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p97 and p47 promote membrane tethering during mitotic Golgi reassembly in cooperation with FTCD

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Thank you for submitting your manuscript entitled "FTCD is a novel tethering factor in p97/p47-mediated Golgi membrane fusion" [EMBOJ-2020- 105853] to The EMBO Journal. Your study has been sent to three reviewers for evaluation, whose reports are enclosed below.

As you can see, the referees concur with us on the general interest of your study. However, they also raise several critical points that need to be addressed before they can support publication here. In particular, referee #1 and #3 request you to test the interaction of FTCD with p57/p97 and with full-length p47 at the endogenous level, as well as to investigate the relationships between FTCD and the other known p47/p97 interactors. In addition, reviewer #3 asks you to further investigate FTCD localization to the Golgi complex (point 3 and 4) and to provide a model of how the FTCD-p97-p47-FTCD complex tethers Golgi membranes (point 9). Also, the referees recommend you to include missing controls and to properly discuss your findings in the light of the existing literature.

Given the overall interest of your study, I am pleased to invite submission of a revised manuscript as indicated in the reports attached herein. I would like to point it out that addressing all referees' points in a conclusive manner, as well as a strong support by the referres, would be essential for publication in The EMBO Journal.

I should also add that it is our policy to allow only a single round of major revision. Therefore, acceptance of your manuscript will depend on the completeness of your responses in this revised version.

We generally grant three months as standard revision time. As we are aware that many laboratories cannot function at full capacity owing to the COVID-19 pandemic, we may relax this deadline. Also, we have decided to apply our 'scooping protection policy' to the time span required for you to fully revise your manuscript and address the experimental issues highlighted herein. Nevertheless, please inform us as soon as a paper with related content published elsewhere.

Before submitting your revision, primary datasets (and computer code, where appropriate) produced in this study need to be deposited in an appropriate public database (see http://msb.embopress.org/authorguide#dataavailability). Please remember to provide a reviewer password if the datasets are not yet public. Include a "Data availability" section even if there are no primary datasets produced in the study.

I thank you for the opportunity to consider this manuscript and look forward to your revision.

Referee #1:

This is an extremely well conceived, organized and executed study mainly based on biochemical approaches

This work presents a very good clean biochemical analysis, one of the best I have seen in a long time. The authors show that the individual components of an FTCD dimer independently bind p47 and p97, forming a complex that is important for tethering Golgi membranes during post-mitotic

reformation. The regulation of the process would be mediated by the release of p47 from the core.

They identified FTCD in a pull-down assay using a recombinant fragment of p47 incubated with liver cytosol. Using recombinant proteins they showed that FTCD interacts with both p47 and p97, while mutagenesis [p97 (V722K, E724K), p47 (E86K, E88K), FTCD (R382A)] then identified the domains important for binding.

EM analysis after siRNA-mediated depletion of FTCD showed a reduction in identifiable Golgi cisternae and an increase in vesicles and tubules. Further, FTCD-KD prevented Golgi reformation during cytokinesis, a phenotype that could be rescued by expression of FTCD-wt but not by mutant R382A, which does not bind p47/p97. These same approaches showed that FTCD is required for the localization of p47 and p97 at the Golgi.
They then showed, using a cisternal regrowth assay, that the WT FTCD, p47, and p97 (but not the mutant forms) can reform cisternae.

Based on these results they hypothesize the formation of a quaternary complex composed of two FTCD units, one of which binds p47 and the other p97, but both using the domain around R382. This hypothesis was supported by sucrose gradient centrifugation and various pull-down assays and by EM (negative staining) of the whole quaternary complex and of various combinations of its components.

Finally, they used aggregation tests with streptavidin beads that bind FTCD and misocalization of FTCD to mitochondria to show that the quaternary complex could act as a tether.

From a general point of view, the paper appears to be well written but there are a few small errors (e.g. "the quantification results" at the end of page 9).

There are some simplifications in the introduction (page 3, line 4; they talk about vesicular transport with review Mellman 1992).
On line 10, in introducing the mitotic fragmentation of the Golgi, reference is made to Zaal's paper dated 1999 according to which part of the Golgi goes into the ER. This hypothesis does not have a general consensus and has been strongly attacked in two papers:
1) Rapid, endoplasmic reticulum-independent diffusion of the mitotic Golgi hazeMagnus A B Axelsson 1, Graham Warren DOI: 10.1091 / mbc.e03-07-0459

2) Golgi enzymes do not cycle through the endoplasmic reticulum during protein secretion or mitosis Julien Villeneuve 1 2, Juan Duran 1 3, Margherita Scarpa 1, Laia Bassaganyas 4 5, Josse Van G DOI: 10.1091 / mbc.E16-08-0560

The only major limit of the manuscript is the lack of demonstration that the endogenous proteins (FTCD and p57/p97) interact: the authors have to validate in cells their pull-down data and show the formation of the complex with endogenous proteins

Another point that should be addressed concerns the relationships between FTCD and the other known interactors of p47/p97: is the FTCD interaction synergic/antagonistic/neutral with that of the other known p47/p97interactors? In addition, in discussing their results, the authors should take into consideration the more general context of the regulatory circuits controlling the p47/p97 complex, such as the role of monoubiquitination (here they only mention it briefly) and the interaction with syntaxin5 (For example, Golgi structure formation, function, and post-translational modifications in mammalian cells. Shijiao Huang, Yanzhuang Wang 1 DOI: 10.12688 /

f1000research.11900.1).
Finally the authors ascribe to the nuclear sequestration of p47 its lack of activity in the Golgi complex in interphase cells. This seems to be an over-simplified wiew as p47 has been shown to play a role out of the nucleus in interphase cells (i.e. dendrite arborization in neurons, PMID: 30783609). It also raises the question as to whether mutations in the NLS of p47 has any impact on its function in the Golgi complex dynamics also in interphase cells.

Specific comments

The authors should provide better description and explanation of the quantitative assessment of the data presented in Figure 7D (Negative staining electron microscopy of the quaternary complex).

Referee #2:

The study by Kaneko et al., details an investigation of the process of Golgi membrane reformation following mitosis and describes the novel finding that the FTCD protein can function as a tethering factor in p97/p47-mediated membrane fusion.
It is now well established that, in higher eukaryotes, the Golgi fragments during mitosis and the many vesicles generated are then distributed between mother and daughter cells before reassembly into the classical stacked Golgi. The role of p97/p47 in this process has been extensively studied and is also well established. One of the remaining questions to be answered is whether tethering of Golgi membranes is necessary prior to membrane fusion and if so, what protein(s) mediate that tethering process.
The study by Kaneko and colleagues reports the identification of FTCD (a Golgi enzyme) as a novel interacting protein for the p97/p47 complex and goes on to show that loss of FTCD leads to Golgi fragmentation. They further demonstrate that FTCD is, most likely, functioning as a tethering factor to facilitate the fusion of post-mitotic Golgi membranes. This could be regarded as a surprising result as many tethering factors are effectors of Rab proteins and are often part of multimeric protein complexes (e.g. HOPS complex or GARP) or conform to a largely coiled-coils based structure (e.g. EEA1 or the Golgins). The FTCD protein does not appear to be regulated by a Rab GTPase and forms octomeric complexes on the membrane.
The data as presented by Kaneko and colleagues is of a very high standard and I have few significant criticisms. Arguably, it could be published without revision but I feel that it could also be strengthened by addressing the following points, mostly by changes to the text:

1. Why does it appear that there is some FTCD in the nucleus? This is most apparent when the HA-tagged FTCD is localized.
2. FTCD does not appear to have a transmembrane domain so what is required to localize the protein to the Golgi? Could it be a rab protein?
3. For the sake of comparison, it would be helpful to show how loss of p47 affects Golgi fragmentation/reassembly in one of the figures.
4. Is there enough FTCD present in a cell to function as a tether for post-mitotic Golgi membranes?

Given that it appears that at least two octomers of FTCD would be required to function as tethers for each pair of Golgi vesicles and that there are "thousands" of Golgi vesicles generated at mitosis, there would have to be many tens of thousands of FTCD present to ensure that the Golgi vesicles can fuse following mitosis. Unless of course, FTCD is necessary for tethering/fusion of just a few hundred vesicles which then create a template for the reassembly of the Golgi complex. To address this question, could the authors perform some immunoEM (or possibly super-resolution light microscopy) on mitotic cells to determine what percentage of Golgi vesicles have FTCD on them?
5. I found some of the micrographs to be a bit small, could they be enlarged within the figures?

Referee #3:

In the manuscript titled, "FTCD is a novel tethering factor in p97/p47-mediated Golgi membrane fusion," Kaneko et al. investigated additional players of p97-p47 machinery that is necessary for SNARE-mediated Golgi reassembly after mitosis. The authors have identified a potential partner protein, FTCD (also known as Golgi 58kDa protein), that can bind to both p97 and p47 via their polyglutamine motifs. Using a commercial monoclonal antibody to FTCD, authors confirmed that FTCD is partially localized to the Golgi. They found that siRNA-mediated depletion of FTCD is detrimental for Golgi reassembly, and mitochondria-targeted FTCD causes artificial aggregation of mitochondria, suggesting that FTCD can tether membranes. Moreover, in vitro studies indicated that FTCD forms a stable complex with p97 and p47. The authors proposed that the FTCD-p97-p47-FTCD complex is a membrane tethering complex that is involved in post-mitotic Golgi reassembly. Although the data is very well presented and mostly backed by proper controls and statistical analysis, some of the critical controls and primary references for FTCD are missing, and authors failed to discuss alternative explanations for their results.
Major comments:
1. In figure 1, a very clean Coomassie staining shows a single band interacting with the N-terminal domain of p47 (1-170). This band was found to be FTCD. While this data is convincing that FTCD can interact with p47, it is essential to repeat the same "fishing" experiment with full-length p47 for this crucial data forms the basis for all further analysis. Also, this is the only experiment that really shows a direct association between p47 and endogenous FTCD, making it all the more important to use full-length FTCD. It is vital to note that additional binding partners have been previously observed in FTCD preparations purified from porcine liver (Beaudet et al, 1976).
2. The authors have done an impressive number of in vitro binding studies using WT and mutant proteins, and the data is impeccable. However, there should be at least some data showing endogenous protein interactions by co-immunoprecipitation. An FTCD IP could be done in WT and p47 or p97 siRNA depleted cells, and interacting proteins probed for, to confirm endogenous interactions. It has been previously shown that FTCD binds directly to vimentin subunits and to polymerized vimentin filaments in vivo and in vitro (Gao and Sztul, 2001). Moreover, FTCD was shown to promote the binding of Golgi elements to vimentin. Alarmingly, this critical interaction is not mentioned or discussed in the current manuscript.
3. In Figures 1F and 2G, the authors concluded that both p47 and p97 bind FTCD on the Golgi. The data, actually, only show that recombinant p47 and p97 molecules can bind FTCD extracted from salt-washed Golgi membranes.
4. As shown previously and in this study, FTCD is Golgi localized. In Figure 4 and Figure 6, however, the HA staining profile is not that of a Golgi localized protein, but when the FTCDwt-HA construct is localized to the mitochondria, HA has a mitochondrial staining profile. This inconsistency of IF staining is odd and should be addressed. Also, FTCD staining in figure 3 colocalized perfectly with the cis-Golgi marker GM130, and in figure 4, FTCD puncta associates with GM130 puncta suggesting all of FTCD is cis-Golgi localized which is at odds with the fact that FTCD is localized

throughout the Golgi as shown in Gao and Sztul, 2001. This also raises questions of the claim that p97 and p47 dissociate from the Golgi in FTCD KD. Indeed, these two proteins separate from GM130. Is there any FTCD localized at the trans-Golgi? Using trans, medial, and cis Golgi marker is vital to claim separation from the Golgi as a whole.
5. In EVI1, the assay needs proper negative controls (gradient run with missing biding partners) to be able to definitively state that 2 FTCD molecules bind to p97/p47.
6. For figure 7, it will be essential to provide a set of negative-stain EM images of each individual protein in the complex either in figure 7 itself or as a supplemental figure.
7. There is a small discrepancy in figure 8. From the earlier figures, it is established that AMP-PNP enhances complex formation, but in this figure, the presence of AMP-PNP (C) does not appear to induce bead aggregation more than p97+p47 (A) or p97wt+p47wt (B). What could be the reason for this?
8. IF alone is insufficient to conclude that mitochondrial aggregation is due to the tethering activity of the complex. As shown by Willett et al, 2013 (PMID: 23462996) and in Wong et al, 2014 (PMID: 25359980), EM images should be provided to show the tethering of mitochondria by the complex (or by the additional membrane component).
9. A model of how the FTCD-p97-p47-FTCD complex tethers Golgi membranes is very important. How would Stx5, VCIP135, and vimentin fit in this model?
10. A number of essential references addressing FTCD-Golgi relationship (PMID: 9837973, 11238446, 16534631, 9677386) are not discussed or even mentioned. It is certainly not acceptable.
a. Hennig et al, 1998 data suggested that FTCD is mediating the interaction of Golgi-derived membranes with microtubules.
b. Gao et al, 1998 demonstarted that FTCD exists in dimeric, tetrameric, and octameric complexes resistant to proteolysis. FTCD appears to be a dynamic component of the Golgi, and a proportion of FTCD molecules cycle between the Golgi and earlier compartments of the secretory pathway.
c. Gao and Sztul, 2001 showed that the expression of FTCD in cultured cells results in the formation of extensive FTCD-containing fibers originating from the Golgi region and is paralleled by a dramatic rearrangement of the vimentin cytoskeleton in a coordinate process in which vimentin filaments and FTCD integrate into chimeric fibers. The assembly of the FTCD/vimentin fibers causes a parallel change in the structure of the Golgi complex and results in Golgi fragmentation into individual elements that are tethered to the FTCD/vimentin fibers.
d. Hagiwara et al, 2006 showed that in addition to the Golgi apparatus, FTCD is localized to the centrosome, more abundantly around the mother centriole. The centrosome localization of FTCD continued throughout the cell cycle and is not disrupted after Golgi fragmentation.
Minor comments:
Consider revising the title. It gives the impression that FTCD is the tether in the complex analogous to p115-GM130 in the p97/p37 complex. The data suggest that FTCD is a binding platform for p97-p47 and p97-p47is playing a "tethering" role.
For figure 6, it would be essential to see few EM images from which cisternal outgrowth was measured, at least as a supplemental figure.

Response to Referee #1

1. **Comment**: From a general point of view, the paper appears to be well written but there are a few small errors (e.g. "the quantification results" at the end of page 9).

Ans: Following Referee's comment, we amended the text.

2. **Comment**: On line 10, in introducing the mitotic fragmentation of the Golgi, reference is made to Zaal's paper dated 1999 according to which part of the Golgi goes into the ER. This hypothesis does not have a general consensus and has been strongly attacked in two papers:
   1) Rapid, endoplasmic reticulum-independent diffusion of the mitotic Golgi hazeMagnus A B Axelsson 1, Graham Warren DOI: 10.1091 / mbc.e03-07-0459
   2) Golgi enzymes do not cycle through the endoplasmic reticulum during protein secretion or mitosis Julien Villeneuve 1 2, Juan Duran 1 3, Margherita Scarpa 1, Laia Bassaganyas 4 5, Josse Van G DOI: 10.1091 / mbc.E16-08-0560

Ans: We appreciate Referee's kind advice. We amended the introduction.

3. **Comment**: The only major limit of the manuscript is the lack of demonstration that the endogenous proteins (FTCD and p57/p97) interact: the authors have to validate in cells their pull-down data and show the formation of the complex with endogenous proteins.
   Comment: Another point that should be addressed concerns the relationships between FTCD and the other known interactors of p47/p97: is the FTCD interaction synergic/antagonistic/neutral with that of the other known p47/p97interactors? In addition, in discussing their results, the authors should take into consideration the more general context of the regulatory circuits controlling the p47/p97 complex, such as the role of monoubiquitination (here they only mention it briefly) and the interaction with syntaxin5 (For example, Golgi structure formation, function, and post-translational modifications in mammalian cells. Shijiao Huang, Yanzhuang Wang 1 DOI: 10.12688 / f1000research.11900.1).

Ans: Since the above comment and request are tightly related, we want to respond to them

together.

Following Referee's suggestion, we performed immunoprecipitation experiments and the results are presented in new Figure 1C and 1D. Briefly, HepG2 cells were solubilized and used for the immunoprecipitation experiments. We first carried out the immunoprecipitation using anti-p47 antibodies. FTCD was precipitated together with p47 (Fig. 1C, lane 2). We also precipitated FTCD and its binding proteins with anti-FTCD antibodies (Fig.1D). p47 and p97, a p47-binding partner, were precipitated together with FTCD (Fig. 1D, the second, third and fifth panels from the top, lane 2). These results strongly suggest that FTCD, p47 and p97 form a complex in cells. We also investigated whether VCIP135 and syntaxin5, p47-interacting proteins, were included in this complex. Only a tiny amount of VCIP135 was coprecipitated (Fig. 1D, top panel, lane 2) and syntaxin5 was not found in the precipitate (the fourth panel from the top, lane 2). To describe these results, we added one paragraph (p5, the 3rd paragraph) in the Results.

Membrane tethering and SNARE-related events are generally assumed to be spatiotemporally linked and, therefore, it is quite likely that FTCD may interact with syntaxin5 via its receptor protein (complex) in Golgi membranes, which is the reason why we have been rushing to identify the receptor of FTCD. In the next paper, we will need to prove the existence of a big complex containing FTCD, its receptor and syntaxin5, for example, using a crosslinker technique. The identification of the receptor of FTCD is also expected to lead to the clarification of the competitive/cooperative relationships among the p97/p47, p97/p37 and NSF membrane fusion pathways during mitotic Golgi reassembly.

In addition, we isolated VCIP135 as a dissociating factor of the p97/p47 complex (Uchiyama et al., JCB, 2002). This strongly suggests that VCIP135 may function in the recycling of the FTCD-p97/p47-FTCD complex after membrane fusion, which is supported by the immunoprecipitation result that a small amount of VCIP135 was coprecipitated (Fig. 1D, top panel, lane 2). In the following paper, we will test the role of VCIP135 as a dissociating factor of the p97/p47 complex as well as a deubiquitinating enzyme in FTCD-mediated membrane tethering using the *in vivo* mitochondria aggregation assay.

4. **Comment :** Finally the authors ascribe to the nuclear sequestration of p47 its lack of activity in the Golgi complex in interphase cells. This seems to be an over-simplified

view as p47 has been shown to play a role out of the nucleus in interphase cells (i.e. dendrite arborization in neurons, PMID: 30783609). It also raises the question as to whether mutations in the NLS of p47 has any impact on its function in the Golgi complex dynamics also in interphase cells.

Ans: In the case of Golgi reassembly at mitosis, a huge amount of p47 must be necessary and, hence, the nuclear sequestration of p47, which enables the speedy supply of a large amount of p47 to thousands of mitotic Golgi fragments, is a convincing idea. Although the nuclear sequestration of p47 is required, it must not be sufficient for the Golgi reassembly at mitosis. The role of mitotic modification should not be underestimated. We hence amended the Discussion to weaken the tone (p21, the 2nd paragraph).

Apart from the above, we want to point out one important thing. We previously reported that microjnjection of p47(S140A), which is unable to be mitotically phosphorylated, allows the cell to keep Golgi stacks during mitosis (JCB, 1067-1079, 2003). In that paper, our collaborator, Dr. Eija Jokitalo, took beautiful EM images of mitotic (late prometaphase) cells with Golgi stacks. Additionally, in an *in vitro* Golgi disassembly assay, the addition of p47(S140A) to mitotic cytosol inhibited cisternal fragmentation almost completely (JCB, 1067-1079, 2003). These suggest that p47(S140A) can overcome the inhibitory effect of mitotic (mono-)ubiquitination on p97/p47-mediated fusion. We never intend to underestimate the significance of ubiquitination, but we are just wondering whether the ubiquitination may be less significant compared with the nuclear sequestration and mitotic phosphorylation of p47.

On the other hand, we agree with Referee's comments: the function of a small amount of p47, which still remains in the cytoplasm during interphase, is very interesting. From the viewpoint of the 'cisternal progenitor model' by Dr. Pfeffer, the expression of p47 mutant lacking NLSs may accelerate protein transport through the Golgi. Moreover, the microinjection of p47 mutant lacking NLSs into interphase cells with fragmented Golgi, e.g., p37-depleted interphase cells, is also interesting: it may result in the Golgi reconstitution? This experiment enables us to test that the ubiquitination is really essential in p97/p47 membrane fusion. We deeply appreciate Referee's interesting suggestion.

5. **Specific comments :** The authors should provide better description and explanation of the quantitative assessment of the data presented in Figure 7D (Negative staining

electron microscopy of the quaternary complex).

Ans: Following Referee's request, we amended the Materials and Methods (p27-p28).

Response to Referee #2

1. **Comment 1**: Why does it appear that there is some FTCD in the nucleus? This is most apparent when the HA-tagged FTCD is localized.

Ans: As Referee pointed out (Comment 4 of Referee #2), a large amount of FTCD must be required for Golgi membrane tethering at mitosis. We hence speculated that some FTCD required at mitosis can be stored in the nucleus during interphase, which is similar to the case of p47. Alternatively, FTCD may have a distinct function in the nucleus.

We amended the Results (p8, the 2nd paragraph; p9, the 3rd paragraph).

2. **Comment 2**: FTCD does not appear to have a transmembrane domain so what is required to localize the protein to the Golgi? Could it be a rab protein?

Ans: This is a very good question. At this moment, we don't have any data indicating the direct interaction of FTCD with a rab protein. We have recently isolated a receptor candidate of FTCD in Golgi membranes. Interestingly, the candidate is reported to bind to several rab proteins. I absolutely agree with Referee's idea that a rab protein should be involved in the p97/p47-mediated Golgi membrane fusion, although no rab protein has yet been reported in the p97/p47 pathway.

3. **Comment 3**: For the sake of comparison, it would be helpful to show how loss of p47 affects Golgi fragmentation/reassembly in one of the figures.

Ans: Following Referee's request, we added one panel to show the effect of p47 siRNA treatment on the Golgi morphology in new Figure 3C and also amended Figure 3D.

4. **Comment 4**: Is there enough FTCD present in a cell to function as a tether for post-mitotic Golgi membranes? Given that it appears that at least two octomers of FTCD would be required to function as tethers for each pair of Golgi vesicles and that there are "thousands" of Golgi vesicles generated at mitosis, there would have to be many tens of thousands of FTCD present to ensure that the Golgi vesicles can fuse following mitosis. Unless of course, FTCD is necessary for tethering/fusion of just a

few hundred vesicles which then create a template for the reassembly of the Golgi complex. To address this question, could the authors perform some immunoEM (or possibly super-resolution light microscopy) on mitotic cells to determine what percentage of Golgi vesicles have FTCD on them?

Ans: We are very grateful to Referee's valuable comments. As shown in new Figure EV1, FTCD mainly localizes to cis-Golgi. Syntaxin5, which is required for the p97/p47-mediated Golgi membrane fusion, is also known to localize to cis-Golgi. Therefore, it is a fascinating idea that FTCD is necessary for tethering/fusion of just a few hundred vesicles which then create a template for the reassembly of the Golgi complex. In order to prove this hypothesis, it must be necessary to identify the receptor of FTCD and its associating rab proteins, which may play important roles in the competitive/cooperative relationships among the p97/p47, p97/p37 and NSF membrane fusion pathways during the Golgi reassembly at mitosis.

5. **Comment 5**: I found some of the micrographs to be a bit small, could they be enlarged within the figures?

Ans: We enlarged the sizes of Figures 4C, 11E, EV3 and EV5C.

Response to Referee #3

1. **Major comment 1**: In figure 1, a very clean Coomassie staining shows a single band interacting with the N-terminal domain of p47 (1-170). This band was found to be FTCD. While this data is convincing that FTCD can interact with p47, it is essential to repeat the same "fishing" experiment with full-length p47 for this crucial data forms the basis for all further analysis. Also, this is the only experiment that really shows a direct association between p47 and endogenous FTCD, making it all the more important to use full-length FTCD. It is vital to note that additional binding partners have been previously observed in FTCD preparations purified from porcine liver (Beaudet et al, 1976).

   Major comment 2: The authors have done an impressive number of in vitro binding studies using WT and mutant proteins, and the data is impeccable. However, there should be at least some data showing endogenous protein interactions by co-immunoprecipitation. An FTCD IP could be done in WT and p47 or p97 siRNA depleted cells, and interacting proteins probed for, to confirm endogenous interactions.

   Major comment 3: In Figures 1F and 2G, the authors concluded that both p47 and p97 bind FTCD on the Golgi. The data, actually, only show that recombinant p47 and p97 molecules can bind FTCD extracted from salt-washed Golgi membranes.

Ans: Since the above comment and request are tightly related, we want to respond to them together.

Following Referee's suggestion, we performed immunoprecipitation experiments and the results are presented in new Figure 1C and 1D. Briefly, HepG2 cells were solubilized and used for the immunoprecipitation experiments. We first carried out the immunoprecipitation using anti-p47 antibodies. FTCD was precipitated together with p47 (Fig. 1C, lane 2). We also precipitated FTCD and its binding proteins with anti-FTCD antibodies (Fig.1D). p47 and p97, a p47-binding partner, were precipitated together with FTCD (Fig. 1D, the second, third and fifth panels from the top, lane 2). These results strongly suggest that FTCD, p47 and p97 form a complex in cells. We also investigated whether VCIP135 and syntaxin5, p47-interacting proteins, were included in this complex. Only a tiny amount of VCIP135 was coprecipitated (Fig. 1D, top panel, lane 2) and

syntaxin5 was not found in the precipitate (the fourth panel from the top, lane 2).

To describe these results, we added one paragraph (p5, the 3rd paragraph) in the Results.

2. **Major comment 2**: It has been previously shown that FTCD binds directly to vimentin subunits and to polymerized vimentin filaments in vivo and in vitro (Gao and Sztul, 2001). Moreover, FTCD was shown to promote the binding of Golgi elements to vimentin. Alarmingly, this critical interaction is not mentioned or discussed in the current manuscript.

Ans: We isolated FTCD from liver tissue. In liver, the amount of vimentin, which is specifically expressed in mesenchymal cells, is too low to be detected by Western blotting (Proteomics, 4, p1135-p1144, 2004). In HepG2, FTCD is very abundant, while vimentin is reported not to exist (Glycobiology, 20, p843-864, 2010). Even if vimentin is expressed in HepG2, its amount is very tiny compared with those of FTCD, p47 and p97. Moreover, as shown in new Figure 3C, FTCD and p47 really work for the Golgi biogenesis in HepG2. These suggest that vimentin may not be directly used in the p97/p47/FTCD-mdiated membrane fusion.

On the other hand, since vimentin exists in Hela cells, we tested the role of vimentin in Hela cells using the *in vivo* mitochondria aggregation assay. As shown in new Figure EV4, we could not show that vimentin is required for the FTCD/p97/p47-mediated membrane tethering. Nevertheless, it is very interesting that FTCD is necessary for the two distinct events of membrane fusion and reconstruction of the cytoskeleton, which suggests that FTCD may be a key molecule spatiotemporally liking these two important events required for Golgi reassembly at mitosis.

Following Referee's request, we also added one paragraph in the Discussion (p22, the 2nd paragraph) .

3. **Major comment 4**: As shown previously and in this study, FTCD is Golgi localized. In Figure 4 and Figure 6, however, the HA staining profile is not that of a Golgi localized protein, but when the FTCDwt-HA construct is localized to the mitochondria, HA has a mitochondrial staining profile. This inconsistency of IF staining is odd and should be addressed.

Ans: As shown in Figure 3A, FTCD mainly localized to the Golgi and was also found in the nucleus. In the case of overexpressed FTCD-HA , it also localized to the Golgi and the nucleus, and the amount of FTCD in the nucleus was increased. In order to demonstrate the colocalization of FTCD-HA with GM130, we added two panels (panels e and f) in new Figure 4C. We also amended the Results (p8, the 2nd paragraph; p9, the 3rd paragraph).

With regard to its nuclear localization, there are several possible explanations. One of them is that the nucleus can function as a reservoir of FTCD. As Referee#2 pointed out (Comment 4 of Referee #2), a large amount of FTCD must be required for Golgi membrane tethering at mitosis. We hence speculated that some FTCD required at mitosis can be stored in the nucleus during interphase, which is similar to the case of p47. Alternatively, FTCD may have a distinct function in the nucleus.

4. **Major comment 4**: Also, FTCD staining in figure 3 colocalized perfectly with the cis-Golgi marker GM130, and in figure 4, FTCD puncta associates with GM130 puncta suggesting all of FTCD is cis-Golgi localized which is at odds with the fact that FTCD is localized throughout the Golgi as shown in Gao and Sztul, 2001. This also raises questions of the claim that p97 and p47 dissociate from the Golgi in FTCD KD. Indeed, these two proteins separate from GM130. Is there any FTCD localized at the trans-Golgi? Using trans, medial, and cis Golgi marker is vital to claim separation from the Golgi as a whole.

Ans: Following Referee's request, we compared the localization of FTCD with those of expressed β-1,2-N-acetyl-glucosaminyltransferase I (NAGT I), a medial/trans-Golgi marker, and α2,6-syalyltransferase (SialylT), a trans-Golgi/TGN marker (J Cell Sci, p1617-p1627. 1995) (Fig. EV1B). Although the best co-localization was obtained with GM130, the staining of FTCD revealed some colocalization with those of NAGT I and Sialyl T and, hence, we cannot exclude the possibility that there is some FTCD localized at the trans-Golgi.

We added new Figure EV1B and some sentences in the Results (p8, the 2nd paragraph).

5. **Major Comment 5**: In EVI1, the assay needs proper negative controls (gradient run with missing binding partners) to be able to definitively state that 2 FTCD molecules bind to p97/p47.

Ans: A negative control is presented in new Figure EV1D (lower panel).

6. **Major comment 6**: For figure 7, it will be essential to provide a set of negative-stain EM images of each individual protein in the complex either in figure 7 itself or as a supplemental figure.

Ans: We added two negative-stain images in new Figure 7D (lower panels).

7. **Major comment 7**: There is a small discrepancy in figure 8. From the earlier figures, it is established that AMP-PNP enhances complex formation, but in this figure, the presence of AMP-PNP (C) does not appear to induce bead aggregation more than p97+p47 (A) or p97wt+p47wt (B). What could be the reason for this?

Ans: In Figure 8A (and the results of quantification: Figure 8B), the binding experiments were performed in the presence of AMP-PNP, which is described in the Figure legend.

8. **Major comment 8**: IF alone is insufficient to conclude that mitochondrial aggregation is due to the tethering activity of the complex. As shown by Willett et al, 2013 (PMID: 23462996) and in Wong et al, 2014 (PMID: 25359980), EM images should be provided to show the tethering of mitochondria by the complex (or by the additional membrane component).

Ans: Following Referee's suggestion, we investigated the ultrastructures of the aggregated mitochondria induced by the expression of FTCDwt-HA-MAO. Electron microscopic images are presented in new Figures 9C and EV2C. In the aggregated mitochondria cluster, mitochondria were adhered closely to each other in a very narrow gap and no additional membrane component was found between them (Fig. 9C, panel b, arrowheads). This indicates that the mitochondria aggregation is caused by the direct tethering between mitochondria, which may be achieved by FTCD-containing tether

protein complexes.

We inserted one paragraph to explain these EM images in the Results (at the end of p14).

9. **Major comment 9**: A model of how the FTCD-p97-p47-FTCD complex tethers Golgi membranes is very important. How would Stx5, VCIP135, and vimentin fit in this model?

Ans: As presented in new Figure 1D, neither syntaxin5 nor VCIP135 was coprecipitated with FTCD. However, membrane tethering and SNARE-related events are generally assumed to be spatiotemporally linked and, therefore, it is quite likely that FTCD may interact with syntaxin5 via its receptor protein (complex) in Golgi membranes, which is the reason why we have been rushing to identify the receptor of FTCD. In the next paper, we will need to prove the existence of a big complex containing FTCD, its receptor and syntaxin5, for example, using a crosslinker technique. The identification of the receptor of FTCD is also expected to lead to the clarification of the competitive/cooperative relationships among the p97/p47, p97/p37 and NSF membrane fusion pathways during mitotic Golgi reassembly.

In addition, we isolated VCIP135 as a dissociating factor of the p97/p47 complex (Uchiyama et al., JCB, 2002). This strongly suggests that VCIP135 may function in the recycling of the FTCD-p97/p47-FTCD complex after membrane fusion. In the following paper, we will test the role of VCIP135 as a dissociating factor of the p97/p47 complex as well as a deubiquitinating enzyme in FTCD-mediated membrane tethering using the *in vivo* mitochondria aggregation assay.

Concerning the role of vimentin, we have already answered. Please refer to '2. Major comment 2'. We added one paragraph in the Discussion (p22, the 2nd paragraph).

Following Referee's suggestion, the model is presented in Figure EV6.

10. **Major comment 10**: A number of essential references addressing FTCD-Golgi relationship (PMID: 9837973, 11238446, 16534631, 9677386) are not discussed or even mentioned. It is certainly not acceptable.
    a. Hennig et al, 1998 data suggested that FTCD is mediating the interaction of Golgi-derived membranes with microtubules.

b. Gao et al, 1998 demonstarted that FTCD exists in dimeric, tetrameric, and octameric complexes resistant to proteolysis. FTCD appears to be a dynamic component of the Golgi, and a proportion of FTCD molecules cycle between the Golgi and earlier compartments of the secretory pathway.
c. Gao and Sztul, 2001 showed that the expression of FTCD in cultured cells results in the formation of extensive FTCD-containing fibers originating from the Golgi region and is paralleled by a dramatic rearrangement of the vimentin cytoskeleton in a coordinate process in which vimentin filaments and FTCD integrate into chimeric fibers. The assembly of the FTCD/vimentin fibers causes a parallel change in the structure of the Golgi complex and results in Golgi fragmentation into individual elements that are tethered to the FTCD/vimentin fibers.
d. Hagiwara et al, 2006 showed that in addition to the Golgi apparatus, FTCD is localized to the centrosome, more abundantly around the mother centriole. The centrosome localization of FTCD continued throughout the cell cycle and is not disrupted after Golgi fragmentation.

Ans: We added one paragraph in the Discussion (p22, the 2nd paragraph) and the above papers in the References.

11. **Minor comment 1**: Consider revising the title. It gives the impression that FTCD is the tether in the complex analogous to p115-GM130 in the p97/p37 complex. The data suggest that FTCD is a binding platform for p97-p47 and p97-p47is playing a "tethering" role.

Ans: We appreciate Referee’s kind comment. Following your suggestion, we revised the title.

12. **Minor comment 2**: For figure 6, it would be essential to see few EM images from which cisternal outgrowth was measured, at least as a supplemental figure.

Ans: Following the Referee's suggestion, we added some EM images in new Figure 6.

1st Revision - Editorial Decision 19th Nov 2020

Thank you for submitting your revised manuscript. The study has been seen by two of the original referees, whose comments are shown below.

As you can see, the referees find that their criticisms have been sufficiently addressed and recommend the manuscript for publication.

However, there are a few editorial issues concerning the text and the figures that I need you to address before I we can officially accept the manuscript for publication.

Referee #2:

The revised manuscript has addressed the concerns I raised previously.
The study by Kondo and colleagues is a well executed investigation of the mechanisms that control tethering of post-mitotic Golgi membranes and as such is an important study that will be of wide interest. I recommend publication.

Referee #3:

The authors have done a nice job addressing all my comments. I support the publication of this thorough research paper.

2nd Revision - Editorial Decision 17th Dec 2020

I am pleased to inform you that your manuscript has been accepted for publication in The EMBO Journal.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Hisao Kondo
Journal Submitted to: EMBO Journal
Manuscript Number: EMBO J-2020-105853

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	in principle, we incresed the sample size as much as possible. We also checked the sample size using statistical methods
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	randomization procedure.
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Our data were obtained within the range of liner proportional change and, hence, are thought to show normal distribution.
Is there an estimate of variation within each group of data?	We checked the standard deviation of each group compared with that of total data.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Done.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes. The cells were purchased from ATCC.

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Yes. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
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