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ACBD3-mediated recruitment of PI4KB to picornavirus RNA replication sites

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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 04 July 2011

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers express interest in your work and are broadly in favour of publication - pending satisfactory revision. To highlight the major additional experiments that need to be done:

- Define which of the viral proteins is directly interacting with ACBD3 (refs 2 and 3): this could probably best be done by in vitro GST pulldown experiments.
- Provide better evidence for your claim that the viral protein competes with giantin for binding to ACBD3 (ref 2).
- Demonstrate that PI4P accumulates at replication complexes (refs 1 and 3).

I also have one additional point that I recognise was not explicitly raised by any of the referees, but that is related to those issues highlighted above. Your data support, but do not directly show that ACBD3 recruits PI4KB to the Golgi or replication complex. I think the paper would be significantly strengthened if you could directly demonstrate that PI4KB localisation is disrupted upon ACBD3 knock-down.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. 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, please visit our website:

http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1:

Sasaki and colleagues report on a very intriguing finding of Aichi viruses, which are the causative agents of gastroentiritic outbreaks across the globe in recent times, hijacking Type III Pi4K beta in order to support viral RNA synthesis. Previously another group had shown that enteroviruses could utilize PI4Kbeta enzymes to generate PI4P lipid enriched replication organelles but how prevalent this requirement for PI4Kb was not clear: was it limited to just enteroviruses and perhaps HCV?

Here Sasaki colleagues beautifully demonstrate that Aichi viruses through a novel pathway hijack PI4Kb enzymes to facilitate their replication. Specifically they show that the Aichi non-structural enzymes target another host protein, ACBD3, and essentially use it as 'bait" to capture PI4KB. Normally the ACBD3 protein interacts with giantin, but the authors find that the Aichi non structural proteins can displace this interaction and in turn enhance ACBD3's interaction with Pi4Kb. They also find that in uninfected cells there is potentially an interaction between Pi4Kb and ACBD3- which is enhanced in infected cells.It would be informative if the authors could demonstrate the presence of PI4P lipids at the replication sites for Aichi virus.

In summary the authors findings will stimulate the design and development of drugs against Pi4Kbeta enzymes as PANVIRAL targets to combat a multitude of viral infections.
The authors findings are relevant to both the fields of virology/human infectious diseases and cell biology in general. ACBD3, a Golgi protein, is critical in regulating assymetric cells division and development (Zhou et al., Cell 2007) and Sasaki et al., finding an interaction of this protein with PI4Kb will no doubt spur interest in the wider cell biology community to look at the role of Phosphoinositide lipids in assymetric cell division.

Referee #2:

This manuscript sought to identify host factors required for Aichi virus (AiV) replication. The host protein ACBD3 was determined to interact with AiV proteins, both in a yeast-two hybrid screen and by coimmunoprecipitation. Furthermore, ACBD3 was shown to colocalize with 2B, 2C, 3A and dsRNA in an immunofluorescence assay indicating that it is most likely a component of the AiV replication complex. ACBD3 interaction with the virus was seen to be important for viral replication in an ACBD3 knockdown experiment and ACBD3 interactions with other host proteins was altered following AiV infection, both indicate a role for ACBD3 in the AiV lifecycle. Since AiV is a member of the Picornaviridae family other cellular factors known to be important in enterovirus infections, namely PI4KB, was also investigated in this manuscript. Not surprisingly, the authors found that PI4KB is also required for AiV replication. Interestingly, however, the PI4KB pathway that is required in poliovirus infection (GBF1-dependent, BFA sensitive) is not the same pathway affected in AiV replication (which was shown to be BFA insensitive). The authors went on to

demonstrate with a yeast-two hybrid screen and by coimmunoprecipitation that PI4KB and ACBD3 interact, a novel finding. Lastly, similar to ACBD3, PI4KB was shown to colocalize with 2B, 2C, 3A and dsRNA in an immunofluorescence assay indicating that it is most likely a component of the AiV replication complex. This paper is well-written and clear. The authors describe a novel host protein- viral protein interaction (ACBD3) and go onto illustrate a novel PI4KB recruitment strategy.
Specific Comments-
1) In a viral system, having proteins with redundant functions is unusual. Although the yeast-two hybrid and coimmunoprecipitation data is convincing (Fig 1), I would argue that only one viral protein is directly interacting with ACBD3 and that the remaining proteins are not directly interacting but rather are part of a complex. If the authors were able to identify which protein (2B, 2BC, 2C, 3A, or 3AB) is directly interacting with ACBD3 it would make this an even stronger finding.
2) Almost all the immunofluorescent images are from 4hpi, where there is definite puncta staining that may be indicative of replication complexes. However, the one image shown at a later timepoint (6hpi, in Fig 3A) puncta staining of both ACBD3 and viral proteins is gone and staining is greatly dispersed, which doesn't support the conclusion that we are observing replication complexes. Interestingly, in the siRNA knockdown experiment, no difference in luciferase activity is seen at 4hpi. The authors should show a timecourse (4-10hpi) of staining with viral proteins to illustrate whether replication complexes can be seen and additionally if ACBD3 plays a role in maintaining the complex or simply helps recruit components of the complex at early timepoints post infection.
3) The conclusion on p10, line1-3 is a reach considering the data presented. Although the ACBD3 interactions with giantin, GM130, and TGN46 are disrupted following infection, the claim that viral protein interaction is "substituting" the host protein interaction cannot be concluded. In order to make this conclusion the authors need to show a competition assay with AiV proteins and giantin, GM130, and TGN46.

Referee #3:

The manuscript by Sasaki et al. identifies phosphatidylinositol 4-kinase IIIb (PI4KB) as an important host factor required for replication of a picornavirus Aichi virus, and provides evidence for a mechanistic model of recruitment of this protein to the viral replication sites. PI4KB has been shown before to participate in replication of other picornaviruses, polio and Coxsackie B3 (CVB3), but Aichi virus apparently uses quite different mechanism of hijacking PI4KB. While polio and CVB3 rely on recruitment of PIK4B through GBF1/Arf1 pathway, some non-structural proteins of Aichi virus interact with another cellular protein, ACBD3, which in turn binds PIK4B and attracts it to the viral replication complexes. Growing evidence support an important role of poshpoinositol 4 phosphate lipids (PI4P) in the formation/functioning of membranous replication complexes of diverse positive strand RNA viruses, and the paper of Sasaki et al. provides a significant contribution into our understanding of the variety of mechanisms employed by different viruses to secure presence of PI4P-generating enzymes on replication complexes. Thus to my opinion the paper is of great interest for a broad virology community as well as for the cell biology researchers. There are a few questions though that need to be clarified before the conclusions of the study are fully justified.

Figs. 1 and 2. The main concern with the data described in these Figures is that many different viral non-structural proteins were identified to interact with the same domain of ACBD3. In yeast two-hybrid screen the authors observed interaction only between 3A and ACBD3, while in co-immunoprecipitation experiments and in mammalian two-hybrid system apparently all viral proteins with membrane binding sequences showed positive interaction, which raises the question of specificity of the assays employed.

The expression data for the constructs used in mammalian two-hybrid system experiments should be provided to support the claim that positive results indeed reflect true interactions and exclude the possibility that negative results were obtained due to low expression/stability of the corresponding fusion proteins.

For co-immunoprecipitation experiments a negative control with a Golgi-targeted membrane-binding protein is required to show that the assay distinguishes between specific interactions and

general pull-down of membrane-associated proteins.

The claim that a very broad range of viral proteins targets one domain of ACBD3 is quite striking and deserves stronger experimental evidence.

Fig.4. A western blot showing giantin during the time course of viral infection is required. The authors proposed a model that viral proteins outcompete giantin for binding of ABCD3, while it could be that giantin is degraded in infected cells. The immunoflourescence images provided are consistent with both models.

Fig.5 While siRNA knock-down of PI4KB data convincingly supports the importance of this protein in replication of Aichi virus, the paper would strongly benefit if it addresses the question if PI4KB activity is actually necessary and thus if Aichi virus shares the requirement for the accumulation of PI4P on replication complexes with other positive strand RNA viruses. An experiment with expression of a PI4P sensor protein such as FAPP1PH-GFP in Aichi virus-infected cells is warranted.

General comment: Firefly luciferase activity data for replicon growth curves should be presented in a unified format, as non-normalized RLU. It is confusing when for example on Fig. 5 three out of four similar graphs show RLU and one normalized activity.

Minor comments:
Fig 1 legend: lines 8-10 are not clear.
Fig. 6C. Schemes for all mutants used should be included. It is very inconvenient for a reader to have to go back to Fig. 2 for most of them.

1st Revision - authors' response 27 September 2011

Answers to the Editor:

- Define which of the viral proteins is directly interacting with ACBD3 (refs 2 and 3): this could probably best be done by in vitro GST pulldown experiments.

We have carried out an in vitro MBP pulldown experiment and have indicated that 2B, 2C, 3A and 3AB can interact with ACBD directly (Figure 1C).

Modifications have been made at page 6, line 20-page 7, line 3; page 20, lines 1-5, 15-16; page 22, line 20-page 23 line 13; page 30, lines 10-12 (one reference has been added).

- Provide better evidence for your claim that the viral protein competes with giantin for binding to ACBD3 (ref 2).

Referee #2 pointed out that this conclusion is a reach considering the data presented (specific comment-3). We agree with this comment. We have deleted this conclusion from the Result section (page 10). We have discussed this possibility as a model for recruitment of PI4KB to the viral RNA replication sites in the Discussion section (page 16).

- Demonstrate that PI4P accumulates at replication complexes (refs 1 and 3).

We have immunostained PI4P in viral RNA-replicating cells, and have demonstrated that PI4P colocalized with the viral proteins, ACBD3, PI4KB, and dsRNA (Figure 8).

Modifications have been made at page 2, lines 11-12, 15; page 5, lines 19, 20; page 13, lines 15-20; page 14, lines 8 and 10; page 20 lines 19-20; page 23, line 21. At page 15, line 4, words “whether PI4P is abundant in the AiV RNA replication sites and” described in the original manuscript have been deleted.

-I also have one additional point that I recognise was not explicitly raised by any of the referees, but that is related to those issues highlighted above. Your data support, but do not directly show that ACBD3 recruits PI4KB to the Golgi or replication complex. I think the paper would be significantly strengthened if you could directly demonstrate that PI4KB localisation is disrupted upon ACBD3 knock-down.

We have carried out this experiment, and the result has been shown in Figure 7B. Modifications have been made at page 13, lines 3-6; page 15, lines 7-9.

Answer to Referee #1:

- It would be informative if the authors could demonstrate the presence of PI4P lipids at the replication sites for Aichi virus.

We have demonstrated this, and the result has been shown in Figure 8.

Answers to Referee #2:

Specific Comments-
1) In a viral system, having proteins with redundant functions is unusual. Although the yeast-two hybrid and coimmunoprecipitation data is convincing (Fig 1), I would argue that only one viral protein is directly interacting with ACBD3 and that the remaining proteins are not directly interacting but rather are part of a complex. If the authors were able to identify which protein (2B, 2BC, 2C, 3A, or 3AB) is directly interacting with ACBD3 it would make this an even stronger finding.

We have performed an in vitro MBP pulldown assay, and have indicated that 2B, 2C, 3A and 3AB can interact with ACBD3 directly (Figure 1C).

2) Almost all the immunofluorescent images are from 4hpi, where there is definite puncta staining that may be indicative of replication complexes. However, the one image shown at a later timepoint (6hpi, in Fig 3A) puncta staining of both ACBD3 and viral proteins is gone and staining is greatly dispersed, which doesn't support the conclusion that we are observing replication complexes. Interestingly, in the siRNA knockdown experiment, no difference in luciferase activity is seen at 4hpi. The authors should show a timecourse (4-10hpi) of staining with viral proteins to illustrate whether replication complexes can be seen and additionally if ACBD3 plays a role in maintaining the complex or simply helps recruit components of the complex at early timepoints post infection.

In this study, we used two transfection methods: electroporation and lipofection. The transfection method used for each experiment has been stated in the manuscript. Viral RNA replication starts more rapidly in the case of electroporation (Figures 5A and C) than lipofection (Figures 3C and 5B). For all the immunofluorescent images shown in Figure 3, we used cells transfected by electroporation. On the other hand, lipofection was used to transfect cells treated with siRNA. At 6 h after electroporation, viral RNA replication is almost completed and an excess amount of viral proteins had been produced. In the image pointed out by the referee #2 (6hpi, in Fig 3A), excess viral proteins are present other than the RNA replication sites. At 4 h after electroporation, viral RNA replicates actively, and this time point is suitable for observation of subcellular localization of viral proteins and viral RNA. In order to show this, we presented only one image obtained at 6 h after transfection.

3) The conclusion on p10, line1-3 is a reach considering the data presented. Although the ACBD3 interactions with giantin, GM130, and TGN46 are disrupted following infection, the claim that viral protein interaction is "substituting" the host protein interaction cannot be concluded. In order to make this conclusion the authors need to show a competition assay with AiV proteins and giantin, GM130, and TGN46.

We agree with this comment. We have deleted this conclusion from the Result section (page 10), and have discussed this possibility as a model for recruitment of PI4KB to the viral RNA replication sites in the Discussion section (A sentence has been added at page 16, lines 9-10).

Answers to Referee #3:

- Figs. 1 and 2. The main concern with the data described in these Figures is that many different viral non-structural proteins were identified to interact with the same domain of ACBD3. In yeast two-hybrid screen the authors observed interaction only between 3A and ACBD3, while in co-immunoprecipitation experiments and in mammalian two-hybrid system apparently all viral proteins with membrane binding sequences showed positive interaction, which raises the question of specificity of the assays employed.

We have performed an in vitro MBP pulldown experiment, and have indicated that 2B, 2C, 3A and 3AB can interact with ACBD3 directly (Figure 1C).

- The expression data for the constructs used in mammalian two-hybrid system experiments should be provided to support the claim that positive results indeed reflect true interactions and exclude the possibility that negative results were obtained due to low expression/stability of the corresponding fusion proteins.

Expression data for viral fusion proteins was previously shown (Ishikawa et al., 2010). We examined expression of ACBD3 and mutant fusion proteins, and confirmed expression of the mutant fusion proteins that exhibited no increase in luciferase activity (data not shown in the manuscript, Figure R1). This has been described in Materials and Methods (page 21, lines 17-21).

- For co-immunoprecipitation experiments a negative control with a Golgi-targeted membrane-binding protein is required to show that the assay distinguishes between specific interactions and general pull-down of membrane-associated proteins.

ACBD3 contains no hydrophobic region, and is recruited to the Golgi through interaction with giantin that contains a membrane-anchoring domain (Sohda et al., 1994, 2001). In this point, we think that PI4KB, which is recruited by other proteins to the Golgi, can be utilized as a control. We have shown an immunoprecipitation data demonstrating that 3A is not coimmunoprecipitated with PI4KB (Figure 6B). In addition, although the data is not shown, we have obtained similar results for 2B and 2C.

- The claim that a very broad range of viral proteins targets one domain of ACBD3 is quite striking and deserves stronger experimental evidence.

We performed an MBP pulldown experiment using ACBD3mut3 and mut4, like that shown in Figure 1C. We obtained the result that mut3, but not mut4, was pulled down with MBP-2C, -3A, and -3AB (Figure R2). This result is consistent with the mammalian two-hybrid data. We did not succeed to detect pulldown of mut3 with MBP-2B. We think, this is because that the ability of 2B to bind to ACBD3 tends to be lower than that of 2C, 3A, and 3AB in this in vitro pulldown experiment as shown in Figure 1C. Thus, we think that the data obtained in the mammalian two-hybrid is reliable.

- Fig.4. A western blot showing giantin during the time course of viral infection is required. The authors proposed a model that viral proteins outcompete giantin for binding of ABCD3, while it could be that giantin is degraded in infected cells. The immunoflourescence images provided are consistent with both models.

We performed a western blotting analysis to detect giantin in cells harvested at 0, 2, 4, and 6 h after electroporation with AiV replicon RNA, and observed no degradation of giantin: no decrease in the amount of giantin detected was observed from 0 to 6 h after electroporation (data not shown in the manuscript; Figure R3). This has been stated at page 9, line 21- page 10, line 2.

- Fig.5 While siRNA knock-down of PI4KB data convincingly supports the importance of this protein in replication of Aichi virus, the paper would strongly benefit if it addresses the question if PI4KB activity is actually necessary and thus if Aichi virus shares the requirement for the accumulation of PI4P on replication complexes with other positive strand RNA viruses. An experiment with expression of a PI4P sensor protein such as FAPP1PH-GFP in Aichi virus-infected cells is warranted.

A PI4KB-specific inhibitor T-00127HEV1 (Arita et al., 2011) inhibited AiV replication (Figure 5A). This strongly suggests that PI4KB activity is actually necessary to AiV replication. In addition, we have shown by immunofluorescence staining that PI4P is present in the viral RNA replication sites (Figure 8).

General comment: Firefly luciferase activity data for replicon growth curves should be presented in a unified format, as non-normalized RLU. It is confusing when for example on Fig. 5 three out of four similar graphs show RLU and one normalized activity.

For transfection of cells with viral replicon RNA, we used two methods: electroporation and lipofection. Electroporation uses 10^6-10^7 cells. On the other hand, because of a difficulty of preparation of a large number of siRNA-treated cells, cells treated with siRNA (10^3-10^4 cells) were transfected by lipofection. The data obtained with electroporation have been presented as RLU, while the data obtained with lipofection have been presented as normalized activity.

Minor comments:
- Fig 1 legend: lines 8-10 are not clear.

We have modified the sentence.

- Fig. 6C. Schemes for all mutants used should be included. It is very inconvenient for a reader to have to go back to Fig. 2 for most of them.

We have added schemes for all mutants in Figure 6C.

FigureR1

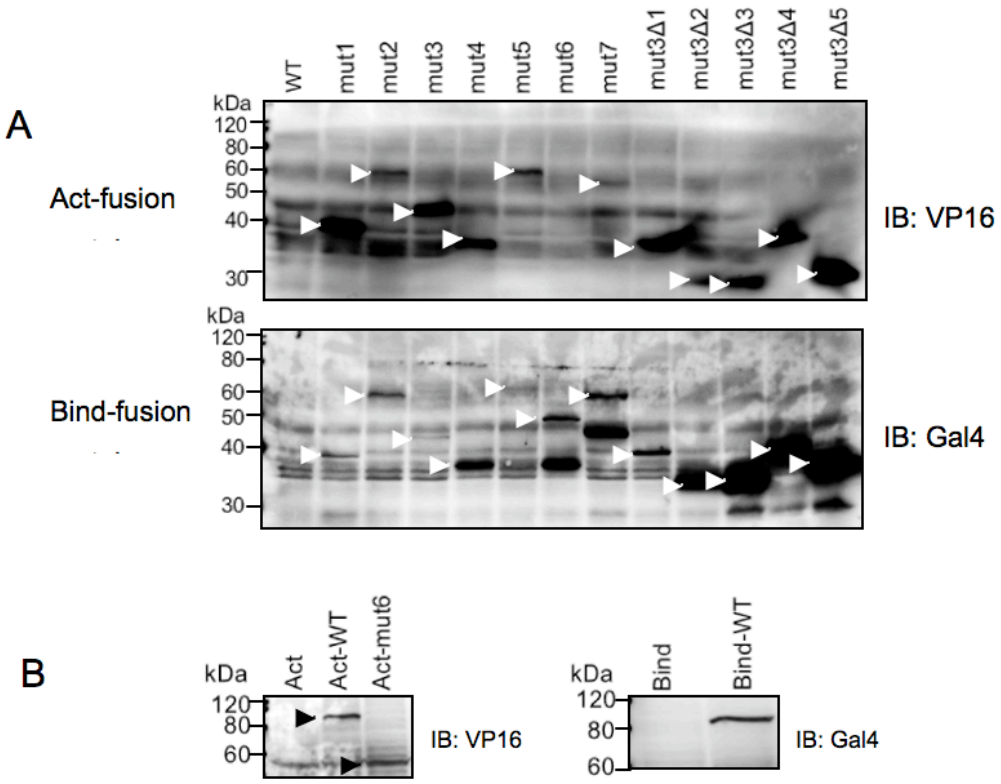

Figure R1 Expression data of the constructs used for the mammalian two-hybrid experiments. (A) Plasmids were transfected into Vero cells using lipofectamine and plus reagents (Invitrogen) (Ishikawa et al., 2010), and at 48 h after transfection, cell lysates were subjected to immunoblotting with anti-VP16 or anti-Gal4 antibody. Expression of ACT-ACBD3(wt), ACT-mut6, and BIND-ACBD3(wt) was not detected in this experiment. (B) pACT-ACBD3(wt), pACT-mut6, and pBIND-ACBD3(wt) were transfected to Vero cells using higher concentrations of lipofectamine and plus reagents than in (A). In this condition, the efficiency of transfection is higher than in (A). Expression of ACT-ACBD3(wt), ACT-mut6, and BIND-ACBD3(wt) was deteced. In the mammalian two-hybrid analyses, ACBD3(wt) induced more remarkable increase in luciferase activity than all of the mutants (Figures 2 and 6), and an increase in luciferase activity for mut6 was also observed (Figure 6C). Therefore, we concluded that negative results obtained for some mutants in the mammalian two-hybrid experiments was not due to low expression/stability of the corresponding fusion proteins.

FigureR2

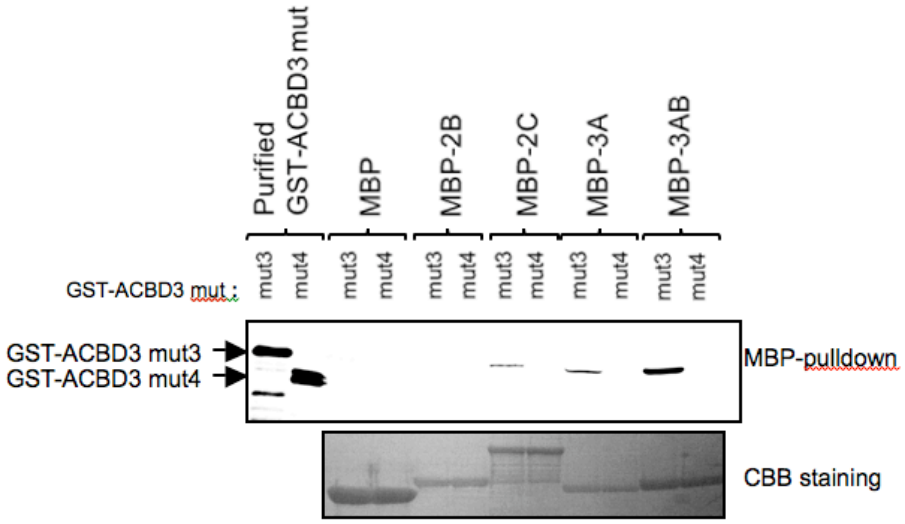

Figure R2 MBP pulldown assay. The experiment was performed as described in Materials and methods with an exception of using GST-ACBD3mut3 or GST-ACBD3mut4 instead of GST-ACBD3. GST-ACBD3mut3 and GST-ACBD3mut4 pulled down with the MBP-fused viral proteins were detected by immunoblotting with anti-GST antibody (upper panel). After immunoblotting, MBP-fused viral proteins on the PVDF membrane were stained with CBB (lower panel).

FigureR3

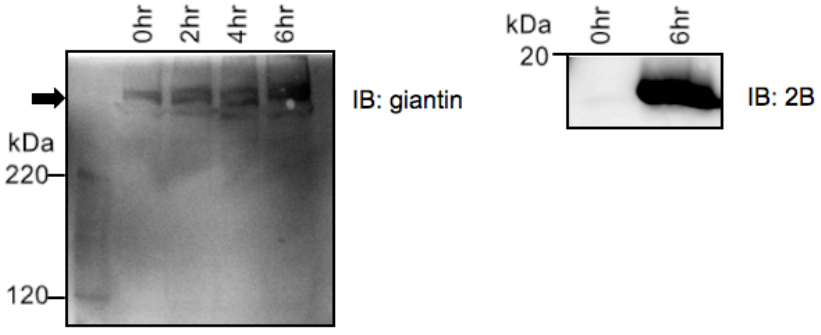

Figure R3 Giantin is not degraded in Aichi virus replicating cells. Vero cells were transfected with the replicon RNA by electroporation, and cell lysates were prepared at 0, 2, 4, and 6 h after transfection, and subjected to immunoblotting with anti-giantin antibody. No decrease in the amount of giantin was observed even at 6 h after electroporation (left panel). Right panel shows the result of immunoblotting with anti-2B antibody to confirm viral RNA replication.

2nd Editorial Decision 11 October 2011

Many thanks for submitting the revised version of your manuscript EMBOJ-2011-78279R. It has now been seen again by the original referees 2 and 3, whose comments are enclosed below. As you will see, both referees find the manuscript to be satisfactorily revised, and are now fully supportive of publication without further revision. I am therefore pleased to tell you that we will be able to accept your manuscript to be published in the EMBO Journal. I do, however, just have a few minor points from the editorial perspective that I need to ask you to deal with first:

- The resolution of some of the immunofluorescence images seems rather low - can you prepare and upload higher resolution figures where possible?

- Some of the blots (particularly figures 1C and 6B) are rather over-contrasted: could you replace these with less highly contrasted versions?

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide the original, uncropped and unprocessed scans of all gels used in the figures (or at least of key data panels)? These should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. Ideally, we would ask for separate files for each figure panel, which you can upload as a single zip file as "source data" - via our online submission system. These will then appear as supplementary files and be directly linked to each relevant main figure. Please let me know if you have any questions about this policy.

- In Figure 5A, I am a bit confused by the SD bars for the 5uM, 5h datapoint: you show an error bar above the data point, but nothing below. I think there must be an error here, so please can you check?
Can you also confirm that the BFA+ and BFA- graphs in 5C lower panel are indeed completely overlapping? I assume this is correct, but would like to be sure!

I look forward to receiving your final revision.
Editor
The EMBO Journal

REFEREE REPORTS

Referee #2:

The authors have made a good effort to address the majority of the previous requests. The observation is interesting and supported by the data. No additional requests are made.

Referee #3:

The authors adequately addressed all the reviewer's concerns and I believe this manuscript should now be accepted for publication.

2nd Revision - authors' response 12 October 2011

- The resolution of some of the immunofluorescence images seems rather low - can you prepare and upload higher resolution figures where possible?

> We have checked the resolution of the images, and uploaded the files again. Figures 7B and 8B (the images for 2C) have been changed.

- Some of the blots (particularly figures 1C and 6B) are rather over-contrasted: could you replace these with less highly contrasted versions?

We have replaced the blot of Figure 1C. On the other hand, the blots in Figure 6B have not been replaced, because these images were hardly processed, as shown in Figure S2, which shows the original data for this figure.

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide the original, uncropped and unprocessed scans of all gels used in the figures (or at least of key data panels)?

We have provided original data for Figures 1C and 6B that you pointed out, in Figures S1 and S2, respectively.

- In Figure 5A, I am a bit confused by the SD bars for the 5uM, 5h datapoint: you show an error bar above the data point, but nothing below. I think there must be an error here, so please can you check?

The error bar below the data point has been really displayed. However, since the standard error extends across from 10^2 to 10^3, the error bar below the data point is very short in logarithmic display.

- Can you also confirm that the BFA+ and BFA- graphs in 5C lower panel are indeed completely overlapping? I assume this is correct, but would like to be sure!

We have confirmed this. The BFA+ and BFA- graphs almost completely overlap.

