis been reported through the use of genome-wide siRNA screens, e.g. PMID: 20190736 and 23263279? If not, can the authors provide an explanation as to why?

Following the reviewer's suggestion, we looked into these two papers and another paper (PMID: 17704769) that comprehensively identified regulators for CME. Unfortunately, we could not find girdin in the hit list in these three papers. It is often pointed out, however, that those genome-wide screens depend on cell lines used and do not necessarily identify all of the genes that are involved in the cellular processes of our interests due to low knockdown efficiency and false positive hits. We would like to note that our current study originated from the data that girdin was identified as one of dynamin-binding proteins in a screen using affinity column chromatography, in which dynamin was used as a bait (Shimpson et al., 2005), providing the conceptual rationale for expecting girdin to be involved in endocytosis.

Although girdin was not identified in those screening studies, one of those studies (PMID: 17704769) has identified cell polarity proteins such as Par-6/Cdc42 as regulators of endocytosis. Our previous work reported that girdin interacts with Par-3/Par-6/atypical Protein Kinase C complex, which is required for the determination of cell polarity during cell migration (Ohara et al., PLoS One, 7:e36681, 2012). We speculate that girdin might be necessary for the convergence of both cell polarization and endocytosis. In the revised manuscript, we cited and referred to these three papers in the Discussion section (page 16, lines 7-14).

Response to Reviewer #2:

One strength of the manuscript is in mapping the domains of girdin that bind to dynamin and its GAP activity. In addition, the role of girdin in cargo selective uptake is clear, as is the mechanism by which this occurs. Less clear is the spatial regulation of clathrin coated pit (CCP) formation. While it is clear that this occurs from multiple experiments, the reason why is simply not clear at this point. This sentiment is echoed by the authors, as they admit "the mechanism by which girdin spatially controls CCP formation in the center of the cells but not the periphery is unknown". I think the manuscript would be strengthened if less emphasis were placed on those findings, so as not to distract from girdins role as a GAP and in cargo selective uptake.

We appreciate the reviewer's comment. Although it was interesting for us to find that the knockdown of girdin led to the defect in CCPs formation in the center of cells, the mechanism is still mysterious. Although Reviewer #1 also mentioned this point and gave us some suggestions for additional proteomic experiments, we unfortunately could not address the issue at this time (please refer to our responses to Reviewer #1, comment 7). Nonetheless, at present, we believe that this is the first protein that was identified to regulate heterogeneous formation of CCPs, which strengthens our manuscript in the research field.

In the revised manuscript, we took into account the reviewer's thoughtful comment and toned down or deleted some description in the original manuscript as follows, so as not to interfere with the construction of our paper:

1) We deleted the phrase "by defining heterogeneous groups of CCPs distributed" from the following sentence in the Result section of the original manuscript (page 12, lines 14-16, in the revised manuscript).

"Thus, these results indicate that, besides competitive mechanism involving dynamin, girdin also regulates spatially controlled endocytosis by defining heterogeneous groups of CCPs distributed in distinct regions of cells, which must be distinguished from its function as a GAP for dynamin 2.

2) We toned down the discussion on the significance of spatial control of CCPs formation by girdin as follows (page 15, lines 15-17, in the revised manuscript):

In our study, we showed that girdin might be involved in the determination of the functional heterogeneity of CCPs.

Minor problems

1. Figure 5G. The co-immunoprecipitation of girdin with adaptin-a is not strong data. The concern is that IgG also pulled down the same proteins. Could this experiment be designed such that it is more convincing?

We appreciate the comment. Following reviewer's suggestion, we repeated the experiment by adding new twists such as preclear procedure to conventional IP protocol. HeLa cell lysate was incubated with protein G beads for 1 hour to remove some non-specific protein G beads-binding proteins, and then performed the co-IP experiment. In addition, we tested a reciprocal IP experiment in which we used anti-α-adaptin antibody to perform co-IP experiment to confirm the endogenous interaction between girdin and AP-2. The new data were presented in Fig. 5G in the revised manuscript. We also confirmed the interaction between AP-2 and exogenous expressing girdin, which was shown below. Although we could still observe some non-specific bands in the control lanes, the difference between control (IgG) and test (anti-girdin or anti-α-adaptin) groups was significant. We hope that the results could meet the reviewer's requirement.

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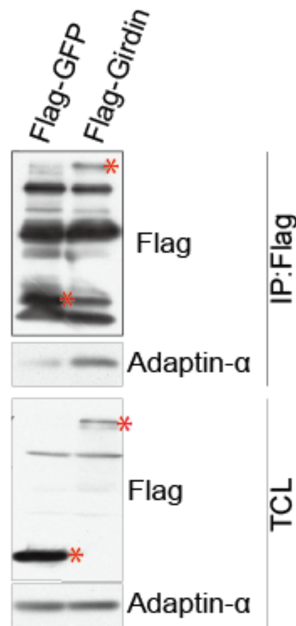

Flag-GFP or Flag-Girdin was transfected into COS7 cells. The cell lysate were incubated with anti-Flag beads (Sigma), followed by Western blot analysis. Flag-fusion proteins are indicated by red asterisks.

2nd Editorial Decision 23 June 2014

Thank you for the submission of your revised manuscript to The EMBO Journal and please accept my apologies for the delay in responding. Thank you for your patience.

Your study was sent back to referees #1 and #2, who now believe that all major concerns have been properly addressed and your manuscript is almost ready for publication (see below). Only a few minor issues will still require your attention -mostly clarifications and further discussion of certain aspects of your data as pointed out by referee #2- but no further experimental evidence is required.

Once these minor issues have been solved, I will be glad to accept your manuscript for publication in The EMBO Journal.

Every paper now includes a 'Synopsis', displayed on the html and freely accessible to all readers. The synopsis includes an image, normally provided as a model/summary figure by the authors or cropped by us from one of the final figures of the manuscript, as well as 2-5 one-short-sentence bullet points that summarize the article. I would appreciate if you could provide these bullet points. If you are also interested in providing a small figure for the synopsis, please keep in mind that final dimensions should be 550 pixels wide X 150-400 pixels (variable) tall.

I would also like to mention that we now encourage the publication of source data, particularly for electrophoretic gels and blots but also for numerical data in graphs, with the aim of making primary data more accessible and transparent to the reader. Although optional at the moment, would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. Raw numerical data for graphs can be provided as Excel (or related) tables. The files will be published online with the article as supplementary "Source Data" files.

If you have any questions regarding this initiative or any other part of the publication process, please let me know.

Thank you very much again for your patience. I am looking forward to seeing the final version of

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your manuscript.

REFEREE REPORTS

Referee #1:

The authors revisions have adequately addressed the concerns I expressed in the initial review and I am now able to recommend the article be published.

Referee #2:

The revised manuscript by Weng et al. addresses many of the reviewer's concerns. Some minor editorial changes would strengthen the manuscript.

The interaction between girdin and dynamin 2 is clear form the in vitro pull down (Figure 1G and H) and the dose dependent measurement of recombinant dynamin 2 GTPase activity in response to varying concentrations of the NT domain of girdin.

However, the guanine nucleotide dependence of girdin and dynamin 2 interaction is still weak. Further, in Figure 2B the effect of girdin knockdown on Tfn uptake seems much greater in the fluorescence-based assay (figure 2B) than in the ELISA assay (Figure 2C). There should be a brief discussion of these differences.

In Figure 4F and 4G, the molar concentration of the EGFR and integrin b1 receptor have an 8-fold difference in concentration. The reason for these differences should be noted in the manuscript.

It is not clear how the Girdin siRNA transfected cells were identified.

It is not clear how many experiments were performed for Figure 5H.

2nd Revision - authors' response 28 June 2014

Response to Reviewer #2:

We are grateful to the reviewer for useful suggestions that have helped us improve our paper. As indicated in our responses, we have taken all of the comments and suggestions into account in the revised version of our paper.

1. The interaction between girdin and dynamin 2 is clear form the in vitro pull down (Figure 1G and H) and the dose dependent measurement of recombinant dynamin 2 GTPase activity in response to varying concentrations of the NT domain of girdin.
However, the guanine nucleotide dependence of girdin and dynamin 2 interaction is still weak. Further, in Figure 2B the effect of girdin knockdown on Tfn uptake seems much greater in the fluorescence-based assay (figure 2B) than in the ELISA assay (Figure 2C). There should be a brief discussion of these differences.

We appreciate the reviewer’s comment. We repeated that experiment several times, which consistently detected an enhanced girdin/dynamin 2 interaction in the lysate from cells loaded with GTPγS, compared to that loaded with GDP (Figure 1A). Therefore, we are confident that the interaction between girdin and dynamin 2 is regulated by nucleotide loading.

The second comment also gets to the point. We also found that the effect of girdin knockdown on Tfn uptake seems greater in the fluorescence-based assay than the ELISA assay. According to our repeated experiments, about 75% HeLa cells transfected with girdin siRNA showed Tfn uptake deficiency (Figure 2B, and E1A, B). In ELISA assay, Tfn uptake decreased by about 30%-50% in girdin knockdown cells. Although speculative, we think that the difference could be attributed to the

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different sensitivity to detect Tfn between these assays. We included this in result section (Page 7, line 10-12).

"The effect of girdin knockdown on Tf uptake was greater in the flurescence-base assay than the ELISA assay (Fig. 2, B, C) which could be due to the different sensitivity between these assays."

<u>2. In Figure 4F and 4G, the molar concentration of the EGFR and integrin b1 receptor have an 8-fold difference in concentration. The reason for these differences should be noted in the manuscript.</u>

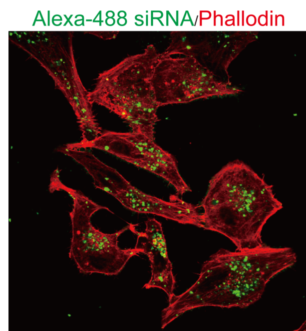
We appreciate the comment. We speculate that the difference in the amount of recombinant proteins required to competitively disrupt girdin/dynamin 2 interaction indicated the difference in the stoichiometry of the reaction between girdin and the cargoes (EGFR and integrin beta 1). We included this in discussion section (Page 16, line 20-23).

"We note that more recombinant integrin β1 is required for competitively disrupting the dynamin 2/girdin interaction compared with EGFR (Fig. 4, F, G), which may reflect the difference in the stoichiometry and affinity between girdin and these cargoes."

<u>3. It is not clear how the Girdin siRNA transfected cells were identified.</u>

In our experience, the transfection efficiency of siRNA is more than 95% in HeLa cells. In response to the reviewer's criticism, we confirmed this by transfecting Alexa 488-conjugated control siRNA into HeLa cells. One day after the transfection, the cells were investigated under confocal microscopy (the picture is shown below), which showed that almost every cell was transfected with the siRNA, which warrants the credibility of our analysis. In addition, in our some experiments, we co-transfected siRNA and plasmids, where GFP or GFP fused plasmid was used as a fill (a transfection marker) to visualize siRNA-transfected cells (See Figure E1A).

Alexa-488 siRNA/Phallodin



<u>4. It is not clear how many experiments were performed for Figure 5H.</u>

The aim of Figure 5H is to show that Tfn endocytosis was inhibited in girdin knockdown cells due to the loss of central CCPs, while the EGF endocytosis was not affected. We repeated this experiment 3 times and then confirmed this finding, the quantification of which has been shown in Figure E5, A and B. We mentioned this in Material and methods section (Page 23, line 9-10)

"All of the experiments were repeated at least 3 times."