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MEK1 inactivates Myt1 to regulate Golgi membrane fragmentation and mitotic entry in mammalian cells

Julien Villeneuve, Margherita Scarpa, Maria Ortega-Bellido and Vivek Malhotra

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(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 29 August 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see, while the referees all consider the study as timely and interesting, they think that MEK1-induced phosphorylation and inhibition of Myt1 needs to be characterised in more mechanistic depth and that the timing and functional role of this regulatory step should be analysed in more detail. The referees put forward a number of suggestions how to address these two issues. However, you also may have alternative ideas to achieve this. We would thus be happy to consider a revised version of the manuscript that addresses the referees' comments in an adequate manner and to their satisfaction. In particular, it will be important to develop the study further with respect to the two points mentioned above. Please do not hesitate to get back to us at any time, in case you would like to consult on any aspect of the revision further.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, 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

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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 COMMENTS

Referee #1

The Golgi apparatus undergoes fragmentation during G2/M phase and reassembles during mitotic exit. It has been reported that the fragmentation requires three kinases: cyclin-dependent kinase 1 (Cdk1), polo-like kinase (Plk) and MAPK/ERK kinase 1 (MEK1), and that the reassembly requires an ER-Golgi membrane-localized kinase Myt1. Myt1 is known to be a direct downstream target of Plk or p90 ribosomal S6 kinase (RSK) in Xenopus embryonic/meiotic cell cycle, and RSK is established as a direct downstream target of the MEK1-ERK pathway. However, there is no study addressing the relationship between MEK1 and Myt1 in mitotic Golgi fragmentation. Myt1 knockdown by siRNA accelerated M phase entry in HeLa cells (Figure 1). Although Myt1 knockdown did not affect the morphology or secretory function of Golgi apparatus in interphase cells (Figure 2), it enhanced mitotic cytosol-induced fragmentation of Golgi in permeabilized interphase cells (Figures 3, 4A and 4B). The mitotic cytosol-induced Golgi fragmentation was reduced by overexpression of wild-type Myt1, treatment with a MEK1 inhibitor PD98059 or immunodepletion of Plk1, but not by treatment with a RSK inhibitor or a Cdk1 inhibitor (Figures 4C, 5A, 6A and 6B). The PD98059-induced reduction in Golgi fragmentation, but not the Plk1 immunodepletion-induced reduction, was cancelled by Myt1 knockdown (Figures 5B and 6C). Moreover, PD98059 treatment or siRNA-mediated MEK1 knockdown delayed M phase entry, and this delay was cancelled by Myt1 knockdown (Figure 7). Also, Myt1 phosphorylation during M phase was affected by PD98059 treatment or siRNA-mediated MEK1 knockdown (Figure 8). These results suggest that MEK1, but not Plk1, RSK or Cdk1, inhibits Myt1 to regulate mitotic Golgi fragmentation. In vitro kinase assay showed that MEK1 does not directly phosphorylate Myt1 (Figure 9). This study thus demonstrates that MEK1 induces Golgi fragmentation through an unknown mechanism involving Myt1 inhibition.

The data presented are solid. This study suggests a novel molecular mechanism that links MEK1 to Myt1, although the details of the mechanism are still unresolved. I recommend publication of this manuscript in EMBO J., if the following points are properly addressed.

1) The authors should address the mechanisms of MEK1-induced inhibition of Myt1 in more detail. The authors only show that MEK1 does not phosphorylate Myt1 in vitro. This result alone, however, is not enough to define the mechanisms.
2) The authors should address whether MEK1-mediated Myt1 inhibition is important for Golgi fragmentation in non-permeabilized cells.

Referee #2

This paper provides evidence for the implication of the membrane-bound kinase Myt1 in Golgi membrane fragmentation. Downregulation of Myt1 by siRNAs does not affect Golgi organization or protein secretion in interphase HeLa cells but potentiates Golgi fragmentation induced by mitotic cytosol in permeabilized cells. Moreover, Myt1 downregulation rescues both impaired Golgi membrane fragmentation and delayed mitosis entry produced by the inhibition or the knockdown of

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MEK1, suggesting that Myt1 functions downstream of MEK1. Further experiments show that inhibitors of Rsk, Plk or Cdk1, three kinases that have been previously reported to phosphorylate Myt1, do not affect Golgi membrane fragmentation induced by mitotic cytosol in permeabilized HeLa cells. The authors propose that Myt1 inhibition by MEK1 is required for mitotic Golgi fragmentation.

This is an interesting story, the manuscript is well written and the conclusions are supported by good quality results. The authors also provide rational explanations for the apparent discrepancies with work published by others on the implication of MEK1 and Myt1 in the regulation of Golgi membrane dynamics.

Specific points:

1. The proposal that Myt1 regulation by MEK1 is not mediated by the downstream kinase Rsk is intriguing. Especially considering that Rsk inhibition does impair mitotic Myt1 phosphorylation in Nocodazole-treated HeLa cells, apparently as efficiently as MEK1 inhibition (Fig 8E). These conclusions are based on the use of a single chemical inhibitor, whose effectiveness in the assay conditions is not demonstrated. It would be important to confirm the differential requirement for MEK1 but not Rsk in Golgi membrane fragmentation using at least one more inhibitor of each.

2. The authors could find no evidence for the direct phosphorylation of Myt1 by MEK1. However, Myt1 regulation could be mediated by MEK1-activated ERK1 or ERK2, and the authors have all tools in hand to test this possibility, just adding Myt1 to the kinase assays of Fig 9A. If Myt1 is phoshorylated by ERK1/2, the next step would be to analyze how siRNA-mediated downregulation of ERK1/2 affects Golgi membrane fragmentation. These experiments should be rather straightforward to perform and potentially very informative.

3. Since all the work reported here is based on HeLa cells, maybe authors should also comment on whether cell-type specific differences could account for some of the discrepancies with other papers.

4. Would be Myt1 required for the MEK1-induced phosphorylation of GRASP55?

5. It seems likely that Myt1 donwregulation would increase Cdk1 activity, which in turn could contribute to the enhanced Golgi fragmentation observed. This should be discussed.

Referee #3

The article investigates a novel angle of MEK1 activity in Golgi fragmentation at the onset of mitosis. There has been a debate in the field on which of MEK1 or CDK1 was involved in Golgi fragmentation. This issue was previously addressed by Kano (2000) (curiously not referenced in this paper). The Kano's group showed that the first disassembly step (Golgi ribbon unlinking and release of the Golgi stacks) was MEK1 dependent whereas the following vesiculation of the Golgi stacks was CDK1 dependent.
Furthermore, the Golgi ribbon unlinking at G2 has been shown to act as a G2/M checkpoint. If it does not happen properly, the cell entry into mitosis inhibited or delayed.

Here the authors show that MEK1 modulates Myt1 activity with an impact on Golgi fragmentation and cell cycle progression.
The authors use a combination of cell cycle synchronization using double thymidine block (S phase) and nocodazole treatment (metaphase) combined with depletion by siRNAs, an assay based on semi-intact HeLa cells incubated with interphase and mitotic cytosols supplemented with several inhibitors and reconstitution assay with purified or IPed proteins and membrane.

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The authors propose that during mitosis, MEK1 leads to phosphorylation (via an unknown kinase) of Myt1 that becomes inactivated. This alleviates its inhibitory role on the mitotic Golgi fragmentation that becomes more efficient.

D) The 4th part of the manuscript aims to illustrate the role of the signaling branch MEK1/Myt1 in cell cycle progression. In doing this, the authors test again the notion that Golgi fragmentation is a checkpoint for cell entry into mitosis.
To do so, the authors use cell synchronization protocol (double thymidine block) to show that:
-Myt1 depletion accelerates cell entry to mitosis by 2 hours.
- MEK1 and Myt1 interact genetically for the control of the cell cycle and that MEK1 is upstream of Myt1.

Taking all these results together, the authors propose that MEK1 activity leads to Myt1 inhibition. As Myt1 is a Golgi fragmentation inhibitor, MEK1 activity allows Golgi fragmentation and cells can enter into mitosis.

The article is interesting and undoubtedly represents a large amount of work that is reflected by its density. This is not helped by the quick writing style and brushing over details and explanation. Furthermore, there are a number of concerns that need to be addressed.

1) Conceptually, I am confused about the timing of Myt1 phosphorylation. It peaks 10h after release from the Thy block (Figure 1B). The cells are then in mitosis, way passed the possible Golgi checkpoint at G2. This is confirmed by Figure 1A and 8A where Myt1 is phosphorylated only in mitotic cells. To have role in controlling cell entry into mitosis via Golgi fragmentation, I would have expected to see Myt1 phosphorylation from 2-6h post release (G2 duration).

MEK1 is involved in this phosphorylation at mitosis. Myt1 is inhibited by PD (MEK1 inhibitor) only in mitosis (Figure 8, Noc treated cells). However, this inhibition by PD is in fact a delay of 2h, so Myt1 is still phosphorylated but later.
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phosphorylation (by 2h). This suggests another phosphorylation event that is MEK1 independent. Is it also inhibitory for Golgi fragmentation? Is it related to CDK1 activation?

4) The role of Myt1 in Golgi fragmentation is shown using the semi intact cell assay incubated with mitotic cytosol whereas its role in cell cycle progression is done with cells release from a thymidine block.
At best, this is an interesting correlation, which could have been remedied by studying the state of the Golgi in cells released from the block. These cells were stained with Dapi and PhosphoH3 antibody. They could have been stained by an anti Golgi antibody. Or HeLa cells expressing ManII-GFP could have been used for these synchronization experiments.
This experiment should be performed to justify the title.

5) Related to point 1(Figure1 and 7), not only the mitotic index should be scored but also the number of cells in G2.

6) How is the RNAi is performed in the cell synchronization experiments? If I understand the text (there is no description in MM or legends for figure), the cells are first synchronized using the double thymidine block protocol and then transfected with relevant siRNA. So at best, they are depleted for 18h+12h post release.
This is considerably shorted than the 48-72h depletion time (Figure 1D and 7A) where cells are simply transfected by siRNAs. These WB show that the oligos CAN deplete Myt1 and MEK1 efficiently, not that they DO so after 24h when transfected in thymidine blocked cells.
How efficient is this?
Is the graph established solely taking into account depleted cells? How is the depletion scored?
Can the cells be depleted before the synchronization?

7) The authors rule out Rsk because Rsk inhibitor does not block Golgi fragmentation. Yet, Rsk inhibitor block Myt1 phosphorylation as shown by the authors and others.
First, Rsk deserves a better introduction as it is rather cryptic and short. Has Rsk been shown to function in mitosis? In Golgi?
Second, is the site on Myt1 phosphorylated by Rsk known? Can this be mutated and this Myt1 version transfected into HeLa cells along the kinase dead version.

8) What about ERKc aa part of this phosphorylation cascade from MEK1 to Myt1?

9) I find that the part of Plk (end page 11 and page 12, Figure 6) should be placed earlier, just after Colanzi et al, 2000, (4th line page 11). Otherwise, the argument of the link between MEK1 and Myt1 is split and does not flow.

1st Revision - authors' response 30 October 2012

Referee #1

The Golgi apparatus undergoes fragmentation during G2/M phase and reassembles during mitotic exit. It has been reported that the fragmentation requires three kinases: cyclin-dependent kinase 1 (Cdk1), polo-like kinase (Plk) and MAPK/ERK kinase 1 (MEK1), and that the reassembly requires an ER-Golgi membrane-localized kinase Myt1. Myt1 is known to be a direct downstream target of Plk or p90 ribosomal S6 kinase (RSK) in Xenopus embryonic/meiotic cell cycle, and RSK is established as a direct downstream target of the MEK1-ERK pathway. However, there is no study addressing the relationship between MEK1 and Myt1 in mitotic Golgi fragmentation. Myt1 knockdown by siRNA accelerated M phase entry in HeLa cells (Figure 1). Although Myt1 knockdown did not affect the morphology or secretory function of Golgi apparatus in interphase cells (Figure 2), it enhanced mitotic cytosol-induced fragmentation of Golgi in permeabilized interphase cells (Figures 3, 4A and 4B). The mitotic cytosol-induced
Golgi fragmentation was reduced by overexpression of wild-type Myt1, treatment with a MEK1 inhibitor PD98059 or immunodepletion of Plk1, but not by treatment with a RSK inhibitor or a Cdk1 inhibitor (Figures 4C, 5A, 6A and 6B). The PD98059-induced reduction in Golgi fragmentation, but not the Plk1 immunodepletion-induced reduction, was cancelled by Myt1

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1) The authors should address the mechanisms of MEK1-induced inhibition of Myt1 in more detail. The authors only show that MEK1 does not phosphorylate Myt1 in vitro. This result alone, however, is not enough to define the mechanisms.

We appreciate the reviewer's concern but anything more on this topic, of significance, requires identification of the MEK1 dependent phosphorylation sites in Myt1. There are potentially 32 phosphorylation sites, and their identification is beyond the scope of this paper. We now include data that shows the involvement of MEK1 dependent Myt1 inhibition in intact cells (point #1 above) and hope this is satisfactory.

2) The authors should address whether MEK1-mediated Myt1 inhibition is important for Golgi fragmentation in non-permeabilized cells.

Yes, and the data from the following experiment are now in the paper. HeLa cells expressing manosidase II-GFP were synchronized in S-phase with a double thymidine block, incubated in normal medium for 8 h and stained with an anti-phospho-histone H3 antibody. 60% of the control siRNA transfected-cells in G2-phase, identified by the specific punctuate phospho-histone H3 staining (Colanzi et al, 2007) had a fragmented Golgi apparatus (Figure 2D). Down regulation of Myt1 increased the percentage of cells with fragmented Golgi to 80% (Figure 2D). Under these experimental conditions, the inhibition of MEK1 with PD in control siRNA transfected cells decreased the percentage of cells with fragmented Golgi to 40%, however as demonstrated in permeabilized cells, the knockdown of Myt1 alleviated the function of MEK1 in the Golgi fragmentation process with a percentage of cell with fragmented Golgi to 80% (Figure 6C).

Referee #2

This paper provides evidence for the implication of the membrane-bound kinase Myt1 in Golgi membrane fragmentation. Downregulation of Myt1 by siRNAs does not affect Golgi organization or protein secretion in interphase HeLa cells but potentiates Golgi fragmentation induced by mitotic cytosol in permeabilized cells. Moreover, Myt1 downregulation rescues both impaired Golgi membrane fragmentation and delayed mitosis entry produced by the inhibition or the knockdown of MEK1, suggesting that Myt1 functions downstream of MEK1. Further experiments show that inhibitors of Rsk, Plk or Cdk1, three kinases that have been previously reported to phosphorylate Myt1, do not affect Golgi membrane fragmentation induced by mitotic cytosol in permeabilized HeLa cells. The authors propose that Myt1 inhibition by MEK1 is required for mitotic Golgi fragmentation.

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Nocodazole-treated HeLa cells, apparently as efficiently as MEK1 inhibition (Fig 8E). These conclusions are based on the use of a single chemical inhibitor, whose effectiveness in the assay conditions is not demonstrated. It would be important to confirm the differential requirement for MEK1 but not Rsk in Golgi membrane fragmentation using at least one more inhibitor of each.

As suggested by the reviewer, we have tested other chemical inhibitors of the kinases. U0126, BI-D1870 and RO-3306 inhibit MEK1, RSK and CDK1, respectively. The effectiveness of the chemical inhibitors was tested against the respective kinases and shown in Figure S1. These chemicals were then tested for their effects on Golgi fragmentation in permeabilized cells. Only UO126 inhibited mitotic cytosol dependent Golgi membrane fragmentation, which confirms the requirement for MEK1 but not RSK and CDK1 in Golgi membrane fragmentation (Figure S2).

2. The authors could find no evidence for the direct phosphorylation of Myt1 by MEK1. However, Myt1 regulation could be mediated by MEK1-activated ERK1 or ERK2, and the authors have all tools in hand to test this possibility, just adding Myt1 to the kinase assays of Fig 9A. If Myt1 is phoshorylated by ERK1/2, the next step would be to analyze how siRNA-mediated downregulation of ERK1/2 affects Golgi membrane fragmentation. These experiments should be rather straightforward to perform and potentially very informative.

We have performed the experiment suggested by the reviewer. Myt1 KD was incubated with purified mitotic MEK1 and pure ERK2 with 32P-ATP. MEK1 phosphorylated ERK2, however, Myt1 was not phosphorylated under these conditions (Figure S3). This corroborates our proposal that MEK1 does not directly phosphorylates Myt1. Additionally, Myt1 phosphorylation by MEK1 is not via its known substrate ERK2.

3. Since all the work reported here is based on HeLa cells, maybe authors should also comment on whether cell-type specific differences could account for some of the discrepancies with other papers.

We briefly describe this possibility in the discussion

4. Would be Myt1 required for the MEK1-induced phosphorylation of GRASP55?

We tested this experimentally. GRASP55 was immunoprecipitated from HeLa cells synchronized in S-phase (thymidine-treated cells) or mitosis (nocodazole-treated cells). Then, using the mitotic phosphoprotein monoclonal-2 (MPM-2) antibody, which recognizes a phosphorylated S/T-P epitope, we tested the MPM-2 reactivity of GRASP55 as reported earlier by Linstedt and colleagues (Jesch et al, 2001). As shown in Figure S4A, GRASP55 immunoprecipitated from mitotic synchronized cells was strongly MPM-2 reactive, in contrast to GRASP55 immunoprecipitated from S-phase synchronized cells. The inhibition of MEK1 with PD reduces the phosphorylation of GRASP55 in mitotic cells as revealed by the decrease of MPM-2 reactivity (Figure S4A). In order to determine whether Myt1 activity is required for the mitotic GRASP55 phosphorylation mediated by MEK1, HeLa cells were transfected with control or Myt1 specific siRNA oligos and synchronized in mitosis by nocodazole treatment in the presence or absence of PD. The downregulation of Myt1 did not affect the MPM-2 reactivity of GRASP55 immunoprecipitated from mitotic cells (Figure S4B), suggesting that MEK1-mediated GRASP55 phosphorylation in mitotic cells does not require Myt1.

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CDK1 is a well-known substrate of Myt1 and it has been reported that CDK1 is required for the fragmentation of isolated Golgi stacks in small tubules and vesicles. Using a Golgi fragmentation assay in permeabilized cell incubated with mitotic cytosol, as previously reported (Acharya et al, 1998) and confirmed in this study, inhibition of CDK1 with olomoucine and RO-3306 did not affect Golgi membrane fragmentation. But we cannot rule out the possibility that an increase in CDK1 activity upon Myt1 downregulation affect fragmentation of the Golgi stacks post MEK1 dependent reaction. This is mentioned in the discussion.

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Furthermore, the Golgi ribbon unlinking at G2 has been shown to act as a G2/M checkpoint. If it does not happen properly, the cell entry into mitosis inhibited or delayed.

We apologize for this mistake. The reference is now included in the introduction

Here the authors show that MEK1 modulates Myt1 activity with an impact on Golgi fragmentation and cell cycle progression.
The authors use a combination of cell cycle synchronization using double thymidine block (S phase) and nocodazole treatment (metaphase) combined with depletion by siRNAs, an assay based on semi-intact HeLa cells incubated with interphase and mitotic cytosols supplemented with several inhibitors and reconstitution assay with purified or IPed proteins and membrane.

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have expected to see Myt1 phosphorylation from 2-6h post release (G2 duration).

MEK1 is involved in this phosphorylation at mitosis. Myt1 is inhibited by PD (MEK1 inhibitor) only in mitosis (Figure 8, Noc treated cells). However, this inhibition by PD is in fact a delay of 2h, so Myt1 is still phosphorylated but later.
I do not understand the relevance of inhibiting/delaying an inhibitor of Golgi fragmentation (Myt1 phosphorylation) once the Golgi is already fragmented. Can this be explained?

During the progression into the mitosis, Myt1 phosphorylation peaks 10 h after release from the double thymidine block. However, as illustrated Figure 1C and 8B the progressive phosphorylation of Myt1 is initiated 6 h after release from the double thymidine block. It means that from this time point Myt1 is progressively inactivated by different kinases in a time dependant manner. Indeed, a partial inhibition of Myt1 might be sufficient at the earliest stage of cell cycle progression for its effect on Golgi fragmentation. The delay in its complete inactivation that occurs 10 h after release could have other consequences such as a defect in CDK1 activity and perhaps on the second step of Golgi membrane fragmentation which is CDK1 dependant. It is important to map the exact sites phosphorylated in Myt1 by various kinases to address this question comprehensively.

2) This suggests that the effect of MEK1 on Myt1 may control Golgi fragmentation (stack vesiculation at prophase/metaphase) but perhaps not the Golgi unlinking occurring at G2, the step that has very carefully been shown to be the one controlling cell entry to mitosis.

Does MEk1/Myt1 operate at mitosis? Yet, it also seems to operate at the earliest stage of Golgi fragmentation and cell cycle progression (as shown by many and stated in the abstract). Can MEK1 have two roles at two different stage of cell cycle. This needs to be addressed.
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Can activation of CDK1 monitored under conditions of MEK1 inhibition?

MEK1, by regulating Myt1 activity is involved in the first step of Golgi fragmentation as shown in this study. This effect is mediated by Myt1 but is CDK1 independent. In order to determine whether MEK1 can regulate CDK1 activity, as suggested by the reviewer, we tested the CDK1 activation under conditions of MEK1 inhibition. HeLa cells were arrested in S-phase with double thymidine block and then cultured in thymidine free medium. In this experiment MEK1 activity was inhibited by incubation of the cells with PD or by transfection with MEK1 specific siRNA oligos. The cells were then collected at the indicated times and western blotted with an anti CDK1 (pY15) and histone H3 (pSer10) antibodies. In control cells, DMSO treated cells or control siRNA transfected cells, CDK1 activity monitored by the dephosphorylation of tyrosine 15, is obvious 10 h post thymidine release. It correlated with Myt1 inhibition and histone H3 phosphorylation (Figure 8B & 8C). However, in PD treated cells and MEK1 siRNA transfected cells, CDK1 activation is delayed by 2 h, like Myt1 inhibition as previously reported (Figure 8B & 8C). Altogether, these results show that MEK1, by inhibiting Myt1 can regulate CDK1 activity. In other words, MEK1 can regulate the first step of Golgi membrane fragmentation via Myt1 but independently of CDK1, but, as MEK1-mediated Myt1 inhibition is equally involved in CDK1 activity, we suggest that MEK1 and Myt1 can operate at later stage of Golgi membrane fragmentation and cell cycle progression.

3) Related to point 2, I do not understand how PD or MEK1 depletion can delay the Myt1 phosphorylation (by 2h). This suggests another phosphorylation event that is MEK1 independent. Is it also inhibitory for Golgi fragmentation? Is it related to CDK1 activation?

Myt1 is inactivated during the mitosis by hyperphosphorylation. Analyzing the amino-acid sequence revealed 32 predicted phosphorylation sites, that corroborate the difference of migration observe by western blot between Myt1 from S-phase and mitotic cells. When the cells are synchronized in S-phase after a double thymidine block and release, Myt1 phosphorylation peaks 10 h after release

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but starts 6 h after release (figure1C and 8B). Some kinases are known to be involved in Myt1 phosphorylation such as Plk, RSK and CDK1. We show in this study that MEK1 is also required for Myt1 phosphorylation, and this event is required for MEK1-mediated Golgi fragmentation. While Myt1 is phosphorylated by Plk, RSK and CDK1, these are not linked to Myt1 dependent Golgi fragmentation. The inactivation of Myt1 by phosphorylation during the progression in the cell cycle requires specific kinases. These coordinate events lead to a complete inactivation of Myt1 essential for CDK1 activation.
In other words, a number of kinases are involved in the activation and inactivation of Myt1. But its inactivation by MEK1 has a role in Golgi fragmentation. We need to map the phosphorylation sites for these kinases, generate the appropriately modified Myt1 and then test the role of phospho-mimetic Myt1 and the non-phosphorylated form for the Golgi membrane fragmentation process

4) The role of Myt1 in Golgi fragmentation is shown using the semi intact cell assay incubated with mitotic cytosol whereas its role in cell cycle progression is done with cells release from a thymidine block.
At best, this is an interesting correlation, which could have been remedied by studying the state of the Golgi in cells released from the block. These cells were stained with Dapi and PhosphoH3 antibody. They could have been stained by an anti Golgi antibody. Or HeLa cells expressing ManII-GFP could have been used for these synchronization experiments.
This experiment should be performed to justify the title.

As asked equally by the reviewer 1, point 2, we have addressed the role of Myt1 in Golgi membrane fragmentation in non-permeabilized cells. Briefly, HeLa cells expressing manosidase II-GFP were synchronized in S-phase with a double thymidine block, incubated in normal medium for 8 h and stained with an anti-phospho-histone H3 antibody. 60% of the control siRNA transfected-cells in G2-phase, identified by the specific punctuate phospho-histone H3 staining (Colanzi et al, 2007) had a fragmented Golgi apparatus (Figure 2D). Down regulation of Myt1 increases the percentage of cells with fragmented Golgi to 80% (Figure 2D). In these experimental conditions, the inhibition of MEK1 with PD in control siRNA transfected cells decreases the percentage of cells with fragmented Golgi to 40%, however as demonstrated in permeabilized cells, the knockdown of Myt1 alleviates the function of MEK1 in the Golgi fragmentation process with a percentage of cell with fragmented Golgi to 80% (Figure 6C).

5) Related to point 1(Figure1 and 7), not only the mitotic index should be scored but also the number of cells in G2.

In order to score the number of cells in G2 for the Figure 1 and 7, we have used the following experimental approach. After a double thymidine block and release, at the indicated time, cells were fixed and stained with a DNA dye, DAPI, and with an anti-phospho-histone H3 antibody. We counted the number of cells in late G2-phase (uncondensed DNA and specific punctate phospho-histone H3 staining, as shown by Corda and colleagues (Colanzi et al., 2007)) and the total number of cells that were in late G2 and M-phase (all the cells that are phospho-histone H3 positive from late G2 to all the mitotic stages). As shown in Figure 1G, in control and Myt1 siRNA transfected cells, 6 h after thymidine release, 70% of phospho-histone H3 positive cells were in G2. Interestingly, 8 h after thymidine release, 40% of phospho-histone H3 positive control cells were still in G2 whereas in Myt1 knockdown cells, only 20% of phospho-histone H3 positive cells were in G2. These results suggested that Myt1 knockdown increases the kinetics of G2-M transition.
The same experimental approach was used to obtain the data shown in Figure 7E and 7H. Figure 7E, the analysis of cells in G2 showed that in PD treated cells, the kinetic of transition from G2 to M-phase was delayed compared with DMSO treated cells. Figure 7H, knockdown of Myt1 alleviated the G2/M-phase transition delay induced by PD treatment.

6) How is the RNAi is performed in the cell synchronization experiments? If I understand the text (there is no description in MM or legends for figure), the cells are first synchronized using the double thymidine block protocol and then transfected with relevant siRNA. So at best, they are depleted for 18h+12h post release.
This is considerably shorted than the 48-72h depletion time (Figure 1D and 7A) where cells are simply transfected by siRNAs. These WB show that the oligos CAN deplete Myt1 and MEK1

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efficiently, not that they DO so after 24h when transfected in thymidine blocked cells.
How efficient is this?
Is the graph established solely taking into account depleted cells? How is the depletion scored?
Can the cells be depleted before the synchronization?

For the cell synchronization experiments in S-phase, the details of siRNA transfection are described in Supplementary Materials and methods under the section entitled "Cell synchronization in S-phase and G2, and Golgi membrane fragmentation in intact cells". To summarize, when the cells are released from thymidine block at 0 min time point, the transfection time was 52 h (6h of seeding, 18h for the first block, 10h of release and 18h for the second block). For the Figure 1D, 5A and and 7A, the depletion efficiency has been scored as shown in Figure 1E, 5B and 7B respectively.
When experiments are performed with siRNA, the graphs established take into account all the cells.

7) The authors rule out Rsk because Rsk inhibitor does not block Golgi fragmentation. Yet, Rsk inhibitor block Myt1 phosphorylation as shown by the authors and others.
First, Rsk deserves a better introduction as it is rather cryptic and short. Has Rsk been shown to function in mitosis? In Golgi?
Second, is the site on Myt1 phosphorylated by Rsk known? Can this be mutated and this Myt1 version transfected into HeLa cells along the kinase dead version.

We now describe RSK in the introduction. RSK is known to regulate diverse cellular processes including cell growth and proliferation, but there is no mention of its role in Golgi membrane function and organization. The amino acids phosphorylated by RSK in Myt1 are known in Xenopus but these phosphorylated amino-acids are not conserved in its human orthologs.

8) What about ERKc aa part of this phosphorylation cascade from MEK1 to Myt1?

We now include data that ERK2 is not required for MEK1 dependent phosphorylation of Myt1 (Figure S3). It is true that Rony Seeger and colleagues have reported the involvement of a novel isoform of ERK called ERK1c in the MEK1 dependent Golgi membrane reorganization. However, the direct test of this proposal is beyond the scope of this paper. We plan to test the potential role of ERK1c in Myt1 phosphorylation in collaboration with Rony.

9) I find that the part of Plk (end page 11 and page 12, Figure 6) should be placed earlier, just after Colanzi et al, 2000, (4th line page 11). Otherwise, the argument of the link between MEK1 and Myt1 is split and does not flow.

The description of Plk has been moved as suggested by the reviewer but placed just after the part entitled "MEK1 regulates Golgi membrane fragmentation via Myt1" as this makes it easier to follow the data shown.

2nd Editorial Decision 13 November 2012

Thank you for submitting your revised manuscript to the EMBO journal. Your study has now been seen referee #3 and the comments are provided below. As you can see, the referee appreciates the introduced changes, but also suggests a number of important text changes. I would like to ask you address these remaining issues in a final round of revision.

Thank you for submitting your interesting manuscript to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

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Referee #3 (Remarks to the Author):

The authors have provided a number of additional experiments that strengthen the paper and clarify a number of points.
However, in my opinion, they still need to make minor but important changes to the text to make it suitable for publication to EMBO J

-I appreciate their introduction on Rsk (page 5) but somehow it does not fit where it is placed. It completely cut the argument. This piece needs to be put somewhere else or better embedded.

-I respectfully disagree with the statement (page 9) that Myt1 depletion does not affect Golgi fragmentation in S cells (Figure 2D). The picture very clearly shows that the Golgi membrane is much more fragmented than in the control, suggesting that in fact Myt1 depletion has an early effect on Golgi fragmentation.
Based on this picture, I do not entirely trust the quantitation n Figure 2D (S-phase). The authors need to change their conclusion!
This is an important point to mention because the authors go back to this statement in the discussion and this needs to be modified as well.

-Myt1 is known to inactivate CDK1 and the authors here show that a delay in Myt1 activation also leads to a lack of CDK1 activation with all the effects known on Golgi fragmentation. This is acknowledged in the text (page 16).
But the authors do not take this result in their model to suggest that in fact Myt1, via CDK1, also impact on the second phase of Golgi fragmentation.

-Last, the discussion is bizarrely constructed. The first part should be extended to incorporate the role of Myt1 on the Golgi organization in S phase, in G2 (their statement) and in mitosis via CDK1. This is important and would fit better with the strong phosphorylation of Myt1 at 10h, well into mitosis.

Furthermore, in the intro, the authors are careful to describe the two phases of Golgi fragmentation, The Golgi ribbon unlinking at G2 followed by Golgi stack fragmentation. Yet, in the rest van de manuscript, the authors only use "Golgi fragmentation" as a generic term without specifying which phase they mean. Defining this would be helpful in improving the manuscript.

2nd Revision - authors' response 14 November 2012

We thank the reviewer for his/her positive remarks. We have rewritten the text extensively and the specific points raised by the reviewer are addressed below.

The authors have provided a number of additional experiments that strengthen the paper and clarify a number of points.
However, in my opinion, they still need to make minor but important changes to the text to make it suitable for publication to EMBO J

-I appreciate their introduction on Rsk (page 5) but somehow it does not fit where it is placed. It completely cut the argument. This piece needs to be put somewhere else or better embedded.

As mentioned above, the overall breakdown of Golgi complex into tubules and vesicles is mediated sequentially by MEK1 and CDK1, respectively. How are these events connected? We have tested the hypothesis that these sequential events are connected by the ER- Golgi complex associated kinase called Myt1, which is expressed only in the metazoans (Liu et al, 1997). Our assumption is

based on the following facts. Myt1 phosphorylates CDK1 on Thr14 and Tyr15 (Mueller et al, 1995) and the Myt1 phosphorylated CDK1 is inactive (Booher et al, 1997). Inactivation of Myt1 is therefore necessary for the activation of CDK1 and entry into mitosis. MEK1 is known to phosphorylate Myt1 via p90RSK (90kDa ribosomal S6 kinase) and this event is required for the entry of Xenopus oocytes into meiosis (Palmer et al, 1998). We now report that MEK1 inactivates Myt1 in mammalian somatic cells. We show that this reaction is required for the process by which Stacks of Golgi cisternae are separated from each other -independent of CDK1- in G2 and promote entry of cells into mitosis. Surprisingly, the MEK1 dependent inactivation of Myt1 is RSK and CDK1 independent. The description of our findings on the involvement of Myt1 in Golgi membrane reorganization and mitotic entry follows.

-I respectfully disagree with the statement (page 9) that Myt1 depletion does not affect Golgi fragmentation in S cells (Figure 2D). The picture very clearly shows that the Golgi membrane is much more fragmented than in the control, suggesting that in fact Myt1 depletion has an early effect on Golgi fragmentation. Based on this picture, I do not entirely trust the quantitation n Figure 2D (S-phase). The authors need to change their conclusion This is an important point to mention because the authors go back to this statement in the discussion and this needs to be modified as well.

The reviewer has misread the figure. The immunofluorescence images shown in Figure 2D are of cells in G2 and not S-phase. The quantitation of Golgi fragmentation is of the cells in S-phase and G2. Therefore, there is no need to change our conclusion.

-Myt1 is known to inactivate CDK1 and the authors here show that a delay in Myt1 activation also leads to a lack of CDK1 activation with all the effects known on Golgi fragmentation. This is acknowledged in the text (page 16).
But the authors do not take this result in their model to suggest that in fact Myt1, via CDK1, also impact on the second phase of Golgi fragmentation.

This was stated clearly in the last paragraph of our discussion. The new text is pasted here. It is known that inactivation of Myt1 is required for the activation of CDK1. The identification of Myt1 in the MEK1 pathway is therefore important because it connects two sequential steps by which the pericentriolar Golgi stacks are first converted into small stacks by MEK1 and then into small tubules and vesicles by CDK1. The challenge now is to identify the kinase required for MEK1 dependent Myt1 phosphorylation and the target(s) of Myt1 on Golgi membranes.

-Last, the discussion is bizarrely constructed. The first part should be extended to incorporate the role of Myt1 on the Golgi organization in S phase, in G2 (their statement) and in mitosis via CDK1. This is important and would fit better with the strong phosphorylation of Myt1 at 10h, well into mitosis.

As mentioned above and shown in Figure 2D, Myt1 is not required for Golgi organization in S-phase. There is no need to change the discussion.

Furthermore, in the intro, the authors are careful to describe the two phases of Golgi fragmentation, The Golgi ribbon unlinking at G2 followed by Golgi stack fragmentation. Yet, in the rest van de manuscript, the authors only use "Golgi fragmentation" as a generic term without specifying which phase they mean. Defining this would be helpful in improving the manuscript

We have now edited the entire text to clarify this issue.

3rd Editorial Decision 16 November 2012

Thank you for submitting your revised manuscript. I appreciate the introduced changes and I am pleased to accept your paper for publication here. Thank you for contributing to the EMBO Journal!

Yours sincerely,

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Editor
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