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A dual role for cell plate-associated PI4Kβ in endocytosis and phragmoplast dynamics during plant somatic cytokinesis

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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 12th February 2018

Thank you for submitting your manuscript (EMBOJ-2017-98853) to The EMBO Journal and my apologies for the delay in getting back to you with a decision. The manuscript has now been reviewed by three expert referees whose comments are provided below. In light of these reports, I am afraid that the study is not a sufficiently strong candidate for publication here.

As you can see, while all referees consider the findings novel and potentially very interesting, the general consensus is that the main conclusions are not adequately supported by the experimental data.
In particular, the referees are mainly concerned that: i) the data showing the physical interaction of MPK4 and PI4K and the proposed model in which PI4K regulates MPK4 are not fully convincing; ii) MPK4 stabilization and microtubule accumulation at the phragmoplast in pi4kb1/pi4kb2 double mutants can be due to delayed or blocked cytokinesis at a specific stage; iii) MPK4 endocytosis at the cell plate is not supported by experimental data; and iv) the rescue experiment using KNOLLE is not conclusive as PI4Kb protein might be stable beyond G2/M phase.

Given these opinions from trusted experts in the field and the large number of essential further experiments with an uncertain outcome that would be needed to convincingly substantiate the proposed mechanism and addressing which would likely extend beyond our usual 3-6 month revision period, I am afraid that we cannot offer to invite a revised version of your manuscript. However, taking into account the potential interest of your findings, I would be willing to reconsider your study as a new submission at a later time if the referees' concerns could be fully addressed and their suggestions implemented.

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REFERE REPORTS

Referee #1:

- general summary and opinion about the principle significance of the study, its questions and findings

In this manuscript, Lin et al., addressed how PI4Kbetas (PI4KB) regulates plant cell cytokinesis. The pi4kβ1 pi4kβ2 double mutant was previously shown to bear cytokinesis defects (Kang et al., Traffic 2011), but the molecular bases for this phenotype were unknown. The authors report that PI4Kβ1 and its product PtdIns4P, localize to the cell plate. PI4KB1 expression during cell division is sufficient to complement the growth phenotype of pi4kβ1 pi4kβ2 double mutant, suggesting that the major function of PI4KBs in plants happens during cell division. In pi4kβ1 pi4kβ2 double mutant s, the authors found a persistent solid phragmoplast, a phenotype that appears to be shared with loss of function mutant of mpk4 kinase (Beck et al., 2011; Kosetsu et al., 2010). The authors show that PI4KB1 and MPK4 genetically and physically interact. Consistent with a delay in phragmoplast maturation, the pi4kβ1 pi4kβ2 double mutant shows a persistent MPK4 localization at the center of the cell plate. Next, the authors propose that cell plate-localized MPK4 is endocytosed by clathrin-mediated endocytosis (CME), which presumably removes MPK4 from the center of the cell plate and recycles it at the edge of the growing cell plate. This endocytic process is presumably impaired in the pi4kβ1 pi4kβ2 double mutant, which could explain the persistent MPK4 localization at the cell plate. The authors then assume that failure to remove MPK4 from the center of the cell plate is causal to the cytokinesis defects observed in the pi4kβ1 pi4kβ2 double mutant.

While the question of the role of PtdIns4P in plant cell cytokinesis is of high interest, I have major concerns about some of the experimental approaches and conclusions. In brief, the physical interaction between PI4Kβ1 and MPK4 is quite convincing, and since the corresponding mutants share phenotypes, the authors seem to favor a model in which MPK4 is regulated by PI4Kβ and its substrate (CF line 186/187). However, I find very little evidence for this scenario (see below). Why do they exclude the alternative model, in which PI4KB would be regulated by MPK4 rather than the other way around? This scenario seems certainly plausible and the same group actually recently showed that MPK6 interacts with and regulates PIP5K6 in pollen tube (Hempel et al., Plant Cell 2017). Indeed, in the current proposed model, it is not clear why MPK4 and PI4Kβ1 would have to physically interact at all. In addition, if aberrant cell division in pi4kβ1 pi4kβ2 are actually blocked at the disk phragmoplast stage, then it is expected that MPK4 and MAP65-3 would be retained at the cell plate (because they are normally present there at this stage) and it is expected that CLC2 should be absent (because it is not normally present there at this stage). I believe the reason for such delay (or blockade) is unclear at present but there is no indication that this should be an effect on MPK4. Overall, I do not think the model proposed in this manuscript is consistent with the data presented for the reasons highlighted bellow.

- specific major concerns essential to be addressed to support the conclusions

1) Cytokinesis is a dynamic process, which undergoes a number of well-established steps (see Smertenko et al., 2016 TIPS). In this manuscript, these stages are never identified and always only one snapshot is presented rather than a time series of the events that happen during cytokinesis. This is problematic, as in the current manuscript, it is impossible to compare the wild-type and the mutant situation in most figures. For example, in Figure 4d, which is pivotal for the story, the wild-type and pi4kβ1 pi4kβ2 double mutant are evidently not at the same stage (in the wild type, the cell plate is touching (or very close) to the plasma membrane, while it is not in the mutant). Therefore, rather than a persistence of the phragmoplast and MPK4 in the pi4kβ1 pi4kβ2 double mutant, it could be that the cells are not at the same stage in the control and mutant condition: i.e. at the disk phragmoplast stage in pi4kβ1 pi4kβ2, and at the ring or even discontinuous phragmoplast stage in the wild type. The authors should perform time-lapse analyses of defective cytokinesis in the pi4kβ1 pi4kβ2 double mutant (see for example Beck et al., 2011 New phytol, Steiner et al., 2016 Mol Plant). The authors should notably perform such analyses for the phragmoplast, MPK4, MAP65-3

and CLC2 and identify the relevant stages in each case. Such analyses will allow to clearly confirm if cytokinesis is delayed (or blocked) at a specific stage of somatic cytokinesis and should allow comparison of cells in similar stages. (Note that in Fig4d, the authors use the fact that nucleus is reformed to conclude that it is a late cytokinesis, but the nuclear envelope starts to reform in the phragmoplast initials stage and is fully completed at the disk phragmoplast stage and therefore before the late cytokinesis stage: i.e. ring and discontinuous phragmoplasts, cf Smertenko et al., 2016 TIPS). In addition, it is important to show the microtubule behaviors during the transition from the aborted phragmoplast and the interphasic cortical microtubules in the daughter cells.

Furthermore, there is an overall lack of quantification of the cytokinesis defects of the pi4kβ1 pi4kβ2 double mutant. What are the proportion/number of aberrant cell division, cell wall stub, multinucleate cells in the pi4kβ1 pi4kβ2 double mutant and how this related to the mpk4 cytokinetic phenotypes (which, from publications, appears to be stronger than that of the pi4kβ1 pi4kβ2 double mutant).

2) There are no data supporting the notion that MPK4 is endocytosed. Indeed, in Figure 5, the authors show that MPK4 colocalized with FM4-64, which likely indicates that MPK4 localize to the TGN. This makes sense considering that MPK4 interacts with PI4KB1, which is itself localized at the TGN (cf Preuss et al., 2006 JCB, Kang et al., 2011 Traffic). The TGN being BFA sensitive it is not surprising to see MPK4-labelled compartments being BFA-sensitive. This does not indicate in any way that MPK4 is endocytosed, but it rather shows that it resides in a BFA-sensitive compartment. The time-lapse presented in Figure 5c is by no mean proof of endocytosis or recycling of MPK4 from or to the cell plate. These are isolated events, with a low number of replicates (from n=6 to n=1) and the quality of the pictures/movie is low. But most importantly, there is no proof that these are genuine endocytic or recycling events, with scission or fusion of membranes. Indeed, the compartment labeled with the arrow could very well be a free moving TGN that happens to pass by the cell plate (especially given the low number of events reported). Finally, the demonstration that MPK4 is indeed endocytosed by clathrin-mediated endocytosis (CME) is provided using TyrA23, which is claimed to be a specific inhibitor of CME. While this drug has been used quite extensively in the past, it was recently showed that TyrA23 is, in fact, a protonophore, which depletes the cell from ATP and rapidly acidified the cytoplasm (Dejonghe et al., 2016 Nat Comm). TyrA23 in turn not only inhibits CME, but all vesicular trafficking events (among other effects). Bearing in mind this paper, TyrA23 cannot be used as an inhibitor of CME and this figure should be removed (indeed, it is not possible to form a BFA body, if the movement of TGN compartments is inhibited by depletion of cellular energy upon TyrA23). For these reasons, the idea that MPK4 is endocytosed to be cleared from the center of the cell plate is not supported by any experiments. There is currently no chemical compound in plants that can be reliably used to inhibit CME. However, inducible genetic systems have been shown to inhibit endocytosis and could be used to show that MPK4 indeed undergo endocytosis in order to be removed from the center of the cell plate (for example based on the inducible expression of auxilin2, see Ortiz-Morea et al., 2016 PNAS). In addition, if the accumulation of MPK4 in BFA bodies is indeed due to its endocytosis, and if MPK4 endocytosis is impaired in the pi4kβ1 pi4kβ2 double mutant, then MPK4 should not be found on BFA bodies in this mutant.

Furthermore, whether there is actually CME at the cell plate has never been formally shown, at least on early stages of cytokinesis. Of note, according to Ito et al., CLC2 is recruited to the cell plate relatively late (at least later than the dynamin-related protein DRP1). Because the pi4kβ1 pi4kβ2 double mutant appears to be blocked at the disk phragmoplast stage, it is not surprising that CLC2 is not found in the mutant. Whether this is because of a specific inhibition of endocytosis by the lack of PtdIns4P or because of a stage transition issue is not resolved and therefore causation cannot be inferred. Again, as explained in point #1, it will be important to make a time-lapse analysis of CLC2 localization in the wild-type and pi4kβ1 pi4kβ2 double mutant.

3) The rescue of the growth defect by KNOLLE does not convincingly show that specific PI4KB expression during cell division is sufficient to rescue most phenotypes related to the double mutant. Indeed, while this promoter is indeed tightly regulated during cell division, what makes KNOLLE protein so tightly expressed, is its degradation following cytokinesis via endocytosis and routing to the vacuole (see for example Boutte et al., 2009 EMBO). It is therefore likely that PI4Kβ1 protein (and its product), may persist post cytokinesis and be stable after cell division. This has actually been shown previously for CPI1 (see Men et al., 2007 Nat Cell Biol). The author should show that

pKNOLLE::Flag-PI4Kβ1 is indeed expressed (at the protein level) only during cytokinesis or change their conclusion accordingly. An additional control would be to look at the phenotype of the root hairs from the pKNOLLE::Flag-PI4Kβ1/pi4kβ1;β2 genotype. Indeed, the pi4kβ1 pi4kβ2 double mutant has a strong root hair phenotype, that is arguably a post-cytokinetic process. If this root hair phenotype is rescued by pKNOLLE::Flag-PI4Kβ1, this would argue that PI4Kβ1 is likely stable and still present after cytokinesis in these plants.

- minor concerns that should be addressed

- The authors state line 367: "Considering i) that the internalization of MPK4-YFP was CME dependent (cf. Fig. 6) and ii) that MPK4-YFP was found to be stabilized at the cell plate in the pi4kβ1 pi4kβ2 double mutant (cf. Fig. 4d), our results indicate that mpk4-2-like cytokinetic defects of the pi4kβ1 pi4kβ2 double mutant are a consequence of mistargeting of MPK4 at the cell plate, likely due to failing CLC2 recruitment and concomitantly reduced CME of MPK4-YFP." This causation is by no mean demonstrated. If the authors want to show causation, they should experimentally remove MPK4 from the center of the cell plate in the pi4kβ1 pi4kβ2 double mutant and show that this can rescue the phenotype. Because this is obviously a very difficult experiment, the author may alternatively change their conclusion and be more cautious.
- Line 88-89, please include a reference to this statement.
- The authors introduce the NACK-PQR pathway in the introduction (line 68-79) but do not introduce the literature on MPK4. This section should be revised to include an introduction on MPK4, which is at the center of the manuscript.
- Line 92, discussion about CESA6 role during cytokinesis is not relevant to the paper and should be discarded. However, a better introduction to the regulation of cytokinesis by MPK4 and other MAPKK like MKK6/ANQ would be helpful.
- Line 115, the number/proportion of cell wall stubs and oblique cell wall should be quantified in the double mutant and complemented lines.
- Line 125, this sentence implies that mCHERRY-PI4Kβ1 is only at the cell plate, but it is also present in dotty structure (cf Fig 1c and d). This should be mentioned, notably as this kinase was previously shown to localize to the TGN (cf Preuss et al., 2006 JCB, Kang et al., 2011 Traffic). The sentence line 117-119 should also be revised accordingly since in fact the localization of PI4Kβs has been described previously.
- Line 153 (related to figure 1e), calcofluor labels cellulose not cell wall. The data presented figure 1e suggest that PtdIns4P appears before deposition of cellulose, not cell wall (for example, it is likely that it would colocalize with a callose dye). It is not clear how relevant is this panel to the story.
- Line 161/162, the number/proportion of multiple nuclei and phragmoplast should be quantified.
- Line 292/293 (related to Figure S6), I am not convinced that PtdIns4P arrives later than MPK4 at the cell plate based on this movie. There seems to be an RFP signal, albeit weak, at the second time point (where MPK4 is first found to localize at the cell plate). This result is rather odd since it was reported by Kotetsu et al., TPC 2010 that MPK4-GFP colocalizes with FM4-64 at the cell plate at early stages, when the nuclei had not yet reformed and that PtdIns4P biosensor also colocalizes with FM4-64 as soon as it appears (Simon et al., Nat Plant 2016). The authors should provide example of several independent movies and show some sort of quantification (for example by making a line scan across the MPK4-decorated cell plate to see whether there is RFP signal or not) and perform colocalization with FM4-64 to know if MPK4 signal will arrive before "vesicle assembly" at the cell division plane.
- Figure 4, the authors should add the time points in panel a. It is puzzling to see the mCherry-PI4KB1 signal disappearing in the 4th picture of the time-lapse and then reappearing in the last panel (are they inverted?).
- Figure 7d, the localization of GFP-MAP65-3 does not significantly differ from its localization in the wild-type as reported by Caillaud et al., (2008 Plant Cell). The authors should be more careful in their conclusion.
- Figure 7e, the authors argue that "Because of robust microtubule bundles in the phragmoplast, it is often difficult to measure the dynamics of phragmoplast microtubules". However, it is again possible to look by time-lapse analysis to MTs behaviors during the transition from the aborted phragmoplast and the interphasic cortical microtubules. In any case, it does make any sense to analyze microtubule dynamics in interphasic cells (differentiated root cells), a stage in which according to the authors, PI4KB1 does not play a significant role. If the authors want to confirm the weak difference in shrinkage they observed, they should consider performing additional experiments

such as oryzalin treatment in both WT and pi4kβ1 pi4kβ2 double mutant. In addition, the authors could check the effect of oryzalin on the persisting phragmoplasts in the pi4kβ1 pi4kβ2 mutant in order to address if CLC and MPK4 would relocalize upon MTs depolymerization.

Referee #2:

The manuscript by Lin et al. provides the first description of a role for a phosphatidylinositol 4-phosphate (PtdIns(4)P kinase) in modifying MAP-kinase (MPK) localization during cytokinesis. Moreover, its relation to cytokinetic downstream events mediated by microtubule-associated protein 65-3 (MAP65-3 also known as PLEIADE) is addressed in this work. While roles for PtdIns(4)P kinases of different families have been reported in other systems than the plant Arabidopsis thaliana (Arabidopsis) analyzed here, this is to my knowledge the first report on a role for PtdIns(4)P kinase/MPK/MAP signaling during cytokinesis in any organism.

Thus, the study is novel, certainly of broad interest to the plant science community but also to some readers working on phospholipid and MAP-kinase signaling as well as cytokinesis in other systems. The findings should therefore to some extent appeal to a broader readership such as the one covered by The EMBO Journal.

The work initially analyses the phenotype of a double mutant defective in the PtdIns(4)P kinase beta 1 and PtdIns(4)P kinase beta 2 (pi4kb1 pi4kb2) as well as its rescue by an mCherry-PI4Kb1 fusion protein expressed from the pPI4Kb1 promoter (pPI4Kb1: mCherry-PI4Kb1). The construct is used for susequent analyses as it proves fully functional. The pi4kb1 pi4kb2 double mutant displays cytokinesis defects and mCherry-PI4Kb1 localizes to the cell plate, a plant-specific cytokinetic membrane structure supporting a function of PI4Kb1 in cytokinesis. Moreover, a fluorescent reporter for PtdIns(4)P confirms its presence in the cell plate (all Fig. 1). While the figures are of very high quality, it would be helpful to obtain quantitative information of cytokinesis-defective cells in the pi4kb1 and pi4kb2 single as well as pi4kb1 pi4kb2 double mutant (Fig 1b).

The authors proceed to characterize the cytokinesis defects of the pi4kb1 pi4kb2 double mutant by employing nuclear markers, localization of tubulin and of the cytokinesis-specific KNOLLE syntaxin by immunofluorescence microscopy. The results reveal the occurrence of multinucleate cells and aberrations in the organization of the phragmoplast, a plant-specific cytokinetic microtubule (MT) array. Again the images are of very high quality, but no quantitative data is provided (Fig. 2)

Lin and colleagues proceed to address the role of plant MPK4 in relation to PI4Kb1 PI4Kb2 by aiming to analyze a pi4kb1 pi4kb2 mpk4-2 triple mutant. In comparison to pi4kb1 pi4kb2 double and mpk4-2 single mutants, no viable homozygous triple mutants are recovered. Interestingly, also pi4kb1 pi4kb2/+ mpk4-2 mutants show a strongly reduced seedling size compared to pi4kb1 pi4kb2 and mpk4-2 mutants, suggesting a synergistic genetic interaction. The latter is not spelled out as such in the manuscript, which it should. Moreover, while the data clearly suggests synergistic genetic interaction of at least one of the PI4Kb s with MPK4 it is not clear which one (or both?) is responsible, as pi4kb1 mpk4-2 doubles etc. were not analyzed. Nonetheless, the data supports a synergistic genetic interaction of either PI4Kb1 and/or PI4Kb2 with MPK4.

The authors further reveal interaction of the 566 N-terminal amino acids of PI4Kb1 with MPK4 by yeast two hybrid (Y2H) assays as well as co-immunoprecipitation of MPK4-myc and PI4Kb1 from Arabidopsis protein extracts. Here, it should be indicated, how often the assays were repeated with similar results (Fig. 3 b,c). The claim that these experiments demonstrate a physical interaction of PI4Kb1 and MPK4 (page 11, line 231) is an overinterpretation as no direct biochemical binding assays or other direct interaction assays like split-YFP, FLIM/FRET experiments were carried out. The latter might have been a possibility, as the authors provide colocalization data of mCherry-PI4Kb1 and MPK4-YFP in Fig. 4a.

The authors further express FLAG-PI4Kb1 under control of the KNOLLE promoter in the pi4kb1

pi4kb2 double mutant, which completely rescues the double mutant phenotype, leading the authors to speculate that "one main function of PI4Kb1 in A. thaliana is the formation of PtdIns(4)P during the G2/M phase to control somatic cytokinesis". To support this conclusion, however, it would have been necessary to address as to whether FLAG-PI4Kb1 is actually only expressed during G2/M by immunofluorescence localization. It is possible that the protein is stable and persists throughout interphase and into cell elongation. This has been reported e.g. for a sterol biosynthesis protein expressed from the KNOLLE promoter (Men et al., 2008, Nat. Cell Biol.).

The authors further report as stabilization of MPK4-YFP and tubulin at the phragmoplast in the pi4kb1 pi4kb2 double mutant. However, it is not clear as to whether the same cytokinetic stages were analyzed and the phragmoplast was imaged in the same plane. It would be helpful to perform this experiment on image 3D image stacks, to include one at least in the supplementary material, so it is clear where in the cell the phragmoplast has which appearance in wild type and mutant, and to include numbers of the image stacks analyzed.

Lin et al. further perform experiments employing the vesicle trafficking inhibitor brefeldin A (BFA) and the translation inhibitor cycloheximide (CHX) from which they conlude that MPK4 is targeted to the cell plate and also underwent endocytosis. However, the authors do not show that CHX does actually completely or almost completely inhibit de novo synthesis of MPK4-YFP under their conditions at this stage of the cell cycle. This could be addressed by performing fluorescence recovery of photo-bleaching (FRAP) analyses of MPK4-YFP in the cell plate under CHX treatment and without. Additional more conclusive data could be obtained by employing photo-activatable (PA) GFP fusions or photo-switchable fusions of MPK4. The data as it is presented in Fig. 5 on its own is not conclusive, yet.

The authors further attempt to address the potential role of clathrin-dependent endocytosis on MPK4-YFP trafficking mainly through application of the rather unspecific inhibitor tyrphostin A23 (Tyr A23) which is known to have many different effects than inhibiting cargo recognition by AP2 associated adaptor proteins in endocytosis (Dejonghe et al., 2016, Nat. Commun.). Application of this inactive analogue Tyr A51 alone is not useful here, as it is not at all clear as to whether the Tyr A23 effect is restrict to the endocytis AP2 coat or very different cellular events (Dejonghe et al., 2016). Here, it would be necessary to involve different genetic and/or pharmacological tools like dynamin-related protein or AP2-coat protein mutants or dominant interference with the clathrin coat using the HUB domain or other proteins, as other authors have done. Otherwise this claim cannot be made.

The authors find that MPK4-YFP is stabilized at the cell plate of the pi4kb1 pi4kb2 double mutant and that its association with CLC2-GFP at the cell plate was largely reduced. They therefore hypothesize that clathrin recruitment is reduced and MPK4-YFP accumulates, which is a reasonable hypothesis, but could have been supported by one or two more markers. The KNOLLE protein and PIN proteins are known to associate with the parental plasma membrane in endocytosis defective mutant cells, where the cell plate has only attached to one parental plasma membrane, yet (e.g. Boutte et al., 2010, EMBO J.; Gadeyne et al., 2014, Cell and several other papers). Does KNOLLE show the same mislocalization in the pi4kb1 pi4kb2 double mutant as in other endocytosis-defective mutants?

The authors continue to address localization of GFP-MAP65-3/PLEIADE in the pi4kb1 pi4kb2 double mutant and find it to potentially over-accumulate at the mutant phragmoplast. Again here 3D-image stacks should be provided (in the supplement), so the reader cannot judge whether this really reflects an accumulation rather that a top view in the mutant versus a median section of the cell in the wild type in Fig. 7a. 3D image stacks in the Supplementary material would be helpful here. Moreover, the panel labeling is mixed up in panel Fig. 7d and needs to be swapped between FM4-64 and MAP65-3.

Finally, the authors address the effect of the pi4kb1 pi4kb2 double mutant on microtubule growth and shrinkage in elongating cells, which is somewhat off the track as most other data deals with cytokinesis. This part may be removed here.

Taken together, this is certainly a novel and interesting study on a topic of general interest. The PI4Kbeta1 MPK4 interaction is already worked out to quite some extent. However, in its current

form the study still requires a larger number of experimental revisions, thinking over as well as rewriting of some of the claims that do not always reflect the data presented, yet.

Major points for revision:

1) Inclusion of quantitative information on cytokinesis-defective cells in the pi4kb1 and pi4kb2 single as well as the pi4kb1 pi4kb2 double mutant (Fig 1b).

2) Inclusion of quantitative data in Fig. 2.

3) Analysis of pi4kb1 mpk4-2 and pi4kb2 mpk4-2 double mutants for genetic interaction, or weakening of the claim to that synergistic genetic interaction occurs for either PI4Kb1 and/or PI4Kb2 with MPK4.

4) Weakening of the claim that the experiments demonstrate a physical interaction of PI4Kb1 and MPK4 (page 11, line 231) or performance and inclusion of direct biochemical binding assays or other direct interaction assays like split-YFP, FLIM/FRET experiments.

5) Performance and inclusion of immunofluorescence labeling of FLAG-PI4Kb1 to test whether this really only persists throughout G2/M when expressed from the KNOLLE promoter.

6) Performance and inclusion of 3D-stack imaging data on MPK4-YFP and tubulin localization at the phragmoplast in the pi4kb1 pi4kb2 double mutant.

7) Performance and inclusion of fluorescence recovery of photobleaching (FRAP) analyses of MPK4-YFP in the cell plate under CHX treatment and without. Optionally, employing photoactivatable (PA) GFP (PA-GFP) fusions or photoswitchable fusions of MPK4 could provide more conclusive data on from where to where the protein is really moving.

8) Additional tools to the relatively unspecific inhibitor tyrphostin A23 (Tyr A23) (Dejonghe et al., 2016, Nat. Commun.) need to be employed to address whether MPK4 localization depends on clathrin-dependent endocytosis e.g. drp1a mutants (e.g. Boutte et al., 2010, EMBO J.) or inducible TPLATE downregulation (Gadeyne et al., 2014, Cell) or another similar genetic tool.

9) In Fig. 7a,b 3D-image stacks should be provided (in the supplement), so the reader can judge whether this really reflects an accumulation rather that a top view in the mutant versus a median section of the cell in the wild type in Fig. 7a.

10) Correct the panel labeling Fig. 7d that needs to be swapped between FM4-64 and MAP65-3.

11) Page 24, Line 494. "Here we found that PtdIns(4)P controls cytokinesis ...". This claim cannot be made as no gain-of-function studies i.e. overexpression studies have been performed, since solely loss-of-function studies were performed. The claim must be weakened to "Here we found that PtdIns(4)P is required for cytokinesis ... " or additional gain-of-function studies need to be included.

12) Page 25, line 507. "Our data uncover that the defective lateral expansion of phragmoplasts in pi4kb1 pi4kb2 double mutants was a consequence of mistrafficking of MPK4 at the cell plate ... ." The study contains no experiment to functionally support this claim. This is just an interpretation based on several correlations. This claim must be weakened

Referee #3:

In the manuscript, entitled "Cell plate-associated PI4Kβ is essential for cytokinetic phragmoplast expansion in Arabidopsis" the authors carefully characterized a cytokinetic defect in a pi4kβ double mutant by beautiful live-imaging and immuno-staining with newly raised antibodies, particularly focusing on PI4KB1&2 and their role MPK4, MAP65-3 and PIP4 behavior during cytokinesis. In

this manuscript, the authors claim that PIP4 accumulation on the developing cell plates decreases in the double mutant, which in turn reduces clathrin-mediated vesicle transport from the cell plates. Then, this reduction of CME induces excessive amount of MPK4 accumulation on the cell plate, thereby affecting phragmoplast microtubules turnover. Although the imaging data itself in the manuscript is very clear, I am not convinced by some biochemical data and interpretation of the data for establishing the roles of pi4kβ in cytokinesis, particularly the relationship between abnormal MPK4 accumulation and the stability of phragmoplast microtubules. The authors should clarify some of their interpretation and should provide additional experiments for more convincing conclusions. The most relevant advance in this paper is the new link between MPK4 and PIP4K. I think that this alone would be enough if this link was more clearly demonstrated.

My biggest concern from is the relationship of over-stabilization of microtubules and excess accumulation of MPK4 on the cell plates. As the authors mentioned in the introduction (line 75-78) "NRK1 finally phosphorylates the microtubule-associated protein 65-1 (NtMAP65-1), thereby decreasing its capacity to bundle microtubules, facilitating turnover and radial expansion of the phragmoplast. All counterparts for the NACK-PQR pathway have been identified also in Arabidopsis", MPK4 potentially induces microtubule destabilization around the cell plates when it is activated. The authors show that in Figure 4d, MPK4 accumulates evenly on the developing cell plates in the pi4kβ double mutant even during the late cytokinesis stage, and in the same figure the authors show over-stabilization of phragmoplast microtubules. I would assume that microtubules are destabilized because of abnormally accumulated MPK4 on the cell plates. This point is the biggest missing link to explain why the cytokinetic defects happens in the pi4kβ double mutant if the authors think over-stabilized phragmoplast induces the defect in expansion of cell plates process. Related to this, I am also wondering about the phosphorylation status of MAP65-3 in the pi4kβ double mutant. Please clarify this point by experiment.
The authors also claim that the kinase activity of MPK4 is not affected by pi4kβ1/2 mutations by immuno-complex kinase assay, but the authors did experiments with whole seedlings as far as I understand from the materials and methods. I think that most of the MPK4 came from interphase cells in this assay and how MPK4 activity changes in dividing cells is therefore not clear. I suggest collecting samples from root tips, like in Kosetsu 2011 Plant Cell, and do an immuno-kinase complex assay with MPK4 kinase activity from dividing cells. In that paper, they succeeded to show meristematic regions have much stronger kinase activity. I assume that this assay will help to understand whether MPK4 has stronger or weaker activity in the pi4kβ double mutant.

The authors hypothesize that MPK4 is a candidate for PI4Kβ- or PtdIns(4)P-dependent regulation, but this connection is not firmly established:
In Figure 3, the authors show genetic interaction and physical interaction between MPK4 and pi4kβ1/2. I do not understand what the authors want to conclude from these data with respect to the cytokinetic defects. The authors assessed the genetic interaction between two kinases by plant growth of multiple mutants, but do not show whether cytokinetic defects were enhanced or not. Moreover, according to Zhang et al. Cell Host Microbe 11: 253-263 (2012), growth defect in the mpk4 mutant is mainly thought to be a result of enhanced salicylic acid signaling. Again, as the authors cited Šašek V, et al. 2014 New Phytologist, over-accumulation of salicylic acid can induce this growth defect. Please explain how these data pertain to understanding the cytokinetic defect in the mpk4 or the pi4kβ1/2 double mutants.
Additionally, I am wondering what the physical interaction between MPK4 and PI4kβ tells about their functional interaction. The authors only show the possibility that PI4Kβ affects MPK4 function, but I think it is at least as reasonable to assume the reverse, i.e. that MPK4 regulates PI4kβ1/2 function. The behavior of PI4kβ1/2 in mpk4 is still unclear from this manuscript, as is the activity of PI4kβ1/2. The alternative - that MPK4 and PIPK4 meet at the phragmoplast where MPK4 activates PIP4K (by phosphorylation), generating PI(4)P and starting to give the cell plate a "plasma membrane" identity and allowing CME to occur (the delayed appearence of PI4P shown in FigS6 is consistent with this). CME would deplete MPK4 from the PI(4)P-containing CP restricting it to the phragmoplast at the growing CP where it should stabilize MTs. (it would be a nice self regulatory loop for MPK4 localization at growing CP)

To explore this possibility, the authors should add data with marker lines in the mpk4-2 mutant. -- Particularly, RFP-FAPP-PH and CLC2-GFP and mCherry-PI4Kβ markers in mpk4 mutant background are essential, also localization at the CP of RFP-FAPP1 in pip4k would be good to see (it should be gone). Phosphorylation status of PIPK4 in WT vs mpk4 or in vitro phosphorylation

assay of PIP4K by MPK4 Some data suggesting that PI(4)P is required for endocytosis - such as KNOLLE localization at PM in pip4k mutants, for example? Or altered CLC/DRP1A dynamics in pip4kP I see that - if not already initiated - these experiments are quite lengthy and and additional biochemistry experiments might be faster although not as convincing on their own.

Minor points:
Line 91, "CME" appeared the first time here but no explanation for the abbreviation
Line 176, a comma is missing between c and f

The authors mention a "specific antibody" line 226, then mention an "aspecific band "in figS2. it is clear that their antibody recognize PIP4KB1 based on the disappearing band in pipk4B1 but it is not specific (just semantics) Fig7c : I really like the orthogonal view depicting a ring-like localization (or not) of MAP65 and TUB. It could be used (if possible with good signal) earlier in the figure for MPK4, PIP4K or FAPP1 localization (or colocalization with FM4) Fig7d: FM4 and GFP-MAP65 legend is inverted Fig7e: The MT dynamic in root elongating cell does not relate in anyway to the rest of the work here, so we cannot conclude that MT shrinkage is also affected at the CP in pipk mutants. This data could go to supplement

Itemized response to reviews:

Editor's summary

[...] In particular, the referees are mainly concerned that:

i) the data showing the physical interaction of MPK4 and PI4K and the proposed model in which PI4K regulates MPK4 are not fully convincing;

Response:

We thank the reviewers a) for pointing out the overreaching interpretation of the interaction tests and b) for suggesting a better model to tie the data together. To point a): While we read the reviewers' comments to mean that the interaction data are generally convincing, reviewer 2 is correct that our yeast-two-hybrid and Co-IP data do not demonstrate a direct physical interaction of PI4Kβ1 and MPK4. We now additionally show phosphorylation of purified recombinant PI4Kβ1 by purified recombinant MPK4 in vitro, supporting a direct physical association of the partners. However, as the other experiments only indicate interaction within a possibly larger protein complex, we have weakened our claim as suggested by reviewer 2. To point b): This is a profound comment, which prompted us to reconsider our data and largely rewrite the entire manuscript. We have followed the advice of all three reviewers to consider a scenario different from that proposed in our original submission. In brief, we are no longer proposing that PI4Kβs regulate the subcellular distribution or membrane-associated trafficking of MPK4 to or from the cell plate, or that MPK4 is a CME cargo. Instead, in line with our aim to delineate the role of PI4Kβs in cytokinesis, we now begin by characterizing the effects of PI4Kβs on membrane trafficking and cytoskeleton during cell plate formation, arrive at the link to MPK4 from the shared phenotypes, demonstrate genetic and physical (...) interaction, and then follow the reviewers' suggested model that MPK4 might influence PI4Kβs and PtdIns4P formation at the cell plate. Based on additional experiments suggested by the reviewers, we find support for this model and now show, e.g., that cell plate-association of the PtdIns4P-reporter FAPP1 is altered in mpk4-2 mutants, confirming regulation of PI4Kβs by MPK4. We are thus glad for the many constructive suggestions. The conceptual change resulted in extensive revision of our text, and a number of critical points raised by the reviewers are no longer central to the story. Therefore, a number of experiments were not performed exactly as suggested, as is outlined in detail in the respective responses below. We have nonetheless performed extensive additional experiments to address both the conceptual and technical issues raised by the reviewers. Our altered chain of argument now culminates in proposing MPK4 as an interacting player in the same pathway as PI4Kβs to control MAP65-3-mediated

phragmoplast dynamics at the cell plate. For details we kindly refer to our responses to the individual reviewer comments.

ii) MPK4 stabilization and microtubule accumulation at the phragmoplast in pi4kβ1/pi4kβ2 double mutants can be due to delayed or blocked cytokinesis at a specific stage;

Response:

We thank you and the reviewers for this insightful comment. To investigate this issue, the reviewers suggested time lapse imaging and we have now performed substantial time lapse experiments additionally using z-stacks to capture the continuous progression of cytokinesis over time. Based on these data we can much better assess the behavior of clathrin, phragmoplast microtubules and of MAP65-3, and report severely altered but still continuous progression of cytokinetic processes in the pi4kβ1 pi4kβ2 double mutant. A more detailed response is given in the responses to individual reviewer comments.

iii) MPK4 endocytosis at the cell plate is not supported by experimental data;

Response:

The reviewers are correct and we apologize for our mistake. We have eliminated the non-conclusive and uninterpretable inhibitor experiments and do no longer claim that MPK4 is a CME cargo.

iv) the rescue experiment using KNOLLE is not conclusive as PI4Kβ protein might be stable beyond G2/M phase.

Response:

We agree that the PI4Kβ1 protein expressed from the KNOLLE promoter might persist. Despite of substantial efforts, we were unable to detect the FLAG-tagged protein in immunostains, so we have toned down the interpretation of this result and no longer claim that the main function of PI4Kβs is in cytokinesis. A number of additional new experiments furthermore indicate roles for PI4Kβ1 outside of cytokinesis, including the fact that a root hair defect of the pi4kβ1 pi4kβ2 double mutant (which is unrelated to cytokinesis) is not rescued by pKNOLLE-driven PI4Kβ1. In the course of additional related experiments, we were able to observe interesting KNOLLE distributions that now allow to better pinpoint the effects of misexpression of PI4Kβ1 on endocytosis at the cell plate. Overall, we found a constructive way to present the pKNOLLE complementation data that is consistent with numerous other new observations (for detail, see below).

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Referee #1:

In this manuscript, Lin et al., addressed how PI4Kβetas (PI4KB) regulates plant cell cytokinesis. The pi4kβ1 pi4kβ2 double mutant was previously shown to bear cytokinesis defects (Kang et al., Traffic 2011), but the molecular bases for this phenotype were unknown. The authors report that PI4Kβ1 and its product PtdIns4P, localize to the cell plate. PI4KB1 expression during cell division is sufficient to complement the growth phenotype of pi4kβ1 pi4kβ2 double mutant, suggesting that the major function of PI4KBs in plants happens during cell division. In pi4kβ1 pi4kβ2 double mutant s, the authors found a persistent solid phragmoplast, a phenotype that appears to be shared with loss of function mutant of mpk4 kinase (Beck et al., New Phytol 2011; Kosetsu et al., Plant Cell 2010). The authors show that PI4KB1 and MPK4 genetically and physically interact. Consistent with a delay in phragmoplast maturation, the pi4kβ1 pi4kβ2 double mutant shows a persistent MPK4 localization at the center of the cell plate. Next, the authors propose that cell plate-localized MPK4 is endocytosed by clathrin-mediated endocytosis (CME), which presumably removes MPK4 from the center of the cell plate and recycles it at the edge of the growing cell plate. This endocytic process is presumably impaired in the pi4kβ1 pi4kβ2 double mutant, which could explain the persistent MPK4 localization at the cell plate. The authors then assume that failure to remove MPK4 from the center of the cell plate is causal to the cytokinesis defects observed in the pi4kβ1 pi4kβ2 double mutant.

While the question of the role of PtdIns4P in plant cell cytokinesis is of high interest, I have major concerns about some of the experimental approaches and conclusions.

Criticism:

In brief, the physical interaction between PI4Kβ1 and MPK4 is quite convincing, and since the corresponding mutants share phenotypes, the authors seem to favor a model in which MPK4 is regulated by PI4Kβ and its substrate (CF line 186/187). However, I find very little evidence for this scenario (see below). Why do they exclude the alternative model, in which PI4KB would be regulated by MPK4 rather than the other way around? This scenario seems certainly plausible and the same group actually recently showed that MPK6 interacts with and regulates PIP5K6 in pollen tube (Hempel et al., Plant Cell 2017). Indeed, in the current proposed model, it is not clear why MPK4 and PI4Kβ1 would have to physically interact at all.

Response:

We agree with the reviewer that a model in which MPK4 acts as an upstream regulator of PI4Kβ1 appears plausible and likely. In fact, while preparing the revision, we noticed the supplement to a recent report on a screen for MAPK-targets, indicating that PI4Kβ1 can be a target for MPK4 (Latrasse et al., Genome Biology 2017). In response to this comment (and

equivalent ones by the other reviewers), we have conceptually changed our story to address the notion that MPK4 may act upstream of PI4Kβ1. Based on additional experiments, we can confirm that PI4Kβ1 is phosphorylated in vitro by MPK4 (Supplementary Fig. 9), and - ultimately - that the levels of PtdIns(4)P are altered at the cell plate in cytokinetic cells from mpk4-2 mutants (Fig. 10e). We are now discussing the likely complex interplay of MPK4, PI4Kβ1 and PtdIns4P during plant cytokinesis in light of the conceptual change suggested by the reviewers and based on substantial new data included in our expanded manuscript.

Criticism:

In addition, if aberrant cell division in pi4kβ1 pi4kβ2 are actually blocked at the disk phragmoplast stage, then it is expected that MPK4 and MAP65-3 would be retained at the cell plate (because they are normally present there at this stage) and it is expected that CLC2 should be absent (because it is not normally present there at this stage). I believe the reason for such delay (or blockade) is unclear at present but there is no indication that this should be an effect on MPK4. Overall, I do not think the model proposed in this manuscript is consistent with the data presented for the reasons highlighted bellow.

Response:

The reviewer is correct in pointing out that a block in cytokinesis might be one possible reason for the effects observed. To address the issue of possibly blocked progression of cytokinesis in the double mutant, we have now performed substantial time lapse imaging experiments. Importantly, these experiments show continuous - not blocked - progression of cytokinesis in both wild type and mutant (although in the mutant phragmoplast progression is disorderd and delayed in time, Fig. 7). We agree with the reviewer that our original model did not comply with the data presented and are thankful for the insightful suggestions. As outlined in the response to the previous comment, we have modified our working hypothesis according to the reviewers' suggestions. Because of the altered logic, we do no longer include data on MPK4 distribution being affected by PtdIns4P or PI4Kβ1, as is also further detailed below.

- specific major concerns essential to be addressed to support the conclusions

Criticism:

1) Cytokinesis is a dynamic process, which undergoes a number of well-established steps (see Smertenko et al., Trends Plant Sci 2016). In this manuscript, these stages are never identified and always only one snapshot is presented rather than a time series of the events that happen during cytokinesis. This is problematic, as in the current manuscript, it is impossible to compare the wild-type and the mutant situation in most Fig.s. For example, in Fig. 4d, which is pivotal for the story, the wild-type and pi4kβ1 pi4kβ2 double mutant are

evidently not at the same stage (in the wild type, the cell plate is touching (or very close) to the plasma membrane, while it is not in the mutant). Therefore, rather than a persistence of the phragmoplast and MPK4 in the pi4kβ1 pi4kβ2 double mutant, it could be that the cells are not at the same stage in the control and mutant condition: i.e. at the disk phragmoplast stage in pi4kβ1 pi4kβ2, and at the ring or even discontinuous phragmoplast stage in the wild type. The authors should perform time-lapse analyses of defective cytokinesis in the pi4kβ1 pi4kβ2 double mutant (see for example Beck et al., New Phytol 2011; or Steiner et al., Mol Plant 2016).

Response:

We thank the reviewer for this insightful comment and the suggestion to perform time lapse experiments. In the pi4kβ1 pi4kβ2 double mutant we regularly observed that no cell plate is formed (Fig. 3 d and Fig. 9 b, c). To understand the consequences of the lesions in the PI4Kβ1 and PI4Kβ2 genes, we were therefore left to study those instances where (partial) cell plates do still form in the mutant. Clearly, the dynamic nature of cytokinesis makes it difficult to judge the stages of individual images, which then cannot be compared. To address this issue, we have performed extensive time lapse imaging of cytokinesis in wild type and pi4kβ1 pi4kβ2 double mutants, using cells displaying roughly comparable cytokinesis (new Figs. 4, 7 and 8). As the imaging plane at which the growing cell plate is captured is an additional issue, we have performed these imaging experiments using z-stacks, resulting in 4D-image series of cytokinesis, which now can be better compared (see also the respective comment by reviewer 2 below). The data presented in the new Fig.s 4, 7 and 8 indicate, e.g., that phragmoplast transition progresses continuously from initiation to disbadning of the phragmoplast in both wild type and mutant and does not arrest at a particular stage. However, in the mutant the process occurs in a disorderly fashion and is significantly delayed (Fig. 7). The new time lapse imaging results furthermore show that in the mutant, the phragmoplast regulator MAP65-3 appears as a solid plate instead of a ring over a prolonged time spanning the entire duration of wild type cytokinesis (Fig. 8). This finding is consistent with the characteristic patterns we had observed before when using individual images. Thus, the suggested additional experiments included in the revised manuscript helped greatly to give a more comprehensive picture.

Criticism:

The authors should notably perform such analyses for the phragmoplast, MPK4, MAP65-3 and CLC2 and identify the relevant stages in each case. Such analyses will allow to clearly confirm if cytokinesis is delayed (or blocked) at a specific stage of somatic cytokinesis and should allow comparison of cells in similar stages. (Note that in Fig4d, the authors use the

fact that nucleus is reformed to conclude that it is a late cytokinesis, but the nuclear envelope starts to reform in the phragmoplast initials stage and is fully completed at the disk phragmoplast stage and therefore before the late cytokinesis stage: i.e. ring and discontinuous phragmoplasts, cf Smertenko et al., 2016 TIPS).

Response:

Time lapse imaging was performed for phragmoplasts (Fig. 7), MAP65-3 (Fig. 8) and CLC2 (Fig. 4), as suggested by the reviewer. We did not perform time lapse imaging for MPK4, because the weak fluorescence signal of the MPK4 marker technically limited prolonged imaging times, and - more importantly - because with the conceptual change in our logic MPK4 distribution was no longer considered critical for our story. Cytokinetic stages are defined by Smertenko et al. Trends Plant Sci (2016) according to characteristic microtubular patterns of the phragmoplast transition (initiation, solid, ring, discontinuous, etc.), and these stages have now been assigned to wild type controls as suggested (Fig. 7a). However, the grossly changed microtubule patterns in the pi4kβ1 pi4kβ2 double mutant (cf. our new Fig. 7) make it impossible to distinguish the stages based on these patterns in the double mutant. To nonetheless provide a fix point of reference for the progression of cell plate formation, we defined the time when the cell plate touches the peripheral plasma membrane as time zero, based on which the timing of microtubules and the other markers CLC2 and MAP65-3 is presented for the the pi4kβ1 pi4kβ2 double mutant. It is in fact a major finding of our work that PI4Kβ isoforms exert an organizing effect on phragmoplast microtubules, and the time lapse imaging suggested by the reviewer have helped greatly to document this effect in relation to wild type controls.

Criticism:

In addition, it is important to show the microtubule behaviors during the transition from the aborted phragmoplast and the interphasic cortical microtubules in the daughter cells.

Response:

Our new time lapse images (Fig. 7) and movies (Movies 3 and 4) cover the behavior of microtubules immediately after completed cytokinesis when microtubules disband from the phragmoplast to form cortical arrays. Importantly, the double mutant displays a delay in microtubule transition, with substantial proportions of microtubules lingering in an irregular pattern at the cell plate even after parts have already moved to a perinuclear or cortical orientation. Thus, as mentioned above, the time lapse imaging helped suppport differences in microtubule behavior between wild type and mutant.

Criticism:

Furthermore, there is an overall lack of quantification of the cytokinesis defects of the pi4kβ1 pi4kβ2 double mutant. What are the proportion/number of aberrant cell division, cell wall stub, multinucleate cells in the pi4kβ1 pi4kβ2 double mutant and how this related to the mpk4 cytokinetic phenotypes (which, from publications, appears to be stronger than that of the pi4kβ1 pi4kβ2 double mutant).

Response:

We apologize that we did not perform sufficient quantitative evaluation of our imaging data before. We have now extracted numerical data from our images and imaging series where possible and are presenting this information alongside the images. We find the informational content of our manuscript much improved for it and thank the reviewers for this (obvious) comment.

Criticism:

2) There are no data supporting the notion that MPK4 is endocytosed. Indeed, in Fig. 5, the authors show that MPK4 colocalized with FM4-64, which likely indicates that MPK4 localize to the TGN. This makes sense considering that MPK4 interacts with PI4KB1, which is itself localized at the TGN (cf A role for the RabA4b effector protein PI-4Kbeta1 in polarized expansion of root hair cells in Arabidopsis thaliana, Preuss et al., 2006 JCB, Electron tomography of RabA4b- and PI-4Kβ1-labeled trans Golgi network compartments in Arabidopsis, Kang et al., 2011 Traffic). The TGN being BFA sensitive it is not surprising to see MPK4-labelled compartments being BFA-sensitive. This does not indicate in any way that MPK4 is endocytosed, but it rather shows that it resides in a BFA-sensitive compartment.

Response:

We agree with the reviewer(s) that we have misinterpreted data related to MPK4 endocytosis, its association with BFA bodies and especially the use of the pharmacological compound, TyrA23. MPK4 signals associating with FM 4-64-labeled dots and with BFA-bodies might indeed reflect interaction of MPK4 with PI4Kβ1 at the periphery of the TGN, as suggested by the reviewer. With the changed conceptual logic of our study, these data were removed from the revised manuscript.

Criticism:

The time-lapse presented in Fig. 5c is by no mean proof of endocytosis or recycling of MPK4 from or to the cell plate. These are isolated events, with a low number of replicates (from n=6 to n=1) and the quality of the pictures/movie is low. But most importantly, there is no proof that these are genuine endocytic or recycling events, with scission or fusion of membranes.

Indeed, the compartment labeled with the arrow could very well be a free moving TGN that happens to pass by the cell plate (especially given the low number of events reported).

Response:

We apologize for the low quality of the movies, which was a consequence of the low fluorescence intensity of the MPK4 marker in our hands. We agree with the reviewer that assessing the movement of MPK4 is not clear from the movies and other data presented in our previous submission, and that this point might require substantial additional experiments. As we have shifted the focus of our work away from the delineation of the exact trafficking of MPK4 at and around the cell plate, these data (also see previous point) were eliminated from the manuscript altogether.

Criticism:

Finally, the demonstration that MPK4 is indeed endocytosed by clathrin-mediated endocytosis (CME) is provided using TyrA23, which is claimed to be a specific inhibitor of CME. While this drug has been used quite extensively in the past, it was recently showed that TyrA23 is, in fact, a protonophore, which depletes the cell from ATP and rapidly acidified the cytoplasm (Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification (Dejonghe et al., 2016 Nat Comm). TyrA23 in turn not only inhibits CME, but all vesicular trafficking events (among other effects). Bearing in mind this paper, TyrA23 cannot be used as an inhibitor of CME and this Supplementary Fig. hould be removed (indeed, it is not possible to form a BFA body, if the movement of TGN compartments is inhibited by depletion of cellular energy upon TyrA23). For these reasons, the idea that MPK4 is endocytosed to be cleared from the center of the cell plate is not supported by any experiments.

Response:

Of course, the reviewer is correct that TyrA23 should not be used to inhibit CME, due to its many unspecific cellular effects. In consequence, we have eliminated the respective data and no longer claim that MPK4 is a CME cargo, a notion not supported by our data.

Criticism:

There is currently no chemical compound in plants that can be reliably used to inhibit CME. However, inducible genetic systems have been shown to inhibit endocytosis and could be used to show that MPK4 indeed undergo endocytosis in order to be removed from the center of the cell plate (for example based on the inducible expression of auxilin2, see Danger-associated peptide signaling in Arabidopsis *requires clathrin, Ortiz-Morea et al., 2016 PNAS). In addition, if the accumulation of MPK4 in BFA bodies is indeed due to its endocytosis, and*

if MPK4 endocytosis is impaired in the pi4kβ1 pi4kβ2 double mutant, then MPK4 should not be found on BFA bodies in this mutant.

Response:

As mentioned above, and following the reviewer's advice, we no longer follow the hypothesis that MPK4 is a cargo for endocytosis. The reviewer is correct in pointing out that the association of MPK4 with BFA-bodies alone does not indicate that MPK4 is endocytosed, but it may rather just associate with membranes following a BFA-sensitive trafficking pathway. As the question of MPK4 trafficking is no longer at the center of our story, we have not performed additional experiments to clarify this point. We respectfully opt to investigate this interesting issue separately and may perform such experiments in the future, in the best of cases in cooperation with experienced experts in the MAPK field.

Criticism:

Furthermore, whether there is actually CME at the cell plate has never been formally shown, at least on early stages of cytokinesis. Of note, according to Ito et al., CLC2 is recruited to the cell plate relatively late (at least later than the dynamin-related protein DRP1). Because the pi4kβ1 pi4kβ2 double mutant appears to be blocked at the disk phragmoplast stage, it is not surprising that CLC2 is not found in the mutant. Whether this is because of a specific inhibition of endocytosis by the lack of PtdIns4P or because of a stage transition issue is not resolved and therefore causation cannot be inferred. Again, as explained in point #1, it will be important to make a time-lapse analysis of CLC2 localization in the wild-type and pi4kβ1 pi4kβ2 double mutant.

Response:

In order to delineate possible reasons for the cytokinetic defects of the pi4kβ1 pi4kβ2 double mutant, we performed additional TEM imaging experiments (Fig. 3). The TEM images of the mutant cells show clusters of non-fused vesicles instead of a developing cell plate, suggesting defects in membrane trafficking at the cell plate. This observation is consistent with previous descriptive reports that PI4Kβ isoforms have a role in secretion at the cell plate (Preuss et al., J Cell Biol 2006; Kang et al., Traffic 2011). However, as a well-characterized role of phosphoinositides in plants is in endocytosis (König et al., 2008; Zhao et al., 2010; Ischebeck et al., 2013), we analyzed effects of the pi4kβ1 pi4kβ2 lesion on CME. The study of CME at the cell plate is difficult, partially because of the imaging angle, which does not invite the use of some advanced imaging techniques, such as spinning disc or TIRF microscopy. Based on our analyses, a role for PI4Kβ1/PtdIns4P in the control of CME is supported by the following new observations: i) KNOLLE displays peripheral plasma membrane association in the mutant, consistent with a CME defect (Fig. 5 a, b); ii) there is

an increased half life of CLC2-GFP at the plasma membrane of interphase cells (Supplementary Fig. 6); iii) there is reduced formation of PIN2-decorated BFA bodies in the mutant (Fig. 5 g, h); and iv) there is reduced internalization of FM 4-64 from the plasma membrane (Fig. 5 i, j). A predominant focus of the CME defect on the cell plate is supported by the further observations that: v) clathrin displays delayed recruitment to the cell plate in the mutant, based on the requested time lapse imaging (Fig. 4 b-d); vi) the abundance of KNOLLE and PIN2 at the cell plate is enhanced in the mutant (Fig. 5 c-f); vii) a change in the intensity of the PtdIns(4)P reporter mCherry-FAPP1-PH was only seen at the cell plate and not at the plasma membrane (Fig. 4 a); and viii) no changes in PtdIns4P levels could be detected in the pi4kβ1 pi4kβ2 double mutant (Supplementary Fig. 4), indicative of a localized and/or transient effect (admittedly, the last is a weak argument). Overall, we are grateful for the constructive suggestions and have performed substantial additional experiments to assess a possible role of PI4Kβs in the control of CME. The abovementioned evidence supports such a role, possibly accounting for some of the cell plate deposition defects observed in the pi4kβ1 pi4kβ2 double mutant.

Criticism:

3) The rescue of the growth defect by KNOLLE does not convincingly show that specific PI4KB expression during cell division is sufficient to rescue most phenotypes related to the double mutant. Indeed, while this promoter is indeed tightly regulated during cell division, what makes KNOLLE protein so tightly expressed, is its degradation following cytokinesis via endocytosis and routing to the vacuole (see for example Endocytosis restricts Arabidopsis KNOLLE syntaxin to the cell division plane during late cytokinesis, Boutte et al., 2009 EMBO). It is therefore likely that PI4Kβ1 protein (and its product), may persist post cytokinesis and be stable after cell division. This has actually been shown previously for CPI1 (see Sterol-dependent endocytosis mediates post-cytokinetic acquisition of PIN2 auxin efflux carrier polarity, Men et al., 2007 Nat Cell Biol). The author should show that pKNOLLE::Flag-PI4Kβ1 is indeed expressed (at the protein level) only during cytokinesis or change their conclusion accordingly. Response:

Response:

We agree with the reviewer(s) that the KNOLLE protein may persist beyond cytokinesis and that our initial intepretation was overreaching. In response to this comment, we have attempted to detect the pKNOLLE-expressed Flag-PI4Kβ1 by immunohistochemistry, but failed repeatedly and using different protocols, antisera etc.. As Western blots will not indicate the persistence only in cytokinetic cells, we therefore cannot provide data on the

persistence of the KNOLLE protein. In consequence, we no longer claim that PI4Kβ1 has its exclusive or even its main function in cytokinesis (see also next comment).

Criticism:

An additional control would be to look at the phenotype of the root hairs from the pKNOLLE::Flag-PI4Kβ1/pi4kβ1;β2 genotype. Indeed, the pi4kβ1 pi4kβ2 double mutant has a strong root hair phenotype, that is arguably a post-cytokinetic process. If this root hair phenotype is rescued by pKNOLLE::Flag-PI4Kβ1, this would argue that PI4Kβ1 is likely stable and still present after cytokinesis in these plants.

Response:

The suggested analysis of root hairs of complemented lines was performed. Root hairs of otherwise normal growing pKNOLLE-PI4Kβ1 complemented plants resembled root hairs of double mutants and were substantially shorter than root hairs from wild type controls or plants expressing PI4Kβ1 under its native promoter (new Supplementary Fig. 1b). Thus, the (post-cytokinetic) root hair defect of the pi4kβ1 pi4kβ2 double mutant was not rescued by pKNOLLE-driven PI4Kβ1. While the root hair data would support that pKNOLLE-PI4Kβ1 might act predominantly during cytokinesis, in the absence of biochemical data on protein persistence we feel more comfortable to tone down our interpretation as recommended by the reviewer. On the other hand, the data provide evidence that PI4Kβ1 has roles also outside of cytokinesis. This notion is also in line with other experimental observations, including the punctate localization of mCherry-PI4Kβ1 at the TGN (Fig. 2 c); reduced formation of PIN2-decorated BFA bodies in the mutant (Fig. 5 g, h); defective FM4-64 endocytosis from the plasma membrane (Fig. 5 i, j); altered plasma membrane life time of CLC2-GFP (Supplementary Fig. 6); and differences in microtubule dynamics detected in interphase cells of the double mutant (Supplementary Fig. 7). In consequence, the text has been rephrased throughout the manuscript to reflect this broader interpretation of the data.

- minor concerns that should be addressed

Criticism:

- The authors state line 367: "Considering i) that the internalization of MPK4-YFP was CME dependent (cf. Fig. 6) and ii) that MPK4-YFP was found to be stabilized at the cell plate in the pi4kβ1 pi4kβ2 double mutant (cf. Fig. 4d), our results indicate that mpk4-2-like cytokinetic defects of the pi4kβ1 pi4kβ2 double mutant are a consequence of mistargeting of MPK4 at the cell plate, likely due to failing CLC2 recruitment and concomitantly reduced CME of MPK4-YFP." This causation is by no mean demonstrated. If the authors want to show causation, they should experimentally remove MPK4 from the center of the cell plate in the pi4kβ1 pi4kβ2 double mutant and show that this can rescue the phenotype. Because this is

obviously a very difficult experiment, the author may alternatively change their conclusion and be more cautious.

Response:

We agree with the reviewer that the proposed experiment might be difficult to perform. In line with our changed working hypothesis, which no longer assumes CME of MPK4, the quoted arguments i) and ii) have been eliminated from the text.

Criticism:

- Line 88-89, please include a reference to this statement.

Response:

Done.

Criticism:

- The authors introduce the NACK-PQR pathway in the introduction (line 68-79) but do not introduce the literature on MPK4. This section should be revised to include an introduction on MPK4, which is at the center of the manuscript.

Response:

The text was revised to include a better description of MPK4, and additional relevant literature was cited.

Criticism:

- Line 92, discussion about CESA6 role during cytokinesis is not relevant to the paper and should be discarded. However, a better introduction to the regulation of cytokinesis by MPK4 and other MAPKK like MKK6/ANQ would be helpful.

Response:

The reference to CesA6 was removed and the text expanded on MPK4 and its upstream kinases, as suggested.

Criticism:

- Line 115, the number/proportion of cell wall stubs and oblique cell wall should be quantified in the double mutant and complemented lines.

Response:

We apologize for the omission and have added image-based quantifications of the requested and many other parameters throughout the entire study.

Criticism:

- Line 125, this sentence implies that mCHERRY-PI4Kβ1 is only at the cell plate, but it is also present in dotty structure (cf Fig 1c and d). This should be mentioned, notably as this kinase was previously shown to localize to the TGN (cf A role for the RabA4b effector protein PI-4Kbeta1 in polarized expansion of root hair cells in Arabidopsis thaliana, Preuss et al., 2006 JCB, Electron tomography of RabA4b- and PI-4Kβ1-labeled trans Golgi network compartments in Arabidopsis, Kang et al., 2011 Traffic). The sentence line 117-119 should also be revised accordingly since in fact the localization of PI4Kβs has been described previously.

Response:

The reviewer is correct in pointing out the punctate signals seen in addition to cell plate-associated signal. We have changed the text accordingly to more precisely describe these fluorescence patterns. To this end, we have performed additional immunolocalization experiments on mCherry-PI4Kβ1 together with the TGN marker ARF1 (new Fig. 2 c), demonstrating colocalization of the signals in both the punctate signals and at the cell plate, consistent with TGN-association of PI4Kβ1.

Criticism:

- Line 153 (related to Fig. 1e), calcofluor labels cellulose not cell wall. The data presented Fig. 1e suggest that PtdIns4P appears before deposition of cellulose, not cell wall (for example, it is likely that it would colocalize with a callose dye). It is not clear how relevant is this panel to the story.

Response:

The reviewer is correct that calcofluor indicates the deposition of cellulose, not "cell wall". We had included this information solely to demonstrate that PtdIns4P appears fully at the cell plate at a time before all cell wall components are deposited. We have now removed this information from the manuscript, together with the reference to CesA6 (see above).

Criticism:

- Line 161/162, the number/proportion of multiple nuclei and phragmoplast should be quantified.

Response:

We apologize for the omission and have added image-based quantifications of the requested and many other parameters throughout the entire study.

Criticism:

- Line 292/293 (related to Supplementary Fig. 6), I am not convinced that PtdIns4P arrives later than MPK4 at the cell plate based on this movie. There seems to be an RFP signal, albeit weak, at the second time point (where MPK4 is first found to localize at the cell plate). This result is rather odd since it was reported by The MAP kinase MPK4 is required for cytokinesis in Arabidopsis thaliana,.Kotetsu et al., TPC 2010 that MPK4-GFP colocalizes with FM4-64 at the cell plate at early stages, when the nuclei had not yet reformed and that PtdIns4P biosensor also colocalizes with FM4-64 as soon as it appears (Simon et al., Nat Plant 2016). The authors should provide example of several independent movies and show some sort of quantification (for example by making a line scan across the MPK4-decorated cell plate to see whether there is RFP signal or not) and perform colocalization with FM4-64 to know if MPK4 signal will arrive before "vesicle assembly" at the cell division plane.

Response:

The reviewer is correct that the interpretation of the FAPP1-reporter fluorescence is difficult to compare to that of the MPK4 marker. In the original submission we may thus have overinterpreted this relation. In our reassessment of numerous image sequences we have now no longer evaluated differences in intensity at each time point between FAPP1 and MPK4 fluorescence; instead, we now individually evaluated the appearance of marker fluorescence over the respective background. Based on this new evaluation, we cannot detect a significant difference in the timing of FAPP2 and MPK4 appearing at the cell plate. Therefore, we have eliminated mention of a possible earlier appearance of PtdIns4P at the cell plate from the text and - in line with the reviewer's suggestion - present the data as temporal coincidence of the markers (Fig. 10 d).

Criticism:

- Fig. 4, the authors should add the time points in panel a. It is puzzling to see the mCherry-PI4KB1 signal disappearing in the 4th picture of the time-lapse and then reappearing in the last panel (are they inverted?).

Response:

We apologize for the omission and have now labeled the cytokinetic stages. We agree that the quality of the image in question was not satisfactory, as the fluorescence of the marker appeared much darker. Therefore, we have removed this panel from the Fig..

Criticism:

- Fig. 7d, the localization of GFP-MAP65-3 does not significantly differ from its localization in the wild-type as reported by MAP65-3 microtubule-associated protein is essential for

nematode-induced giant cell ontogenesis in Arabidopsis, Caillaud et al., (2008 Plant Cell). The authors should be more careful in their conclusion.

Response:

We are aware of the work by Caillaud et al., Plant Cell 2008, and agree that the pattern for GFP-MAP65-3 in the mpk4-2 background appears similar to that reported in that paper. However, in our hands, we never observed the pattern reported by Caillaud et al. in wild type cells and only found the relaxed localization of the GFP-MAP65-3 marker in the mpk4-2 mutants background. We do agree with the reviewer that this situation is puzzling. As this aspect is no longer central to our story, we have eliminated this information from the manuscript.

Criticism:

- Fig. 7e, the authors argue that "Because of robust microtubule bundles in the phragmoplast, it is often difficult to measure the dynamics of phragmoplast microtubules". However, it is again possible to look by time-lapse analysis to MTs behaviors during the transition from the aborted phragmoplast and the interphasic cortical microtubules. In any case, it does make any sense to analyze microtubule dynamics in interphasic cells (differentiated root cells), a stage in which according to the authors, PI4KB1 does not play a significant role. If the authors want to confirm the weak difference in shrinkage they observed, they should consider performing additional experiments such as oryzalin treatment in both WT and pi4kβ1 pi4kβ2 double mutant. In addition, the authors could check the effect of oryzalin on the persisting phragmoplasts in the pi4kβ1 pi4kβ2 mutant in order to address if CLC and MPK4 would relocalize upon MTs depolymerization.

Response:

We agree with the reviewer that information on microtubule patterns at the cell plate would be very valuable. We have therefore performed time lapse imaging of microtubules during cytokinesis in wild type and double mutant as suggested. In the mutant we observed a delay and irregular patterns during phragmoplast dissociation (new Fig. 7). We thank the reviewer for suggesting this experiment. With rectifying our previously too narrow interpretation of pKNOLLE-driven complementation of the mutant (where expression of PI4Kβ1 may not be restricted to cytokinesis due to persisting protein), we are now including the notion that PI4Kβ1 has functions also outside of cytokinesis (see also previous comments above). This notion is in line with punctate localization of mCherry-PI4Kβ1 at the TGN (Fig. 2c); with defective FM4-64 endocytosis from the plasma membrane in the double mutant (Fig. 5 i, j); and with altered plasma membrane life time of CLC2 in interphase cells of the double mutant (Supplementary Fig. 6). In light of this expanded view, we do find it relevant to retain the data

on microtubule dynamics in interphase cells (Supplementary Fig. 7). Together with the time lapse imaging of the phragmoplasts (Fig. 7), the data present a more rounded picture of how microtubule dynamics might be influenced by PI4Kβs. Additional suggested experiments using oryzalin were also performed and in initial control experiments yielded the unexpected result that PI4Kβ1 and the PtdIns4P reporter mRFP-FAPP1-PH both changed their localization upon oryzalin-mediated destabilization of microtubules. This finding might indicate that the interplay between PI4Kβ1, PtdIns4P, microtubules and other regulatory players like MPK4 or MAP65-3 is complex possibly at more levels than we can resolve in this first description of effects of PI4Kβ isoforms on microtubules. We will likely study this unexpected (and drastic) effect in the future. For now, the interpretation of oryzalin application on PI4Kβ-dependent processes will be very difficult in light of the effects of oryzalin and microtubule destabilization on PI4Kβ. Therefore, we respectfully ask your understanding that the suggested oryzalin treatments were not further pursued for the revision of this manuscript.

Overall, we thank reviewer 1 for the many insightful and helpful comments, which prompted us to perform a additional experiments that we feel have substantially improved the quality of our work.

Referee #2:

The manuscript by Lin et al. provides the first description of a role for a phosphatidylinositol 4-phosphate (PtdIns(4)P kinase) in modifying MAP-kinase (MPK) localization during cytokinesis. Moreover, its relation to cytokinetic downstream events mediated by microtubule-associated protein 65-3 (MAP65-3 also known as PLEIADE) is addressed in this work. While roles for PtdIns(4)P kinases of different families have been reported in other systems than the plant Arabidopsis thaliana (Arabidopsis) analyzed here, this is to my knowledge the first report on a role for PtdIns(4)P kinase/MPK/MAP signaling during cytokinesis in any organism.

Thus, the study is novel, certainly of broad interest to the plant science community but also to some readers working on phospholipid and MAP-kinase signaling as well as cytokinesis in other systems. The findings should therefore to some extent appeal to a broader readership such as the one covered by The EMBO Journal.

The work initially analyses the phenotype of a double mutant defective in the PtdIns(4)P kinase beta 1 and PtdIns(4)P kinase beta 2 (pi4kβ1 pi4kβ2) as well as its rescue by an mCherry-PI4Kβ1 fusion protein expressed from the pPI4Kβ1 promoter (pPI4Kβ1: mCherry-PI4Kβ1). The construct is used for susequent analyses as it proves fully functional. The pi4kβ1 pi4kβ2 double mutant displays cytokinesis defects and mCherry-PI4Kβ1 localizes to

the cell plate, a plant-specific cytokinetic membrane structure supporting a function of PI4Kβ1 in cytokinesis. Moreover, a fluorescent reporter for PtdIns(4)P confirms its presence in the cell plate (all Fig. 1). While the Fig.s are of very high quality, it would be helpful to obtain quantitative information of cytokinesis-defective cells in the pi4kβ1 and pi4kβ2 single as well as pi4kβ1 pi4kβ2 double mutant (Fig 1b).

Criticism:

The authors proceed to characterize the cytokinesis defects of the pi4kβ1 pi4kβ2 double mutant by employing nuclear markers, localization of tubulin and of the cytokinesis-specific KNOLLE syntaxin by immunofluorescence microscopy. The results reveal the occurrence of multinucleate cells and aberrations in the organization of the phragmoplast, a plant-specific cytokinetic microtubule (MT) array. Again the images are of very high quality, but no quantitative data is provided (Fig. 2)

[...] 1) Inclusion of quantitative information on cytokinesis-defective cells in the pi4kβ1 and pi4kβ2 single as well as the pi4kβ1 pi4kβ2 double mutant (Fig 1b).

[...] 2) Inclusion of quantitative data in Fig. 2.

Response:

We apologize for the omission and have added image-based quantifications of the requested and many other parameters throughout the entire study.

Criticism:

Lin and colleagues proceed to address the role of plant MPK4 in relation to PI4Kβ1 PI4Kβ2 by aiming to analyze a pi4kβ1 pi4kβ2 mpk4-2 triple mutant. In comparison to pi4kβ1 pi4kβ2 double and mpk4-2 single mutants, no viable homozygous triple mutants are recovered. Interestingly, also pi4kβ1 pi4kβ2/+ mpk4-2 mutants show a strongly reduced seedling size compared to pi4kβ1 pi4kβ2 and mpk4-2 mutants, suggesting a synergistic genetic interaction. The latter is not spelled out as such in the manuscript, which it should. Moreover, while the data clearly suggests synergistic genetic interaction of at least one of the PI4Kβ s with MPK4 it is not clear which one (or both?) is responsible, as pi4kβ1 mpk4-2 doubles etc. were not analyzed. Nonetheless, the data supports a synergistic genetic interaction of either PI4Kβ1 and/or PI4Kβ2 with MPK4.

[...] 3) Analysis of pi4kβ1 mpk4-2 and pi4kβ2 mpk4-2 double mutants for genetic interaction, or weakening of the claim to that synergistic genetic interaction occurs for either PI4Kβ1 and/or PI4Kβ2 with MPK4.

Response:

We thank the reviewer for this comment and have modified the text accordingly to now state clearly that there is a synergistic genetic interaction between one or both PI4Kβ isoforms with MPK4. We now also present additional genotypes. However, as the selection of the strongly impaired mutants is very tedious and PI4Kβ1 and PI4Kβ2 have previously been described as functionally redundant (Preuss et al., J Cell Biol 2006), we have chosen to not attempt to isolate all possible genotypes to delineate, whether PI4Kβ1 or PI4Kβ2 preferentially interact with MPK4. In consequence, we have weakened our claim as suggested by the reviewer and now state that the synergistic genetic interaction of MPK4 occurs for either PI4Kβ1 and/or PI4Kβ2. Because of the functional redundancy reported before, which is also apparent from the double mutant phenotype, we feel that this aspect is not detrimental to our argument.

Criticism:

The authors further reveal interaction of the 566 N-terminal amino acids of PI4Kβ1 with MPK4 by yeast two hybrid (Y2H) assays as well as co-immunoprecipitation of MPK4-myc and PI4Kβ1 from Arabidopsis protein extracts. Here, it should be indicated, how often the assays were repeated with similar results (Fig. 3 b,c). The claim that these experiments demonstrate a physical interaction of PI4Kβ1 and MPK4 (page 11, line 231) is an overinterpretation as no direct biochemical binding assays or other direct interaction assays like split-YFP, FLIM/FRET experiments were carried out. The latter might have been a possibility, as the authors provide colocalization data of mCherry-PI4Kβ1 and MPK4-YFP in Fig. 4a.

[...] 4) Weakening of the claim that the experiments demonstrate a physical interaction of PI4Kβ1 and MPK4 (page 11, line 231) or performance and inclusion of direct biochemical binding assays or other direct interaction assays like split-YFP, FLIM/FRET experiments.

Response:

The reviewer is correct that our yeast-two-hybrid and Co-IP data do not indicate a direct physical interaction between PI4Kβ1 and MPK4 (these experiments were each performed three times). While we now report phosphorylation of purified recombinant PI4Kβ1 by purified recombinant MPK4 in vitro, suggesting direct physical association, we have nonetheless weakened our claim as suggested. In consequence, we now state that PI4Kβ1 and MPK4 may both be elements of a larger protein complex, which may mediate the positive signals in the yeast-two-hybrid and Co-IP experiments. We did not perform additional FRET/FLIM experiments, because the intensity of the MPK4 marker is very low and would likely not give reliable data, and because the phosphorylation detected in vitro is strong evidence in itself.

Criticism:

The authors further express FLAG-PI4Kβ1 under control of the KNOLLE promoter in the pi4kβ1 pi4kβ2 double mutant, which completely rescues the double mutant phenotype, leading the authors to speculate that "one main function of PI4Kβ1 in A. thaliana is the formation of PtdIns(4)P during the G2/M phase to control somatic cytokinesis". To support this conclusion, however, it would have been necessary to address as to whether FLAG-PI4Kβ1 is actually only expressed during G2/M by immunofluorescence localization. It is possible that the protein is stable and persists throughout interphase and into cell elongation. This has been reported e.g. for a sterol biosynthesis protein expressed from the KNOLLE promoter (Sterol-dependent endocytosis mediates post-cytokinetic acquisition of PIN2 auxin efflux carrier polarity, Men et al., 2008, Nat. Cell Biol.).

[...] 5) Performance and inclusion of immunofluorescence labeling of FLAG-PI4Kβ1 to test whether this really only persists throughout G2/M when expressed from the KNOLLE promoter.

Response (partly redundant with the response to a comment by reviewer 1):

We thank the reviewer for this insightful comment, which addresses an issue also raised by reviewer 1. We agree that the KNOLLE protein may persist beyond cytokinesis and that our initial intepretation was overreaching. As we have not been able to detect the pKNOLLE-expressed Flag-PI4Kβ1 by immunohistochemistry (see response to reviewer 1 above), we cannot provide direct data on the persistence of the KNOLLE protein. Analysis of root hairs of complemented lines was performed as suggested by reviewer 1, and the root hairs of otherwise normal growing pKNOLLE-PI4Kβ1 complemented plants were substantially shorter than root hairs from wild type controls or plants expressing PI4Kβ1 under its native promoter (new Supplementary Fig. 1b). These data suggest that the post-cytokinetic root hair defect of the pi4kβ1 pi4kβ2 double mutant was not rescued by pKNOLLE-driven PI4Kβ1, consistent with a role of PI4Kβ1 outside of cytokinesis (and with non-persistence of the expressed protein). In the absence of biochemical data on protein persistence we have therefore chosen to tone down our interpretation, and we no longer claim that expression of PI4Kβ1 restricted to cytokinesis would fully complement the pi4kβ1 pi4kβ2 double mutant. This notion is further supported by other new experiments, including the punctate localization of mCherry-PI4Kβ1 at the TGN (Fig. 2 c); reduced formation of PIN2-decorated BFA-bodies in the mutant (Fig. 5 g, h); the defective FM4-64 endocytosis from the plasma membrane in the double mutant (Fig. 5 i, j); different life time of CLC2 at the plasma membrane (Supplementary Fig. 6); and the differences in microtubule dynamics detected in interphase cells of the double mutant (Supplementary Fig. 7).

Criticism:

The authors further report as stabilization of MPK4-YFP and tubulin at the phragmoplast in the pi4kβ1 pi4kβ2 double mutant. However, it is not clear as to whether the same cytokinetic stages were analyzed and the phragmoplast was imaged in the same plane. It would be helpful to perform this experiment on image 3D image stacks, to include one at least in the supplementary material, so it is clear where in the cell the phragmoplast has which appearance in wild type and mutant, and to include numbers of the image stacks analyzed.

[...] 6) Performance and inclusion of 3D-stack imaging data on MPK4-YFP and tubulin localization at the phragmoplast in the pi4kβ1 pi4kβ2 double mutant.

Response (partly redundant with the response to a comment by reviewer 1):

We thank the reviewer for this insightful comment and the suggestion to obtain z-stacks for 3D imaging. Both the dynamic nature of cytokinesis and the three-dimensional orientation of the forming cell plate make it difficult to judge the stages of individual images, which then cannot be compared. With regard to the particular experiment suggested in this comment, we have not included the exact requested data, because the monitoring of MPK4 localization is no longer presented as part of this story. Nonetheless, this comment was very helpful, because it prompted us to obtain z-stack data for other markers, where the same problem is also imminent: In the course of time lapse imaging (suggested by reviewer 1) of cytokinesis in wild type and pi4kβ1 pi4kβ2 mutants, we also recorded z-stacks, resulting in 4D-image series of CLC2-GFP, of phragmoplast microtubules and of MAP65-3 during cytokinesis, which can now be better compared (Fig.s 4, 7 and 8). We are reporting the 4D time lapse series in the main text only for MAP65-3, because we found the simpler median confocal sections clearer for CLC2-GFP and microtubules. We have however added instructive examples for 3D images for CLC2-GFP in Supplemental Fig. 5 and for microtubules as part of Fig. 7. For instance, the data allow to judge the position of the growing cell plate over time together with the distribution of the phragmoplast regulator MAP65-3, which appears as a solid plate instead of a ring over a prolonged time spanning the entire duration of wild type cytokinesis (Fig. 8). This finding explains the characteristic patterns we observed before when using individual images. Thus, the suggested additional experiments helped to address the important issue of relative timing of the observed effects for several markers. As this information is critical for our story, we have positioned the 4D data in the main part of the manuscript and not in the supplement.

Criticism:

Lin et al. further perform experiments employing the vesicle trafficking inhibitor brefeldin A (BFA) and the translation inhibitor cycloheximide (CHX) from which they conlude that MPK4 is targeted to the cell plate and also underwent endocytosis. However, the authors do not

show that CHX does actually completely or almost completely inhibit de novo synthesis of MPK4-YFP under their conditions at this stage of the cell cycle. This could be addressed by performing fluorescence recovery of photo-bleaching (FRAP) analyses of MPK4-YFP in the cell plate under CHX treatment and without. Additional more conclusive data could be obtained by employing photo-activatable (PA) GFP fusions or photo-switchable fusions of MPK4. The data as it is presented in Fig. 5 on its own is not conclusive, yet.

[...] 7) Performance and inclusion of fluorescence recovery of photobleaching (FRAP) analyses of MPK4-YFP in the cell plate under CHX treatment and without. Optionally, employing photoactivatable (PA) GFP (PA-GFP) fusions or photoswitchable fusions of MPK4 could provide more conclusive data on from where to where the protein is really moving.

Response (partly redundant with the response to a comment by reviewer 1):

As stated also in the previous comment, we have altered the chain of arguments presented in our manuscript, and the focus has shifted away from the analysis of MPK4 as a CME cargo and its localization. The use of CHX to analyze cell plate-associated proteins is not trivial, because in our hands CHX blocks cytokinesis, so we have opted to not pursue this line of experiments. As moreover this particular aspect and the Fig. in question have been eliminated from the manuscript, we are not including the requested 3D-stack or FRAP experiments for the weak MPK4 marker in the revised manuscript.

Criticism:

The authors further attempt to address the potential role of clathrin-dependent endocytosis on MPK4-YFP trafficking mainly through application of the rather unspecific inhibitor tyrphostin A23 (Tyr A23) which is known to have many different effects than inhibiting cargo recognition by AP2 associated adaptor proteins in endocytosis (Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification (Dejonghe et al., Nat Commun 2016). Application of this inactive analogue Tyr A51 alone is not useful here, as it is not at all clear as to whether the Tyr A23 effect is restrict to the endocytis AP2 coat or very different cellular events (Dejonghe et al., Nat Commun 2016). Here, it would be necessary to involve different genetic and/or pharmacological tools like dynamin-related protein or AP2-coat protein mutants or dominant interference with the clathrin coat using the HUB domain or other proteins, as other authors have done. Otherwise this claim cannot be made.

[...] 8) Additional tools to the relatively unspecific inhibitor tyrphostin A23 (Tyr A23) (Mitochondrial uncouplers inhibit clathrin-mediated endocytosis largely through cytoplasmic acidification, Dejonghe et al., 2016, Nat. Commun.) need to be employed to address whether MPK4 localization depends on clathrin-dependent endocytosis e.g. drp1a

mutants (e.g. Boutte et al., EMBO J 2010) or inducible TPLATE downregulation (Gadeyne et al., Cell 2014) or another similar genetic tool.

Response (partly redundant with the response to a comment by reviewer 1):

We agree with the reviewer(s) that we have misinterpreted data related to MPK4 endocytosis, its association with BFA bodies and especially the use of the pharmacological compound, TyrA23. We have altered the interpretion of MPK4 signals associating with FM 4-64-labeled dots and with BFA-bodies, which we agree might indeed reflect interaction of MPK4 with PI4Kβ1 at the TGN, as suggested by reviewer 1 (see above). Of course, TyrA23 should not be used to inhibit CME due to its many unspecific effects. In consequence, we have eliminated the respective data and no longer claim that MPK4 is a CME cargo, a notion not supported by our data. As we have shifted the focus of our study away from the trafficking of MPK4 at and around the cell plate, the requested additional experiments were not performed. Alternatively, to delineate a role for PI4Kβ isoforms in CME, we have now included evidence from a range of other experiments, including i) peripheral plasma membrane association of KNOLLE in the mutant, consistent with a CME defect (Fig. 5 a, b); ii) increased half life of CLC2 at the plasma membrane of interphase cells of mutant cells (Supplementary Fig. 6); iii) reduced formation of PIN2-decorated BFA bodies in the mutant (Fig. 5 g, h); and iv) reduced internalization of FM 4-64 from the plasma membrane of mutant cells (Fig. 5 i, j). A predominant focus of the CME defect at the cell plate is supported by the further observations that: v) clathrin displays delayed recruitment to the cell plate in the mutant, based on the requested time lapse imaging (Fig. 4 b-d), vi) in the mutant the abundance of KNOLLE and PIN2 is enhanced at the cell plate (Fig. 5 c-f); and vii) a change in the intensity of the PtdIns(4)P reporter mCherry-FAPP1-PH was only seen at the cell plate and not at the plasma membrane (Fig. 4a). Based on the extensive additional experiments, we conclude that PI4Kβ isoforms contribute to the control of CME, which may partially account for the cytokinetic defects of the pi4kβ1 pi4kβ2 double mutant.

Criticism:

The authors find that MPK4-YFP is stabilized at the cell plate of the pi4kβ1 pi4kβ2 double mutant and that its association with CLC2-GFP at the cell plate was largely reduced. They therefore hypothesize that clathrin recruitment is reduced and MPK4-YFP accumulates, which is a reasonable hypothesis, but could have been supported by one or two more markers. The KNOLLE protein and PIN proteins are known to associate with the parental plasma membrane in endocytosis defective mutant cells, where the cell plate has only attached to one parental plasma membrane, yet (e.g., Boutte et al., EMBO J 2010; Gadeyne

et al., Cell 2014 and several other papers). Does KNOLLE show the same mislocalization in the pi4kβ1 pi4kβ2 double mutant as in other endocytosis-defective mutants?

Response:

We thank the reviewer for this insightful comment and the suggested experiments. While we have removed the MPK4 data from the manuscript, we have followed the suggestions and analyzed the localization of KNOLLE in the pi4kβ1 pi4kβ2 mutant in more detail. In consequence, in the double mutant we observed association of KNOLLE fluorescence with the plasma membrane, a pattern characteristic for endocytosis defects (Fig. 5 a, b). The effect is further enhanced in the presence of BFA, and we have included these data and respective quantifications. We have furthermore observed increased PIN2 fluorescence at the cell plate (Fig. 5 c, d) and reduced formation of PIN2-BFA bodies (Fig. 5 g,h), consistent with a cell plate-centered defect in endocytosis of KNOLLE and PIN2 during cytokinesis.

Criticism:

The authors continue to address localization of GFP-MAP65-3/PLEIADE in the pi4kβ1 pi4kβ2 double mutant and find it to potentially over-accumulate at the mutant phragmoplast. Again here 3D-image stacks should be provided (in the supplement), so the reader cannot judge whether this really reflects an accumulation rather that a top view in the mutant versus a median section of the cell in the wild type in Fig. 7a. 3D image stacks in the Supplementary material would be helpful here. Moreover, the panel labeling is mixed up in panel Fig. 7d and needs to be swapped between FM4-64 and MAP65-3.

[...] 9) In Fig. 7a,b 3D-image stacks should be provided (in the supplement), so the reader can judge whether this really reflects an accumulation rather that a top view in the mutant versus a median section of the cell in the wild type in Fig. 7a.

Response (partly redundant with the response to a comment by reviewer 1):

We thank the reviewer for this insightful comment and the suggestion to obtain 3D-stacks. To address this issue, we have performed time lapse imaging experiments using z-stacks, as suggested by reviewer 2, resulting in 4D-image series of cytokinesis, which now can be compared. Importantly, the 3D-stacks clarify the angle of aspect. The data presented in the new Fig. 8 indicate that cytokinesis observed in wild type and mutant progresses continuously and completely and does not arrest at a particular stage. However, in the mutant, MAP65-3 does not disband from the phragmoplast after the cell plate has contacted the peripheral plasma membrane, MAP65-3 persists for an extended period of time and does not completely clear the central zone opf the cell plate (Fig. 8). In consequence, in the mutant the phragmoplast regulator MAP65-3 appears as a solid plate instead of a ring over a prolonged time spanning the entire duration of wild type cytokinesis. This explains the

patterns we had observed before when using individual images. Thus, the suggested additional 3D-imaging helped greatly to resolve this important issue. We have therefore positioned the 4D data in the main part of the manuscript and not in the supplement.

Criticism:

Finally, the authors address the effect of the pi4kβ1 pi4kβ2 double mutant on microtubule growth and shrinkage in elongating cells, which is somewhat off the track as most other data deals with cytokinesis. This part may be removed here.

Response:

In light of the failure of pKNOLLE-driven PI4Kβ1 expression to rescue the root hair phenotype of pi4kβ1 pi4kβ2 double mutants, and in the absence of data on the persistence of the pKNOLLE-driven PI4Kβ1 complementation of the double mutant, we have no conclusive evidence that PI4Kβs only act at the cell plate during cytokinesis. Instead, a number of new experimental data support the notion that PI4Kβs are not restricted in localization to the cell plate and have functions also outside cytokinesis, including the punctate localization of mCherry-PI4Kβ1 at the TGN (Fig. 2 c); reduced internalization of PIN2 into BFA-bodies (Fig. 5 g, h); the defective FM4-64 endocytosis from the plasma membrane in the double mutant (Fig. 5 i, j); the increased lifetime of CLC2 at the plasma membrane (Supplementary Fig. 6), etc.. The differences in microtubule dynamics detected in interphase cells of wild type and double mutant (Supplementary Fig. 7) further support the notion that PI4Kβs act outside cytokinesis, and therefore we would like to retain the data. As clear data on microtubule dynamics are difficult to obtain for phragmoplast microtubules, we have included new time lapse imaging demonstrating altered behavior of phragmoplast microtubules during the transition to cortical arrays at the end of cytokinesis, as suggested by reviewer 1 (new Fig. 7). With this combination of results, we feel that the microtubule data from interphase and cytokinetic cells are complementary and provide evidence for altered microtubules in the pi4kβ1 pi4kβ2 double mutant.

[...] Taken together, this is certainly a novel and interesting study on a topic of general interest. The PI4Kβeta1 MPK4 interaction is already worked out to quite some extent. However, in its current form the study still requires a larger number of experimental revisions, thinking over as well as rewriting of some of the claims that do not always reflect the data presented, yet.

Major points for revision:

10) Correct the panel labeling Fig. 7d that needs to be swapped between FM4-64 and MAP65-3.

Response:

With the refocused content of the manuscript, the former Fig. 7d has been eliminated.

Criticism:

11) Page 24, Line 494. "Here we found that PtdIns(4)P controls cytokinesis ...". This claim cannot be made as no gain-of-function studies i.e. overexpression studies have been performed, since solely loss-of-function studies were performed. The claim must be weakened to "Here we found that PtdIns(4)P is required for cytokinesis ... " or additional gain-of-function studies need to be included.

Response:

We agree with the reviewer that we cannot make the claim of PtdIns4P "controlling" cytokinesis. We have altered the text as suggested by the reviewer.

Criticism:

12) Page 25, line 507. "Our data uncover that the defective lateral expansion of phragmoplasts in pi4kβ1 pi4kβ2 double mutants was a consequence of mistrafficking of MPK4 at the cell plate ... ." The study contains no experiment to functionally support this claim. This is just an interpretation based on several correlations. This claim must be weakened

Response:

With the working hypothesis suggested by the reviewers that MPK4 acts upstream of PI4Kβs, we are no longer including a discussion of mistrafficking of MPK4 in the pi4kβ1 pi4kβ2 double mutant.

Referee #3:

In the manuscript, entitled "Cell plate-associated PI4Kβ is essential for cytokinetic phragmoplast expansion in Arabidopsis" the authors carefully characterized a cytokinetic defect in a pi4kβ double mutant by beautiful live-imaging and immuno-staining with newly raised antibodies, particularly focusing on PI4KB1&2 and their role MPK4, MAP65-3 and PIP4 behavior during cytokinesis. In this manuscript, the authors claim that PIP4 accumulation on the developing cell plates decreases in the double mutant, which in turn reduces clathrin-mediated vesicle transport from the cell plates. Then, this reduction of CME induces excessive amount of MPK4 accumulation on the cell plate, thereby affecting phragmoplast microtubules turnover. Although the imaging data itself in the manuscript is very clear, I am not convinced by some biochemical data and interpretation of the data for establishing the roles of pi4kβ in cytokinesis, particularly the relationship between abnormal

MPK4 accumulation and the stability of phragmoplast microtubules. The authors should clarify some of their interpretation and should provide additional experiments for more convincing conclusions. The most relevant advance in this paper is the new link between MPK4 and PIP4K. I think that this alone would be enough if this link was more clearly demonstrated.

Criticism:

My biggest concern from is the relationship of over-stabilization of microtubules and excess accumulation of MPK4 on the cell plates. As the authors mentioned in the introduction (line 75-78) "NRK1 finally phosphorylates the microtubule-associated protein 65-1 (NtMAP65-1), thereby decreasing its capacity to bundle microtubules, facilitating turnover and radial expansion of the phragmoplast. All counterparts for the NACK-PQR pathway have been identified also in Arabidopsis", MPK4 potentially induces microtubule destabilization around the cell plates when it is activated. The authors show that in Fig. 4d, MPK4 accumulates evenly on the developing cell plates in the pi4kβ double mutant even during the late cytokinesis stage, and in the same Fig. the authors show over-stabilization of phragmoplast microtubules. I would assume that microtubules are destabilized because of abnormally accumulated MPK4 on the cell plates. This point is the biggest missing link to explain why the cytokinetic defects happens in the pi4kβ double mutant if the authors think over-stabilized phragmoplast induces the defect in expansion of cell plates process.

Response:

As all three reviewers have suggested (see also comment by reviewer 3 further down), we have adjusted our logic to test the working hypothesis that MPK4 acts as an upstream regulator of PI4Kβs. The recent report that PI4Kβ1 is a possible phosphorylation target for MPK4 (supplement to Latrasse et al., Genome Biology 2017), further prompted us to focus on this line of argument. With this conceptual shift we have modified the presentation of the data included in the manuscript. Specifically, the data of the former Fig. 4d have been eliminated and we are no longer including the stabilization of MPK4 at the cell plate of double mutant cells, which might be due to delayed cytokinesis at a specific stage. However, we fully agree with the reviewer that stabilization of MPK4 at the cell plate of double mutant cells is puzzling in combination with the accompanying stabilization of phragmoplast microtubules. While the observation in question is quite clear, it is currently unknown why in the pi4kβ1 pi4kβ2 double mutant MPK4 might not destabilize microtubules as expected. One explanation is that MPK4 requires PtdIns4P and/or PI4Kβs to exert its function on microtubules, and that these players are absent in the double mutant. We anticipate that substantial additional experiments will have to be performed to sort this out and better

delineate the complex interrelations between PI4Kβs, MPK4 and microtubules at the cell plate, which are likely not direct (see Supplementary Fig. 9) nor simply linear (e.g., considering the effects of oryzalin on PI4Kβ/FAPP1 localization mentioned above). Importantly, this aspect only arose based on the new primary observations reported in this study. While we are already working on this aspect, we find it more realistic to try to resolve these next questions in a future separate study. Therefore, we respectfully opt to not address this point in this first and already quite voluminous description of the role of PI4Kβs in cell plate formation and the discovery of the functional link to MPK4.

Criticism:

Related to this, I am also wondering about the phosphorylation status of MAP65-3 in the pi4kβ double mutant. Please clarify this point by experiment.

Response:

We agree with the reviewer that in the context of elucidating the PtdIns4P-dependent MPK4 function (see previous point) it will be of great interest to study the effects on the MPK4 target, MAP65-3. These experiments are inherently difficult to perform and interpret, in particular as more kinases than MPK4 might phosphorylate MAP65-3. As this issue has moved out of the focus of this manuscript, we respectfully choose not to perform this suggested experiment.

Criticism:

The authors also claim that the kinase activity of MPK4 is not affected by pi4kβ1/2 mutations by immuno-complex kinase assay, but the authors did experiments with whole seedlings as far as I understand from the materials and methods. I think that most of the MPK4 came from interphase cells in this assay and how MPK4 activity changes in dividing cells is therefore not clear. I suggest collecting samples from root tips, like in Kosetsu 2011 Plant Cell, and do an immuno-kinase complex assay with MPK4 kinase activity from dividing cells. In that paper, they succeeded to show meristematic regions have much stronger kinase activity. I assume that this assay will help to understand whether MPK4 has stronger or weaker activity in the pi4kβ double mutant.

Response:

We thank the reviewer for this insightful comment, which we heeded, albeit possibly not quite as intended by the reviewer. With the reversed argument that PI4Kβs may act downstream of MPK4, the immediate question to address was whether MPK4 controls PI4Kβs and/or PtdIns4P production. Thus, we have performed experiments equivalent to those requested, but not to test for PI4Kβ-dependent MPK4 function, but rather inversely to test for MPK4-

dependent PI4Kβ function. As an outcome of these experiments, we found that the association of the PtdIns4P reporter mRFP-FAPP1-PH with the cell plate increased in mpk4-2 mutants, suggesting a functional impairment - not activation - of PI4Kβ by MPK4. While at first this finding appears counterintuitive, because the pi4kβ1 pi4kβ2 double mutant and the mpk4-2 mutant both display similar cytokinetic defects and show synergistic genetic interaction (Fig. 9), the phenotypes might be explained by assuming that PtdIns4P and/or PI4Kβs are required for MPK4 to exert its effects on microtubules. With this assumption, both the absence of MPK4 and the absence of PI4Kβs/PtdIns4P would result in the same phenotypic outcome. This notion would be consistent with the - no longer included - stabilization of MPK4 at the cell plate with concomitant stabilization - not destabilization - of the phragmoplast (see also the response to the previous comment). The interpretation is also consistent with the report that another MPK4 target, patellin 2,loses its ability to bind PtdIns4P at the cell plate upon phosphorylation by MPK4 (Suzuki et al., Plant Cell Physiol 2016). If reduced binding of cell plate-associated proteins to PtdIns4P is a part of cytokinetic progression, then it might be envisioned that MPK4 also mediates reduced formation of PtdIns4P at the cell plate, as we observe. Obviously, there are still substantial unknowns, which we will try to address in the future. We already invested substantial effort in elucidating the molecular basis for regulation of PtdIns4P production by MPK4. For instance, we performed in vitro phosphorylation assays and can confirm phosphorylation of purified recombinant PI4Kβ1 protein by purified recombinant MPK4 in vitro (Supplementary Fig. 9a), as was recently described in the supplement to Latrasse et al., Genome Biology 2017. However, further in vitro tests for effects of the phosphorylation on the lipid kinase activity of PI4Kβ1 (an experiment we have previously performed successfully for the effect of MPK6 on the phosphoinositide kinase PIP5K6 (Hempel et al., Plant Cell 2017)) did not indicate an effect of the phosphorylation on PI4K-activity (Supplementary Fig. 9b). As activity of PI4Kβ1 was not apparently affected by MPK4, we next tested for PI4Kβ1 localization in the mpk4-2 background and again did not detect a difference to wild type controls (Supplementary Fig. 9c). With the two obvious parameters (activity and localization) thus not influenced by MPK4, the reason for the enhanced formation of PtdIns4P production at the cell plate of mpk4-2 mutants remains currently unclear. It is possible, for instance, that PI4Kβ1 acts as a required binding partner for MPK4 and MAP65-3 in addition to its role in endocytosis. In consequence, PI4Kβs might influence the precise localization of MAP65-3, as shown in Fig. 8, whereas MPK4 controls MAP65-3 activity. In this scenario, the genetic elimination of both PI4Kβs and of MPK4 might result in the additive cytokinetic phenotype of the mpk4-2 pi4kβ1 pi4kβ2 triple mutant shown in Fig. 9. Again, we feel that the interplay between the partners involved is likely not direct and may involve additional proteins, as was also suggested by reviewer 2 (see comment about the possibly not-direct interaction between PI4Kβ1 and MPK4). It is also

possible that our in vitro tests lack essential components, and negative data cannot be proven. On a positive note, we show a physical and functional link between MPK4 and PI4Kβ, demonstrate that PtdIns4P production is influenced by MPK4, and raise a number of new and interesting questions. However, it will likely take considerable additional effort to address in detail how MPK4 might mechanistically control PI4Kβ function.

Criticism:

The authors hypothesize that MPK4 is a candidate for PI4Kβ- or PtdIns(4)P-dependent regulation, but this connection is not firmly established: In Fig. 3, the authors show genetic interaction and physical interaction between MPK4 and pi4kβ1/2. I do not understand what the authors want to conclude from these data with respect to the cytokinetic defects. The authors assessed the genetic interaction between two kinases by plant growth of multiple mutants, but do not show whether cytokinetic defects were enhanced or not. Moreover, according to Zhang et al., Cell Host Microbe 2012, growth defect in the mpk4 mutant is mainly thought to be a result of enhanced salicylic acid signaling. Again, as the authors cited Šašek et al., New Phytol 2014, over-accumulation of salicylic acid can induce this growth defect. Please explain how these data pertain to understanding the cytokinetic defect in the mpk4 or the pi4kβ1/2 double mutants.

Response:

We thank the reviewer for this insightful comment, which from our perspective refers to yet another level of complexity, namely that MPK4 and MAP65-3 have additional functions in plant defense that appear unrelated to their roles in cytokinesis. It is correct that plants carrying genetic lesions in the genes for MPK4 or MAP65-3 display altered defence characteristics against pathogens as well as altered levels of salicylic acid (Petersen et al., Cell 2000; Quentin et al., J Exp Bot 2016). Similar findings have in fact been reported also for the pi4kβ1 pi4kβ2 double mutant (Sasek et al., New Phytol 2014; Antignani et al., Plant Cell 2015), although salicylic acid is only responsible for the dwarf phenotype of rosettes, but not roots (Sasek et al., New Phytologist 2014), and our findings pertain to the root model. In any case, a major question arising is how different roles of the proteins are orchestrated (e.g., cytokinesis vs. defence). We think that this issue is related to the function of all these proteins in a joint complex controlling cytokinesis, as indicated by the yeast-two-hybrid, Co-IP and colocalization data (Fig. 10), which suggest interaction between MPK4 and PI4Kβ1. Physical interaction of MPK4 with MAP65-3 has long been known (Kosetsu et al., Plant Cell 2010), supporting the existence of a protein complex containing MPK4, PI4Kβ2 and MAP65-3 involved in controlling cytokinesis. The functional link with regard to cytokinesis is supported by the genetic interaction data (Fig. 9), for which we now also show the requested

quantifications of additive effects on the incidence of multinucleated cells etc. (Fig. 9 b, c). If such a protein complex is required for correct progression of cytokinesis, then a missing partner might dissolve the complex and result in cytokinetic defects, as we see in the mpk4-2, pi4kβ1 pi4kβ2 or map65-3 mutants. Importantly, the other partners now released from the complex might then display added functionality in their respective alternative roles, e.g. in defence. Thus, it may be that the correlation between salicylic acid accumulation and enhanced defence with failed cytokinesis might be due to a functional shift of the respective proteins towards their respective alternative roles in defence. We have included a brief discussion along these lines. Of course, this model is highly speculative, and other explanations might be possible.

Criticism:

Additionally, I am wondering what the physical interaction between MPK4 and PI4kβ tells about their functional interaction. The authors only show the possibility that PI4Kβ affects MPK4 function, but I think it is at least as reasonable to assume the reverse, i.e. that MPK4 regulates PI4kβ1/2 function. The behavior of PI4kβ1/2 in mpk4 is still unclear from this manuscript, as is the activity of PI4kβ1/2. The alternative - that MPK4 and PIPK4 meet at the phragmoplast where MPK4 activates PIP4K (by phosphorylation), generating PI(4)P and starting to give the cell plate a "plasma membrane" identity and allowing CME to occur (the delayed appearence of PI4P shown in FigS6 is consistent with this). CME would deplete MPK4 from the PI(4)P-containing CP restricting it to the phragmoplast at the growing CP where it should stabilize MTs. (it would be a nice self regulatory loop for MPK4 localization at growing CP)

Response:

The reviewer is correct in pointing out these very plausible possibilities. As already noted in the responses to reviewers 1 and 2, we have followed this thought and modified our working hypothesis accordingly. As PtdIns4P is enhanced at the cell plate of mpk4-2 mutants (Fig. 10 e) and PI4Kβ1 can be a substrate for MPK4 (Latrasse et al., Genome Biology 2017), which we could verify experimentally (Supplementary Fig. 9a), it is reasonable to assume that MPK4 regulates PI4Kβ1 by phosphorylation, and that this modification might result in reduced (not increased) formation of PtdIns4P. Please note that the previous Supplementary Fig. 6, has been reevaluated based on the comment by reviewer 1, and that we no longer claim that PtdIns4P appears at the cell plate later than PI4Kβ1/MPK4. With a positive role of PI4Kβ isoforms in membrane trafficking at the cell plate and a - possibly coordinating - role in binding and recruiting MPK4 into a complex controlling phragmoplast microtubules, PI4Kβ

isoforms might aid the integration of membrane trafficking and microtubule dynamics at the cell plate. We are now discussing this - still speculative - possibility in the revised manuscript.

Criticism:

To explore this possibility, the authors should add data with marker lines in the mpk4-2 mutant. -- Particularly, RFP-FAPP-PH and CLC2-GFP and mCherry-PI4Kβ markers in mpk4 mutant background are essential, also localization at the CP of RFP-FAPP1 in pip4k would be good to see (it should be gone). Phosphorylation status of PIPK4 in WT vs mpk4 or in vitro phosphorylation assay of PIP4K by MPK4. Some data suggesting that PI(4)P is required for endocytosis - such as KNOLLE localization at PM in pip4k mutants, for example? Or altered CLC/DRP1A dynamics in pip4kP. I see that - if not already initiated - these experiments are quite lengthy and and additional biochemistry experiments might be faster although not as convincing on their own.

Response:

We have performed most of the requested experiments (except for CLC2 in mpk4-2), and data on RFP-FAPP1 (Fig. 10 e) and mCherry-PI4Kβ1 in the mpk4-2 mutant (Supplementary Fig. 9c) have all been included, as well as data on the intensity of mCherry-FAPP1-PH at the cell plate of the pi4kβ1 pi4kβ2 mutant (Fig. 4a). We also tested phosphorylation of PI4Kβ1 by MPK4 (in vitro, not in the mutant background; Supplementary Fig. 9 a) and are now providing extensive data on the requirement of PtdIns4P for endocytosis, including KNOLLE localization at the plasma membrane (Fig. 5a, b), altered CLC2 dynamics in the pi4kβ1 pi4kβ2 double mutant (Fig. 4 b-d), and other avidence described in more detail in previous responses, as requested. Overall, we thank the reviewer for these manifold suggestions, because these experiments have greatly enhanced our view on the function of PI4Kβ1.

Minor points:

Criticism:

Line 91, "CME" appeared the first time here but no explanation for the abbreviation

Response:

The abbreviation CME is now introduced properly at first mention.

Criticism:

Line 176, a comma is missing between c and f

Response:

, added.

Criticism:

The authors mention a "specific antibody" line 226, then mention an "aspecific band "in figS2. it is clear that their antibody recognize PIP4KB1 based on the disappearing band in pipk4B1 but it is not specific (just semantics)

Response:

The text has been modified accordingly.

Criticism:

Fig7c : I really like the orthogonal view depicting a ring-like localization (or not) of MAP65 and TUB. It could be used (if possible with good signal) earlier in the Fig. for MPK4, PIP4K or FAPP1 localization (or colocalization with FM4)

Response:

We thank the reviewer for the encouraging words. While we agree with the reviewer that the 3D-SIM analysis would be interesting also for other aspects of the study and in particular for MPK4, we have not applied this method to the MPK4 marker due to technical limitations. The 3D-SIM analysis requires a certain level of fluroescence signal intensity, as the illumination is performed over an extended period of time. The weak signal obtained for MPK4 was not conducive to its analysis by 3D-SIM. As furthermore the MPK4 localization is no longer at the center of our argument, we are not including the respective data.

Criticism:

Fig7d: FM4 and GFP-MAP65 legend is inverted

Response:

Fig. 7d was eliminated alltogether, because the MAP65-3 pattern in the mpk4-2 mutant may also have been wild type-like, as was pointed out by reviewer 1.

Criticism:

Fig7e: The MT dynamic in root elongating cell does not relate in anyway to the rest of the work here, so we cannot conclude that MT shrinkage is also affected at the CP in pipk mutants. This data could go to supplement.

Response:

The reviewer is correct that the analysis of microtubular dynamics in interphase cells does not pertain to microtubule behavior at the cell plate. However, as was pointed out by reviewers 1 and 2, the mutant complementation experiments with pKNOLLE-driven PI4Kβ1 are not conclusive, so we no longer claim that the functionality of PI4Kβs is restricted to

cytokinetic cells. In fact, we are now providing numerous additional data demonstrating that PI4Kβ1 also has a role in interphase cells (as redundantly listed above). In light of this altered interpretation, we would prefer to retain the altered microtubule dynamics in interphase cells of pi4kβ1 pi4kβ2 double mutants as part of the manuscript, and have moved the data to the supplement as suggested here (Supplementary Fig. 6).

2nd Editorial Decision 24th September 2018

Thank you for submitting your revised manuscript on a role for PI4Kβ in regulating MPK localization during cytokinesis in Arabidopsis thaliana to The EMBO Journal. My apologies for the extended duration of the review process due to the delayed delivery of one report.
Your study has been sent to the three original referees for evaluation, and we have now received reports from them, which are enclosed below for your information.

As you can see, the referees concur with us on the overall interest of your findings. However, they also raise few points that need to be addressed before they can support publication in The EMBO Journal. In particular, referees #1 requests you to test the localization of pKNOLLE:FLAG-PI4Kbeta in the pi4kb1;2 mutant background by immunofluorescence microscopy using your anti-PI4Kbeta1 antibody. This should then be compared to the pi4kb1;2 mutant (as a control) to test whether PI4Kbeta1 remains restricted to dividing cells. Also, referee #2 and #3 point out that the conclusions on CME defects seen in interphase cells need to be mitigated and the potential role of TGN defects in the cytokinetic phenotype discussed in depth. Referee #3 is the most critical one. Please note that while his/her concerns about novelty and quantification (point 2) are per se well taken, these would in our view not preclude publication. In addition, we do not request you to address point 5 from this referee. We instead agree with point 1 by referee #3 that some of the conclusions on cytokinesis are obtained from interphase cells. Thus, we request you to clearly indicate every time observations made only in interphase are used to interpret mitotic phenotypes and these explanations are based on assumptions (i.e. the same phenotypes are present throughout the entire cell cycle).

Addressing these issues as suggested by the referees is required to warrant publication in The EMBO Journal. Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS

Referee #1:

This is my re-review of a manuscript I had reviewed for the EMBO Journal at the beginning of this year. At the time, it was rejected due to a larger number of changes requested by all three reviewers, although all reviewers agreed that the work was very interesting and, in principle, of general interest. Therefore, this is treated as a new submission and reviewer comments are not attached. As I could see during the re-review, the authors have largely taken the common comments of the reviewers into consideration and have experimentally addressed them as outlined below. Accordingly, the new manuscript submitted under a different title includes substantial improvements.

Lin et al. provide the first description of a role for a phosphatidylinositol 4-phosphate (PtdIns(4)P kinase) in modifying MAP-kinase (MPK) localization during cytokinesis. Moreover, its relation to cytokinetic downstream events mediated by microtubule-associated protein 65-3 (MAP65-3 also known as PLEIADE) is addressed in this work. While roles for PtdIns(4)P kinases of different families have been reported in other systems than the plant Arabidopsis thaliana (Arabidopsis) analyzed here, this is to my knowledge the first report on a role for PtdIns(4)P kinase/MPK/MAP signaling during cytokinesis in any organism.

The work initially analyses the phenotype of a double mutant defective in the PtdIns(4)P kinase beta 1 and the PtdIns(4)P kinase beta 2 (pi4kb1 pi4kb2) as well as its rescue by an mCherry-PI4Kb1 fusion protein expressed from the pPI4Kb1 promoter (pPI4Kb1: mCherry-PI4Kb1). The construct is used for subsequent analyses as it proves fully functional. In response to my and other reviewers' comments, the authors now have added quantitative data and statistical analyses of the cytokinesis, cell wall orientation and root meristem defects in Figure 1c., quantitatively describing the

phenotypes and their rescue.

The pi4kb1 pi4kb2 double mutant displays cytokinesis defects and mCherry-PI4Kb1 localizes to the cell plate, a plant-specific cytokinetic membrane structure, supporting a function of PI4Kb1 in cytokinesis. Moreover, a fluorescent reporter for PtdIns(4)P confirms its presence in the cell plate (new Fig. 2). Additional data has now been provided on mCherry-PI4Kb1and ARF1 localization at different stages of cytokinesis (Fig. 2c).

The new Figure 3 now analyses the cytokinesis defects of the pi4kb1 pi4kb2 double mutant in more detail by additional transmission electron microscopy experiments, revealing cell wall stabs and unfused vesicles during late cytokinesis.

In response to one of the other reviewers, the authors include quantitative live imaging analysis of a PtdIns(4)P reporter (2xmCherry-FAPP1-PH) at the cell plate as well as of the timing of clathrin light chain 2 (CLC2)-GFP recruitment to the cell plate in both the wild type and the pi4kb1 pi4kb2 double mutant. The quantitative and statistical analyses reveal significantly delayed CLC2-GFP recruitment and a reduced accumulation of the PtdIns(4)P reporter at the cell plate, suggesting reduced PtdIns(4)P production during cell division and reduced or delayed endocytosis from the cell plate.

In response to my and other reviewers' comments the authors have now tested the cell plate-localized KNOLLE and PIN2 proteins for their localization in the pi4kb1 pi4kb2 double mutant and, additionally, their occurrence in agglomerations of endocytosed material induced by the vesicle trafficking inhibitor brefeldin A (BFA) under concomitant cycloheximide (CHX) treatment (new Figure 5). An accumulation of KNOLLE observed at the lateral plasma membrane is indicative of an endocytosis defect albeit not as clearly visible as in other previously described endocytosis defective mutants. Nonetheless, the relatively stronger accumulation of both cargoes at the cell plate relative to internal compartments in the pi4kb1 pi4kb2 double mutant compared to wild type upon BFA and CHX co-treatment further suggested that endocytosis of both cargoes is reduced in this double mutant. All data has been quantified and subjected to statistical analysis.

The data on tyrphostin A23 treatment has been removed from the manuscript, because the inhibitor has additional effects to those observed on endocytosis. Instead, the data on CLC2-GFP and endocytic cargo accumulation presented in Figs. 4 and 5 has been included.

The authors further described the occurrence of multinucleated cells in the pi4kb1 pi4kb2 double mutant and quantitatively describe aberrancies in phragmoplast formation in the double mutant (Fig. 6) by anti-KNOLLE and anti-tubulin immunofluorescence labeling of root meristems and subsequently employ quantitative live imaging of phragmoplast formation employing an mCherry-TUBULINA5 (TUA5) reporter, revealing delayed and perturbed phragmoplast formation in the double mutant (Figure 7).

They extend these analyses to live imaging of the MICROTUBULE ASSOCIATED PROTEIN 65-3/PLEIADE fused GFP in presence of the endocytic, plasma membrane and cell plate marker FM4-64, revealing a longer persistence of GFP-MAP65-3 in the center of the cell plate in the pi4kb1 pi4kb2 double mutant than in the wild type. Here, the authors provided 3D-stacks of GFP-MAP65-3 localization to support their point - as requested.

MAP65-3 had previously been shown to be a target molecule for phosphorylation by the MAP-kinase MPK4. Lin and colleagues therefore proceed to address the role of MPK4 in relation to PI4Kb1 PI4Kb2 by analysing a pi4kb1 pi4kb2 mpk4-2 triple mutant. In comparison to pi4kb1 pi4kb2 double and mpk4-2 single mutants, no viable homozygous triple mutants are recovered. Interestingly, also pi4kb1 pi4kb2/+ mpk4-2 mutants show a strongly reduced seedling size compared to pi4kb1 pi4kb2 and mpk4-2 mutants, suggesting a synergistic genetic interaction (Fig. 9). In this improved manuscript, the authors also provide quantitative analyses of the number of multinucleated cells in the pi4kb1 pi4kb2 and mpk4-2 mutants as well as the pi4kb1 pi4kb2/+ mpk4-2 mutant, revealing a strongly enhanced phenotype with respect to the number of multinucleated cells in the pi4kb1 pi4kb2/+ mpk4-2 mutant strongly suggesting synergistic (NOT ADDITIVE) genetic interaction of MAPK4 with PIK4b1 and/or PIK4b2 during cytokinesis in vivo (Fig. 9). While this data does not infer epistatsis, it strongly suggests that in vivo action of MAPK4

with PIK4b1 and/or PIK4b2 converges at a common biological process (e.g. phragmoplast formation) and obviously in both cases directly or indirectly affects MAP65-3.

The authors further reveal interaction of the 566 N-terminal amino acids of PI4Kb1 with MPK4 by yeast two hybrid (Y2H) assays as well as co-immunoprecipitation of MPK4-myc and PI4Kb1 from Arabidopsis protein extracts. The claim that these experiments demonstrate a physical interaction of PI4Kb1 and MPK4 now has been weakened but data showing in vitro phosphorylation of GST-PI4Kb1 by GST-MPK-4 has been added (Figure S9a). mCherry-PI4Kb1 and MPK4-YFP do colocalize at the cell plate in vivo (Fig. 10c), but mpk4-2 mutation does not alter mCherry-PI4Kb1 cell plate localization (Figure S9c). A very slight difference in localization of the PtdIns(4)P reporter 2xmCherry-FAPP1-PH is observed at the cell plate of the mpk4-2 mutant, but it is questionable as to whether this reflects direct action of MPK4 on PI4Kb1 in vivo and the difference appears only marginally significant.

Nevertheless, this manuscript reveals that PI4Kb1 and MPK4 can interact in yeast, in plant extracts by co-immunoprecipitation, potentially directly because MPK4 can phosphorylate PI4Kb1 in vitro, they co-localize during cell plate formation and genetic evidence suggest that in Arabidopsis their action certainly converges on a common target namely phragmoplast formation and MAP63-5 localisation.

These findings have been worked out very well and all experiments have been performed very thoroughly. As the first study addressing a role for PtdIns(4)P kinase and MPK/MAP signaling interaction during cytokinesis, the work is novel, certainly of broad interest to the plant science community but also to some readers working on phospholipid and MAP-kinase signaling as well as cytokinesis in other systems. The findings should therefore appeal to a broader readership such as the one covered by The EMBO Journal.

The only remaining caveat of the study is that it cannot, yet, mechanistically address whether it is PI4Kb1 that regulates MPK4 or vice versa, or whether action of both converges at a common biological process or target protein. Resolving this would involve several more years of work and does not appear to be reasonable to request at this stage given the novelty and extensive work efforts presented by the current work.

Taken together, this is a very well conducted, comprehensive and highly interesting study that requires minor revisions prior to publication.

Minor revisions:

1) Two reviewers requested to demonstrate that the FLAG-PI4Kb1 protein expressed from the KNOLLE promoter is indeed only expressed in dividing cells. The authors argue that they could not detect the protein by immunofluorescence microscopy using an anti-FLAG antibody (although several exist that work in immunofluorescence microscopy in other systems). However, did the authors test their own anti-PI4Kb1antibody in immunofluorescence (IF)microscopy. Since the pKNOLLE:FLAGPI4Kb1 fusion was generated in the pi4kb1 pi4kb2 mutant background the double mutant alone could serve as a good specificity control in IF and because the pKNOLLE promoter is rather strong it may be possible to detect the fusion with the anti-PI4Kb1antibody.

2) I suggest removal of Fig. 10e, because the observed effect is very weak (reproducible?) marginally significant and it does also not resolve the question who mechanistically acts on whom here (i.e. MPK4 on PI4Kb1or vice versa or on a common target). This is a bit of a loose end towards this otherwise nice story.

3) Abstract: Mutant names should be given in italics.

4) Abstract: "acting in a common pathway" should be removed in the last sentence, because the synergistic interaction could as well indicate action of two different pathways converging on the same target protein or biological process i.e. phragmoplast dynamics which could be influenced by endocytosis and phragmoplast microtubule organization. Whether this occurs via one common or

two different pathways is not really shown, yet.

5) Line 119: This needs to be rewritten, because the authors have not shown physical interaction of PI4b1 and MPK4 in vivo and this could be misunderstood in the previous version.

This should read: "Based on similar cytokinetic defects of the pi4kb1 pi4kb2 and mpk4 mutants, their genetic interaction and in vitro physical interaction of PI4b1 and MPK4, we propose ... ."

6) Figure 1 legend and elsewhere (e.g. page 26 line 524. "root tissue patterning" I would refrain from using this word in this context as it is pre-occupied by pattern formation of hair and non-hair cells in the root epidermis. What the authors refer to are the irregularities of cell division orientation in epidermal cells and I would exactly call them like this. This could read "The irregularities in the pattern of root epidermal cell division orientation is also complemented by ... ."

7) Figure 3. Please, insert how many cells/roots were observed by TEM in the figure legend or the methods.

8) Page 15, lines 318-320. The sentence is a bit strong because the authors cannot specifically remove function of PI4Kb1 and PI4Kb2 at the cell plate to really prove this point. Therefore, I would rather write "are likely required" rather than "are required".

9) Page 17, lines 360, 362 (and elsewhere in the manuscript): Wild type should not be written in italics (throughout the manuscript).

10) What happened to the data on MPK4-YFP localization in pi4kb1 pi4kb2 which was removed from the manuscript. Could it not be substantiated?

11) page 25, line 496. This needs to be changed, because the authors do not show physical interaction of PI4Kb1 and MPK4 in Arabidopsis as the title suggest. Suggestion: "Arabidopsis PI4Kb1 and MPK4 interact genetically, co-imunoprecipitate, interact in yeast and directly in vitro".

12) page 26, line 541: Please, remove "in vivo" here, because co-immunoprecipitation from a plant extract is not an "in vivo" method. It maybe replaced by "from plant protein extracts".

13)Remove "according to additive cytokinetic defects" and replace thos by "during cytokinesis". The effect presented in Fig. 9c reveals an enhancement of the number of multinucleate cells. pi4kp1 pi4kb2 = 5%, mpk4-2 = 10%, but pi4kp1 pi4kb2/+ mpk4-2 = 40%. Since 40% is clearly more than 15% this is certainly more than additive considered from a quantitative genetics view and certainly not additive when employing the older "mendelian" definition from a developmental geneticists point of view that an additive phenotype is one where two completely different phenotypes are combined. Hence, this is clearly a phenotypic enhancement and factually, even better, a synthetic lethality of the fully homozygous triple mutant, clearly suggesting synergistic interaction and it helps the authors interpretation! (although they might have preferred to see an epistatic relationship)

Referee #2:

The new version of this manuscript has been much improved and I want to thank the authors for taking into account the points raised during the previous round of review. This paper is a very nice combination of genetic, cell biology and biochemistry, which will certainly make date in the field of plant cytokinesis, intracellular trafficking and phosphoinositides. The new live imaging data constitute a very nice addition to the story.
While I don't have any new experiments to suggest, I still would like the authors to be a bit more cautious with some of their statements/conclusions, mainly concerning the following two points:
- Clathrin/CME defects v.s. TGN defects. As stated, the yeast pik1 mutant affects clathrin recruitment at the Golgi and hence induces many protein sorting phenotypes in this compartment. It is therefore likely that the Arabidopsis pi4kB1B2 double mutant has also defects in TGN trafficking (as also supported by Kang et al., 2011). Note that, like in yeast, these phenotypes may include

defects in clathrin recruitment at the TGN. I think overall, it will be difficult to completely untangle whether the cytokinesis defects are caused by trafficking defects at the PM, at the TGN, at the cell plate or a combination thereof. This may be possible to address in the future, perhaps by using experiments allowing to specifically deplete PI4P at the TGN vs PM vs cell plate, or identifying PI4KB mutant versions that localize at the TGN but not the cell plate or vice versa. However, these kinds of experiments clearly extend beyond the scope of the manuscript. One may not exclude that TGN defects alone could contribute to the observed cytokinesis defects. Rather than insisting on CME, I think the authors should discuss this possibility. It would also help, if the authors would analyze the relative quantity of PtdIns4P sensor and CLC2-GFP at the TGN, and not only at the cell plate and PM. This quantification may be obtained from the images already acquired to quantify the presence of these proteins at the cell plate and should therefore not require new experiments.
- My second point is regarding the relationship between PI4KBs and MPK4. This part of the manuscript has been largely clarified from the previous version, nonetheless, the authors still conclude that "The experiments suggest MPK4 as an upstream regulator of PI4Kβ1 at the cell plate". I am not sure such statement is clearly proven and even useful. I would leave it at "functional interplay between MPK4, PI4KB1 and MAP65-3". I believe it is still unclear why MPK4 and PI4KB1 interacts. This could be sorted out by (for example) refining the molecular mechanisms of the MPK4/PI4KB1 interaction in order to obtain point mutants of MPK4 and PI4KB1 which specifically cannot interact which each other. Again, this goes beyond the scope of this manuscript, and I would therefore encourage the authors to be more careful and leave some space for future studies.

- This is a minor point but in order to analyze the presence of PtdIns4P at the cell plate, the authors used the FAPP1 PtdIns4P sensor, which is also known to bind to the ARF1 protein. The decreased labeling could therefore be due to limiting PtdIns4P and/or miss-localization of ARF1 (at TGN and/or cell plate). The authors could address this point by analyzing ARF1 localization in pi4kB1B2 double mutant (using their anti-ARF1 antibody) or additional PtdIns4P sensors, which localization are independent of ARF1. In any case, it could be helpful if this point could at least be discussed.

Referee #3:

The manuscript of Lin et al. has been completely reformulated and changed in its logics. Additionally, substantial amount of key data for the previous conclusions was deleted and numerous new data was added. For this reason, a complete re-evaluation as a new manuscript was needed.
The current manuscript, "A dual role for PI4Kβ in endocytosis and phragmoplast dynamics during plant somatic cytokinesis" describes mainly two roles of PI4Kβ during cytokinesis, namely clathrin-mediated endocytosis on cell plates and phragmoplast stability. The authors added beautiful live-imaging data and some quantification for imaging data to support their conclusions. However, the findings and major points of the manuscript are not of great conceptual novelty, particularly the PIP4P-dependent clathrin-mediated endocytosis, because the cytokinetic defect phenotype of the double pi4kβ1 pi4kβ2 mutant has been previously described (Kang et al. 2011) and PIP4P-induced clathrin-mediated endocytosis has been shown several times in other organisms, as the authors cited in the discussion: animal cells (Burke, Inglis et al., 2014, de Graaf, Zwart et al., 2004, Kapp-Barnea, Ninio-Many et al., 2006) and yeast (Audhya, Foti et al., 2000, Yamamoto, Wada et al., 2018). Additionally, it is not mechanistically straightforward to link the vesicle-fusion defect phenotype in the cell plates with the described function of PI4K in endocytosis. Moreover, this CME aspect is also not clear mechanistically both in the interphase and the cytokinesis.
Regarding the over-stabilized phragmoplast phenotype in the pi4kβ double mutant, I agree this is a new phenotype to understand plasticity and dynamics of the phragmoplast, but the link to NACK-PQR pathway that regulates MAP65-3 is not established well enough to be of interest to more general readers.
Overall, the authors tried to explain the cytokinetic defect phenotype, but many of the explanations are conjectures from the phenotype observed in interphase (for example Figure 5 e,g,i Supplemetnal Fig 6,7).
Therefore, I still think that the authors should rethink the interpretation and/or provide additional data to support their conclusions. A more detailed discussion follows below.

1) The link between PtdIns(4)P production and phenotypes are not clear both in the interphase and the cytokinesis

The role claimed for the PI(4)P at the cell plate is (too) often assumed from data at the plasmamembrane of non-cytokinetic cells

CLC2 persistence at the fused cell plate (i.e. PM?) post-cytokinesis (Fig4 &S5)
CLC2 increased residency during interphase (root elongation zone S6)
Reduced KN endocytosis from the PM during late telophase (Fig5)
Reduced PIN2 endocytosis from basal PM post cytokinesis (Fig5)
Reduced FM4-64 internalization (Fig5) in root epidermal cells
All those experiment rather demonstrate a role for PI(4)P (even though not significantly reduced) in endocytosis at the PM rather than at the cell plate

One of the main claims in the new manuscript is the dynamics of CLC2-GFP in the interphase or the cytokinesis as shown in Fig 4 (This figure is lacking proper captions, there are no a and c) and Supplemental Fig 6. The data is clear, persistent retaining of CLC2-GFP on the PM during the interphase and retarded CLC2-GFP accumulation on the cell plates. But I could not understand the logic of the statement at line 257-8 "In any case, the strong phenotype of the pi4kβ1 pi4kβ2 double mutant indicates that even a minor contribution to cellular PtdIns(4)P production exerts a relevant effect." I am not convinced whether this phenotype is due to the amount of PtdIns(4)P because the amount was not changed in the pi4kβ double mutant shown in Supplemental Fig 4, as the authors mentioned. I am afraid that the authors may describe some secondary effects, not from the quantitative change of PtdIns(4)P, for example abnormal auxin distribution or different cellular growth rates.

2) The quantification of intensity ratio between the plasma membrane and the cell plates is not convincing

The authors used the PI(4)P reporter mCherry-FAPP1 to quantify the relative abundance of PI(4)P between the PM and the CP during cytokinesis.
This method is used to compare intensities ratio between PM and CP in WT versus the pi4kβ1 pi4kβ2 double mutant (fig4A) or mpk4 mutant (Fig10E) and convey a strong message for this story (there are differences in PI(4)P at the CP of the pi4kβ1 pi4kβ2 and mpk4)

However there are discrepancies that seem to invalidate the method:
While the reporter intensities might vary across samples, the ratios should remain fairly reproducible. The difference between genotypes is rather low (4A WT=1,1 pipkB1/2=0,9 / 10E WT=0,9 mpk4=1) yet there seems to be a high variability between experiments, as witnessed by the WT variation across experiment (values higher or lower than 1 suggesting opposite repartition of PI4P between PM and CP!)
Also, the opposite phenotype of the pi4kβ1 pi4kβ2 and mpk4 contrasts with the otherwise shared phenotypes.

I am not so convinced whether this quantification method is able to measure genuine differences. As the authors showed in nice TEM data in Fig3, the phragmoplast is more dispersed in the pi4kβ double mutant, which should cause the biosensor signal to be diffused as well. Moreover, this marker appears to have high background as seen in the pictures. For these reasons, I do not think this ratio change is convincing enough to support their model.

3) Does endocytosis defect exert the fusion defect in cell plates as shown in figure 3?
The authors claimed defect in endocytosis is one of the reasons to block normal cell plate formation. I agree the balance of endocytosis and exocytosis is absolutely important to form the cell plate but why would defects in endocytosis, which removes or trims excess material, block the fusion of vesicles in the double mutant as shown in figure3? This is not easy to understand.

4) The stage description is not clear
In Figure 5 e, the authors presented PIN2-GFP localization on the CP in WT and the double mutant of pik4β. It is unclear to me how the authors distinguish the PM and newly developed PM from CP.

Is there some definition?

5) Abnormal localization of MAP65-3
As beautifully shown in Figure 8, GFP-MAP65-3 is over-stabilized in the double mutant. But, mechanistically, this phenomena is not satisfactorily explained, particularly the link from PI4Kβ. I guess the link between MPK4 and MAP65-3 is of high interest for readers, especially those who are working on plant cytokinesis. Furthermore, the behavior of PI4Kβ and MAP65-3 in the mpk4 mutant should be compared. I agree this is a novel finding, but I think the authors should clarify and provide additional data to establish the link for example using synchronized suspension cells like in Sasabe et al. (2006).

Minor points

In the abstract, most of the mutant name are not in Italic. Please change them into Italic eg. mpk4-2, pi4kβ1,2.

In the figure legends Fig 1(c) Data are mean {plus minus} SD. (bars?)
For Figure 2, captions are lacking in the figure. The top panels on the left should be annotated as "a" and the right panel should include "c" somewhere in the figure.

In figure 10B I am wondering if the molecular weight marker labeling is correct because the predicted molecular weight of MPK4-myc is much less. I agree some protein shows different molecular weight from expected size but the difference in the panel is quite big. Is it possible to check also by anti-MPK4 (like A6979 from sigma)?

In supplemental Fig9, corresponding CBB stained gel image is absolutely necessary as a protein loading control.

Line 690 pi4kb1 pi4kb2 should be written "pi4kβ1 pi4kβ2."

Most of the cases, the authors mentioned two authors when the authors have citations in the text. I do not think this style is common. Is it EMBO journal style?

Line 975 I assume the authors meant degree not temperature, C should be removed.

2nd Revision - authors' response 26th October 2018

Lin et al. "A dual role for cell plate-associated PI4Kβ in endocytosis and phragmoplast dynamics during plant somatic cytokinesis"

EMBOJ-2018-100303R

Itemized response to reviews:
Editor's summary:

[...] As you can see, the referees concur with us on the overall interest of your findings. However, they also raise few points that need to be addressed before they can support publication in The EMBO Journal. In particular, referees #1 requests you to test the localization of pKNOLLE:FLAG-PI4Kbeta in the pi4kb1;2 mutant background by immunofluorescence microscopy using your anti-PI4Kbeta1 antibody. This should then be compared to the pi4kb1;2 mutant (as a control) to test whether PI4Kbeta1 remains restricted to dividing cells. Also, referee #2 and #3 point out that the conclusions on CME defects seen in interphase cells need to be mitigated and the potential role of TGN defects in the cytokinetic phenotype discussed in depth. Referee #3 is the most critical one. Please note that while his/her concerns about novelty and quantification (point 2) are per se well taken, these would in our view not preclude publication. In addition, we do not request you to address point 5 from this referee. We instead agree with point 1 by referee #3 that some of the conclusions on cytokinesis are obtained from interphase cells. Thus, we request you to clearly indicate every time observations made only in interphase are used to interpret mitotic phenotypes and these explanations are based on assumptions (i.e. the same phenotypes are present throughout the entire cell cycle).

Response:

We have performed all requested experiments and made all requested changes to the best of our ability. The responses to the individual points raised by the reviewers are described in more detail below.

Editor:

Addressing these issues as suggested by the referees is required to warrant publication in The EMBO Journal. Given the overall interest of your study, I would like to invite you to revise the manuscript in response to the referee reports.

REFEREE REPORTS.

Referee #1:

Summary/comments:

This is my re-review of a manuscript I had reviewed for the EMBO Journal at the beginning of this year. At the time, it was rejected due to a larger number of changes requested by all three reviewers, although all reviewers agreed that the work was very interesting and, in principle, of general interest. Therefore, this is treated as a new submission and reviewer comments are not attached. As I could see during the re-review, the authors have largely taken the common comments of the reviewers into consideration and have experimentally addressed them as outlined below. Accordingly, the new manuscript submitted under a different title includes substantial improvements.

Lin et al. provide the first description of a role for a phosphatidylinositol 4-phosphate (PtdIns(4)P kinase) in modifying MAP-kinase (MPK) localization during cytokinesis. Moreover, its relation to cytokinetic downstream events mediated by microtubule-associated protein 65-3 (MAP65-3 also known as PLEIADE) is addressed in this work. While roles for PtdIns(4)P kinases of different families have been reported in other systems than the plant Arabidopsis thaliana (Arabidopsis) analyzed here, this is to my knowledge the first report on a role for PtdIns(4)P kinase/MPK/MAP signaling during cytokinesis in any organism.

The work initially analyses the phenotype of a double mutant defective in the PtdIns(4)P kinase beta 1 and the PtdIns(4)P kinase beta 2 (pi4kb1 pi4kb2) as well as its rescue by an mCherry-PI4Kb1 fusion protein expressed from the pPI4Kb1 promoter (pPI4Kb1: mCherry-PI4Kb1). The construct is used for subsequent analyses as it proves fully functional. In response to my and other reviewers' comments, the authors now have added quantitative data and statistical analyses of the cytokinesis, cell wall orientation and root meristem defects in Figure 1c., quantitatively describing the phenotypes and their rescue.

The pi4kb1 pi4kb2 double mutant displays cytokinesis defects and mCherry-PI4Kb1 localizes to the cell plate, a plant-specific cytokinetic membrane structure, supporting a function of PI4Kb1 in cytokinesis. Moreover, a fluorescent reporter for PtdIns(4)P confirms its presence in the cell plate (new Fig. 2). Additional data has now been provided on mCherry-PI4Kb1and ARF1 localization at different stages of cytokinesis (Fig. 2c).

The new Figure 3 now analyses the cytokinesis defects of the pi4kb1 pi4kb2 double mutant in more detail by additional transmission electron microscopy experiments, revealing cell wall stabs and unfused vesicles during late cytokinesis.

In response to one of the other reviewers, the authors include quantitative live imaging analysis of a PtdIns(4)P reporter (2xmCherry-FAPP1-PH) at the cell plate as well as of the timing of clathrin light chain 2 (CLC2)-GFP recruitment to the cell plate in both the wild type and the pi4kb1 pi4kb2 double mutant. The quantitative and statistical analyses reveal significantly delayed CLC2-GFP recruitment and a reduced accumulation of the PtdIns(4)P reporter at the cell plate, suggesting reduced PtdIns(4)P production during cell division and reduced or delayed endocytosis from the cell plate.

In response to my and other reviewers' comments the authors have now tested the cell plate-localized KNOLLE and PIN2 proteins for their localization in the pi4kb1 pi4kb2 double mutant and, additionally, their occurrence in agglomerations of endocytosed material induced by the vesicle trafficking inhibitor brefeldin A (BFA) under concomitant cycloheximide (CHX) treatment (new Figure 5). An accumulation of KNOLLE observed at the lateral plasma membrane is indicative of an endocytosis defect albeit not as clearly visible as in other previously described endocytosis defective mutants. Nonetheless, the relatively stronger accumulation of both cargoes at the cell plate relative to internal compartments in the pi4kb1 pi4kb2 double mutant compared to wild type upon BFA and CHX co-treatment further suggested that endocytosis of both cargoes is reduced in this double mutant. All data has been quantified and subjected to statistical analysis.

The data on tyrphostin A23 treatment has been removed from the manuscript, because the inhibitor has additional effects to those observed on endocytosis. Instead, the data on CLC2-GFP and endocytic cargo accumulation presented in Figs. 4 and 5 has been included.

The authors further described the occurrence of multinucleated cells in the pi4kb1 pi4kb2 double mutant and quantitatively describe aberrancies in phragmoplast formation in the double mutant (Fig. 6) by anti-KNOLLE and anti-tubulin immunofluorescence labeling of root meristems and subsequently employ quantitative live imaging of phragmoplast formation employing an mCherry-TUBULINA5 (TUA5) reporter, revealing delayed and perturbed phragmoplast formation in the double mutant (Figure 7).

They extend these analyses to live imaging of the MICROTUBULE ASSOCIATED PROTEIN 65-3/PLEIADE fused GFP in presence of the endocytic, plasma membrane and cell plate marker FM4-64, revealing a longer persistence of GFP-MAP65-3 in the center of the cell plate in the pi4kb1 pi4kb2 double mutant than in the wild type. Here, the authors provided 3D-stacks of GFP-MAP65-3 localization to support their point - as requested.

Comment:

MAP65-3 had previously been shown to be a target molecule for phosphorylation by the MAP-kinase MPK4. Lin and colleagues therefore proceed to address the role of MPK4 in relation to PI4Kb1 PI4Kb2 by analysing a pi4kb1 pi4kb2 mpk4-2 triple mutant. In comparison to pi4kb1 pi4kb2 double and mpk4-2 single mutants, no viable homozygous triple mutants are recovered. Interestingly, also pi4kb1 pi4kb2/+ mpk4-2 mutants show a strongly reduced seedling size

compared to pi4kb1 pi4kb2 and mpk4-2 mutants, suggesting a synergistic genetic interaction (Fig. 9). In this improved manuscript, the authors also provide quantitative analyses of the number of multinucleated cells in the pi4kb1 pi4kb2 and mpk4-2 mutants as well as the pi4kb1 pi4kb2/+ mpk4-2 mutant, revealing a strongly enhanced phenotype with respect to the number of multinucleated cells in the pi4kb1 pi4kb2/+ mpk4-2 mutant strongly suggesting synergistic (NOT ADDITIVE) genetic interaction of MAPK4 with PIK4b1 and/or PIK4b2 during cytokinesis in vivo (Fig. 9). While this data does not infer epistatsis, it strongly suggests that in vivo action of MAPK4 with PIK4b1 and/or PIK4b2 converges at a common biological process (e.g. phragmoplast formation) and obviously in both cases directly or indirectly affects MAP65-3.

Response:

We have altered the text to more precisely define the genetic interaction of MPK4 and PI4Kbetas as a synergistic, not additive, effect, as suggested.

Further summary:

The authors further reveal interaction of the 566 N-terminal amino acids of PI4Kb1 with MPK4 by yeast two hybrid (Y2H) assays as well as co-immunoprecipitation of MPK4-myc and PI4Kb1 from Arabidopsis protein extracts. The claim that these experiments demonstrate a physical interaction of PI4Kb1 and MPK4 now has been weakened but data showing in vitro phosphorylation of GST-PI4Kb1 by GST-MPK-4 has been added (Figure S9a). mCherry-PI4Kb1 and MPK4-YFP do colocalize at the cell plate in vivo (Fig. 10c), but mpk4-2 mutation does not alter mCherry-PI4Kb1 cell plate localization (Figure S9c).

Comment:

A very slight difference in localization of the PtdIns(4)P reporter 2xmCherry-FAPP1-PH is observed at the cell plate of the mpk4-2 mutant, but it is questionable as to whether this reflects direct action of MPK4 on PI4Kb1 in vivo and the difference appears only marginally significant.

Response:

We agree with the reviewer that the difference in 2xmCherry-FAPP1-PH at the cell plate is slight. In response to this comment, we have now added a remark stating that it remains to be seen whether the slight difference observed has true biological significance. Our quantitative analysis was included in response to a reviewer suggestion and - respectfully - we see no reason to exclude these data from the manuscript. (please also see the response to the respective comment 2 by reviewer 3).

Further summary:

Nevertheless, this manuscript reveals that PI4Kb1 and MPK4 can interact in yeast, in plant extracts by co-immunoprecipitation, potentially directly because MPK4 can phosphorylate PI4Kb1 in vitro, they co-localize during cell plate formation and genetic evidence suggest that in Arabidopsis their action certainly converges on a common target namely phragmoplast formation and MAP63-5 localisation.

These findings have been worked out very well and all experiments have been performed very thoroughly. As the first study addressing a role for PtdIns(4)P kinase and MPK/MAP signaling interaction during cytokinesis, the work is novel, certainly of broad interest to the plant science community but also to some readers working on phospholipid and MAP-kinase signaling as well as cytokinesis in other systems. The findings should therefore appeal to a broader readership such as the one covered by The EMBO Journal.

The only remaining caveat of the study is that it cannot, yet, mechanistically address whether it is PI4Kb1 that regulates MPK4 or vice versa, or whether action of both converges at a common biological process or target protein. Resolving this would involve several more years of work and does not appear to be reasonable to request at this stage given the novelty and extensive work efforts presented by the current work.

Taken together, this is a very well conducted, comprehensive and highly interesting study that requires minor revisions prior to publication.

Minor revisions:

Comment:

1) Two reviewers requested to demonstrate that the FLAG-PI4Kb1 protein expressed from the KNOLLE promoter is indeed only expressed in dividing cells. The authors argue that they could not detect the protein by immunofluorescence microscopy using an anti-FLAG antibody (although several exist that work in immunofluorescence microscopy in other systems). However, did the authors test their own anti-PI4Kb1antibody in immunofluorescence (IF)microscopy? Since the pKNOLLE:FLAGPI4Kb1 fusion was generated in the pi4kb1 pi4kb2 mutant background the double mutant alone could serve as a good specificity control in IF and because the pKNOLLE promoter is rather strong it may be possible to detect the fusion with the anti-PI4Kb1antibody.

Response:

We thank the reviewer for this suggestion and we have performed the requested experiments. While the anti-PI4Kb1 antibody detected the protein in Westerns (e.g., Fig. S2a), we were not able to obtain interpretable images by immunofluorescence microscopy. This was tested using wild type material as well as partially complemented plants expressing the pKNOLLE:FlagPI4Kb1 fusion in the pi4kb1 pi4kb2 double mutant background. Obviously, we also detected unspecific signals in the requested pi4kb1 pi4kb2 controls. These requested controls are now included as Fig. S2c, documenting that the antibody appears not suitable for immunofluorescence. While this outcome of this control experiment did not yield the desired information, please note that we are not claiming that PI4Kb isoforms act only during cytokinesis. Our data rather indicate that the function of PI4Kbs is also important in interphase cells, as also evidenced the observations that i) there is an increased half life of CLC2-GFP at the plasma membrane of interphase cells; ii) there is reduced formation of PIN2-decorated BFA bodies in the mutant; and iii) there is reduced internalization of FM 4-64 from the plasma membrane. The notion that there is a role for PI4Kbs also apart from the cell plate is now clearly stated in the text, along with the individual mention which experiments pertain to cytokinetic or to interphase cells. (as requested by reviewers 1 and 3).

Comment:

2) I suggest removal of Fig. 10e, because the observed effect is very weak (reproducible?) marginally significant and it does also not resolve the question who mechanistically acts on whom here (i.e. MPK4 on PI4Kb1or vice versa or on a common target). This is a bit of a loose end towards this otherwise nice story.

Response:

We thank the reviewer for this suggestion, but we do not feel comfortable with leaving out the data. The experiment to test the abundance of PI4P at the cell plate had been suggested by the reviewers, highlighting that this is an open question arising while following the logic of our results. While we agree that the difference might be slight and possibly not even biologicall relevant (see our response above), we feel that showing the data closes an informational gap and will help to further resolve the role of PI4P in future experiments. We respectfully opt to retain the data of Fig. 10 e as part of our manuscript.

Comment:

3) Abstract: Mutant names should be given in italics.

Response:

Done.

Comment:

4) Abstract: "acting in a common pathway" should be removed in the last sentence, because the synergistic interaction could as well indicate action of two different pathways converging on the same target protein or biological process i.e. phragmoplast dynamics which could be influenced by endocytosis and phragmoplast microtubule organization. Whether this occurs via one common or two different pathways is not really shown, yet.

Response:

We thank the reviewer for this suggestion and have changed the phrasing.

Comment:

5) Line 119: This needs to be rewritten, because the authors have not shown physical interaction of PI4b1 and MPK4 in vivo and this could be misunderstood in the previous version.

This should read: "Based on similar cytokinetic defects of the pi4kb1 pi4kb2 and mpk4 mutants, their genetic interaction and in vitro physical interaction of PI4b1 and MPK4, we propose ... ."

Response:

We thank the reviewer for this detailed suggestion and have changed the phrasing as suggested.

Comment:

6) Figure 1 legend and elsewhere (e.g. page 26 line 524. "root tissue patterning" I would refrain from using this word in this context as it is pre-occupied by pattern formation of hair and non-hair cells in the root epidermis. What the authors refer to are the irregularities of cell division orientation in epidermal cells and I would exactly call them like this. This could read "The irregularities in the pattern of root epidermal cell division orientation is also complemented by ... ."

Response:

We thank the reviewer for this detailed suggestion and have changed the phrasing as suggested.

Comment:

7) Figure 3. Please, insert how many cells/roots were observed by TEM in the figure legend or the methods.

Response:

The requested information was added in the figure legend.

Comment:

8) Page 15, lines 318-320. The sentence is a bit strong because the authors cannot specifically remove function of PI4Kb1 and PI4Kb2 at the

cell plate to really prove this point. Therefore, I would rather write "are likely required" rather than "are required".

Response:

We thank the reviewer for this suggestion and have changed the phrasing as suggested.

Comment:

9) Page 17, lines 360, 362 (and elsewhere in the manuscript): Wild type should not be written in italics (throughout the manuscript).

Response:

We have removed italics from all "wild types".

Comment:

10) What happened to the data on MPK4-YFP localization in pi4kb1 pi4kb2 which was removed from the manuscript. Could it not be substantiated?

Response:

The data on the altered MPK4-YFP localization in the pi4kb1 pi4kb2 double mutant was eliminated from the manuscript because we had decided to present a story focusing on the notion that MPK4 controls PI4Kbs. Clearly, the fact that MPK4 phosphorylates PI4Kb1 makes a strong case for this scenario, which was additionally proposed in the reviews to your original submission. We are aware that this is possibly simplifying, and the reviewer is correct that reciprocal regulatory effects of PI4Kbs on MPK4 function are a possibility. In fact, we feel that a bi-directional regulatory circuit of

MPK4 and PI4Kbs is a fascinating and very interesting topic. Our data to this effect, which had been part of our original submission, have not in any way been insubstantiated, and we are still following this train of thought. However, given the large volume of additional data that would be required to convincingly demonstrate such further regulatory steps, we opted to remove this information from the manuscript and possible use it as a basis for a future separate study.

Comment:

11) page 25, line 496. This needs to be changed, because the authors do not show physical interaction of PI4Kb1 and MPK4 in Arabidopsis as the title suggest. Suggestion: "Arabidopsis PI4Kb1 and MPK4 interact genetically, co-imunoprecipitate, interact in yeast and directly in vitro".

Response:

We thank the reviewer for this suggestion and have changed the phrasing as suggested.

Comment:

12) page 26, line 541: Please, remove "in vivo" here, because co-immunoprecipitation from a plant extract is not an "in vivo" method. It maybe replaced by "from plant protein extracts".

Response:

We thank the reviewer for this suggestion and have changed the phrasing as suggested.

Comment:

13)Remove "according to additive cytokinetic defects" and replace thos by "during cytokinesis". The effect presented in Fig. 9c reveals an enhancement of the number of multinucleate cells. pi4kp1 pi4kb2 = 5%, mpk4-2 = 10%, but pi4kp1 pi4kb2/+ mpk4-2 = 40%. Since 40% is clearly more than 15% this is certainly more than additive considered from a quantitative genetics view and certainly not additive when employing the older "mendelian" definition from a developmental geneticists point of view that an additive phenotype is one where two completely different phenotypes are combined. Hence, this is clearly a phenotypic enhancement and factually, even better, a synthetic lethality of the fully homozygous triple mutant, clearly suggesting synergistic interaction and it helps the authors interpretation! (although they might have preferred to see an epistatic relationship)

Response:

We thank the reviewer for this insightful analysis of our genetic data and have changed the phrasing as suggested.

Referee #2:

Summary:

The new version of this manuscript has been much improved and I want to thank the authors for taking into account the points raised during the previous round of review. This paper is a very nice combination of genetic, cell biology and biochemistry, which will certainly make date in the field of plant cytokinesis, intracellular trafficking and phosphoinositides. The new live imaging data constitute a very nice addition to the story.
While I don't have any new experiments to suggest, I still would like the authors to be a bit more cautious with some of their statements/conclusions, mainly concerning the following two points:

Comment:

- Clathrin/CME defects v.s. TGN defects. As stated, the yeast pik1 mutant affects clathrin recruitment at the Golgi and hence induces many protein sorting phenotypes in this compartment. It is therefore likely that the Arabidopsis pi4kB1B2 double mutant has also defects in TGN trafficking (as also supported by Kang et al., 2011). Note that, like in yeast, these phenotypes may include defects in clathrin recruitment at the TGN. I think overall, it will be difficult to completely untangle whether the cytokinesis defects are caused by trafficking defects at the PM, at the TGN, at the cell plate or a combination thereof. This may be possible to address in the future, perhaps by using experiments allowing to specifically deplete PI4P at the TGN vs PM vs cell plate, or identifying PI4KB mutant versions that localize at the TGN but not the cell plate or vice versa. However, these kinds of experiments clearly extend beyond the scope of the manuscript. One may not exclude that

TGN defects alone could contribute to the observed cytokinesis defects. Rather than insisting on CME, I think the authors should discuss this possibility. It would also help, if the authors would analyze the relative quantity of PtdIns4P sensor and CLC2-GFP at the TGN, and not only at the cell plate and PM. This quantification may be obtained from the images already acquired to quantify the presence of these proteins at the cell plate and should therefore not require new experiments.

Response:

We thank the reviewer for this insightful assessment and we agree that a more cautious phrasing is required. In response to this comment, we have performed the requested analyses of PI4P sensor signal and CLC2-GFP at the TGN. The data indicate changes in PI4P sensor (decreased) and in CLC2-GFP signals (increased) at the TGN and we thank the reviewer for pointing out this important aspect. The additional data are included in the revision and we have altered our text to adopt more cautious phrasing. Specifically, the text has been altered to now include discussion of the notion that the cytokinetic defects observed might not strictly be a consequence of altered CME and/or PI4P at the cell plate but also of altered PI4P production at the TGN, as pointed out by the reviewer.

Comment:

- My second point is regarding the relationship between PI4KBs and MPK4. This part of the manuscript has been largely clarified from the previous version, nonetheless, the authors still conclude that "The experiments suggest MPK4 as an upstream regulator of PI4Kβ1 at the cell plate". I am not sure such statement is clearly proven and even useful. I would leave it at "functional interplay between MPK4, PI4KB1 and MAP65-3". I believe it is still unclear why MPK4 and PI4KB1 interacts. This could be sorted out by (for example) refining the molecular mechanisms of the MPK4/PI4KB1 interaction in order to obtain point mutants of MPK4 and PI4KB1 which specifically cannot interact which each other. Again, this goes beyond the scope of this manuscript, and I would therefore encourage the authors to be more careful and leave some space for future studies.

Response:

We thank the reviewer for pointing this out. The text has been rephrased accordingly. The change is in our best interest, as we have in fact observed effects possibly indicating a reciprocal control of MPK4 by PI4Kbs (please also see our response to the respective comment by reviewer 1). While we find this notion highly interesting, we do not cover this aspect in the revised version of our manuscript. Instead, we plan to pursue this interesting aspect as part of a future separate study, as already explained above.

Comment:

- This is a minor point but in order to analyze the presence of PtdIns4P at the cell plate, the authors used the FAPP1 PtdIns4P sensor, which is also known to bind to the ARF1 protein. The decreased labeling could therefore be due to limiting PtdIns4P and/or miss-localization of ARF1 (at TGN and/or cell plate). The authors could address this point by analyzing ARF1 localization in pi4kB1B2 double mutant (using their anti-ARF1 antibody) or additional PtdIns4P sensors, which localization are independent of ARF1. In any case, it could be helpful if this point could at least be discussed.

Response:

We agree with the reviewer that this information might be useful for the interpretation of the data. As we do not currently have an option to detect PI4P in the pi4kb1 pi4kb2 double mutant in vivo by reporters other than FAPP1, we performed the proposed immunofluorescence experiments. The immunofluorescence analysis of ARF1 distribution in wild type vs. the pi4kb1 pi4kb2 double mutant did not show any differences in the ARF1 distribution pattern. This control supports the notion that the FAPP1-PH sensor used to detect PI4P might in fact report the distribution of PI4P and not that of an undesired unspecific binding partner, with altered distribution in the pi4kb1 pi4kb2 double mutant. We have included this additional control in the supplementary data. We also included a brief discussion of the caveats of using the FAPP1-PH sensor, in particular about possible non-specific binding to ARF1, as requested.

Referee #3:

The manuscript of Lin et al. has been completely reformulated and changed in its logics. Additionally, substantial amount of key data for the previous conclusions was deleted and numerous

new data was added. For this reason, a complete re-evaluation as a new manuscript was needed.
The current manuscript, "A dual role for PI4Kβ in endocytosis and phragmoplast dynamics during plant somatic cytokinesis" describes mainly two roles of PI4Kβ during cytokinesis, namely clathrin-mediated endocytosis on cell plates and phragmoplast stability. The authors added beautiful live-imaging data and some quantification for imaging data to support their conclusions. However, the findings and major points of the manuscript are not of great conceptual novelty, particularly the PIP4P-dependent clathrin-mediated endocytosis, because the cytokinetic defect phenotype of the double pi4kβ1 pi4kβ2 mutant has been previously described (Kang et al. 2011) and PIP4P-induced clathrin-mediated endocytosis has been shown several times in other organisms, as the authors cited in the discussion: animal cells (Burke, Inglis et al., 2014, de Graaf, Zwart et al., 2004, Kapp-Barnea, Ninio-Many et al., 2006) and yeast (Audhya, Foti et al., 2000, Yamamoto, Wada et al., 2018).
Additionally, it is not mechanistically straightforward to link the vesicle-fusion defect phenotype in the cell plates with the described function of PI4K in endocytosis. Moreover, this CME aspect is also not clear mechanistically both in the interphase and the cytokinesis. Regarding the over-stabilized phragmoplast phenotype in the pi4kβ double mutant, I agree this is a new phenotype to understand plasticity and dynamics of the phragmoplast, but the link to NACK-PQR pathway that regulates MAP65-3 is not established well enough to be of interest to more general readers.
Overall, the authors tried to explain the cytokinetic defect phenotype, but many of the explanations are conjectures from the phenotype observed in interphase (for example Figure 5 e,g,i Supplemetnal Fig 6,7).
Therefore, I still think that the authors should rethink the interpretation and/or provide additional data to support their conclusions. A more detailed discussion follows below.

Comment:

1) The link between PtdIns(4)P production and phenotypes are not clear both in the interphase and the cytokinesis

The role claimed for the PI(4)P at the cell plate is (too) often assumed from data at the plasmamembrane of non-cytokinetic cells

CLC2 persistence at the fused cell plate (i.e. PM?) post-cytokinesis (Fig4 &S5)
CLC2 increased residency during interphase (root elongation zone S6)
Reduced KN endocytosis from the PM during late telophase (Fig5)
Reduced PIN2 endocytosis from basal PM post cytokinesis (Fig5)
Reduced FM4-64 internalization (Fig5) in root epidermal cells
All those experiment rather demonstrate a role for PI(4)P (even though not significantly reduced) in endocytosis at the PM rather than at the cell plate

Response:

We agree with the reviewer that several of our experiments do not indicate a role of PI4P or PI4Kbs at the cell plate. However, this point has been clearly stated in several places in the manuscript. Importantly, in response to previous reviewer comments on our original submission, we are no longer claiming that the function of PI4P or PI4Kbs is restricted to the cell plate or to cytokinesis, as some of our experiments clearly show roles outside of cytokinesis/in interphase cells. Obviously, all analyses performed on interphase cells, such as the study of CLC2-GFP or microtubule dynamics or the endocytosis tests using FM 4-64 or PIN2-GFP in root cells, will only indicate effects in interphase cells. We understand from the reviewer's comment that this point has not been sufficiently clear, so we have changed the manuscript text to better indicate what can and cannot be concluded from each experiment shown. Specifically, we are now individually indicating for each relevant experiment whether it has been performed with interphase cells or with cytokinetic cells. Also, the interpretation of the results now more clearly states if an experimental finding pertains to a role of PI4Kbs or PI4P in cytokinesic cells or in interphase cells, as was suggested by the editor.

Comment:

One of the main claims in the new manuscript is the dynamics of CLC2-GFP in the interphase or the cytokinesis as shown in Fig 4 (This figure is lacking proper captions, there are no a and c) and Supplemental Fig 6. The data is clear, persistent retaining of CLC2-GFP on the PM during the interphase and retarded CLC2-GFP accumulation on the cell plates. But I could not understand the logic of the statement at line 257-8 "In any case, the strong phenotype of the pi4kβ1 pi4kβ2 double

mutant indicates that even a minor contribution to cellular PtdIns(4)P production exerts a relevant effect." I am not convinced whether this phenotype is due to the amount of PtdIns(4)P because the amount was not changed in the pi4kβ double mutant shown in Supplemental Fig 4, as the authors mentioned. I am afraid that the authors may describe some secondary effects, not from the quantitative change of PtdIns(4)P, for example abnormal auxin distribution or different cellular growth rates.

Response:

We apologize for the missing captions; these might have been "lost" in pdf conversion, as our original Figure had these captions and we did not catch the omission in the merged pdf of our manuscript. We agree with the reviewer that the failure to biochemically detect changes in PI4P levels in the pi4kb1 pi4kb2 double mutant is puzzling. However, if i) a main function of these players is during cytokinesis, and ii) the overall contribution of PI4Kb isoforms to PI4P production may be small, then we might see a localized cytokinetic effect, even though we do not measure a detectable difference in PI4P when bulk-analyzing the entire root. While a localized role of PI4Kbs in cytokinetic cells is supported by the pi4kb1 pi4kb2 double mutant phenotype, which shows a strong cytokinetic defect, the reviewer is correct that other explanations are possible and that these possibilities must be discussed. At this point, we would like to limit the discussion to alternative effects that are based on data, such as the new information on changes in PI4P and CLC2-GFP at the TGN (see response to the comment by reviewer 2), rather than speculate on potential roles of PI4P in the control of auxin transport or plant growth that are currently unsupported by the abailable data. In response to this comment, we have therefore included additional discussion allowing a more inclusive interpretation of our observations, which now accomodates more alternative explanations of the effects.

Comment:

2) The quantification of intensity ratio between the plasma membrane and the cell plates is not convincing

The authors used the PI(4)P reporter mCherry-FAPP1 to quantify the relative abundance of PI(4)P between the PM and the CP during cytokinesis. This method is used to compare intensities ratio between PM and CP in WT versus the pi4kβ1 pi4kβ2 double mutant (fig4A) or mpk4 mutant (Fig10E) and convey a strong message for this story (there are differences in PI(4)P at the CP of the pi4kβ1 pi4kβ2 and mpk4)

However there are discrepancies that seem to invalidate the method:
While the reporter intensities might vary across samples, the ratios should remain fairly reproducible. The difference between genotypes is rather low (4A WT=1,1 pipkB1/2=0,9 / 10E WT=0,9 mpk4=1) yet there seems to be a high variability between experiments, as witnessed by the WT variation across experiment (values higher or lower than 1 suggesting opposite repartition of PI4P between PM and CP!)
Also, the opposite phenotype of the pi4kβ1 pi4kβ2 and mpk4 contrasts with the otherwise shared phenotypes. I am not so convinced whether this quantification method is able to measure genuine differences. As the authors showed in nice TEM data in Fig3, the phragmoplast is more dispersed in the pi4kβ double mutant, which should cause the biosensor signal to be diffused as well. Moreover, this marker appears to have high background as seen in the pictures. For these reasons, I do not think this ratio change is convincing enough to support their model.

Response:

We thank the reviewer for insightfully pointing out this experimental shortcoming. We agree with the reviewer that the interpretation of the quantifications is rather difficult. It is certainly correct that the intensities of the reporter - even though a published, genetically stable reporter line is used - is somewhat variable. In the absence of other means to detect and - possibly - quantify the intensities of the reporter at various subcellular compartments, we are but left to use the tools that are currently available. As these experiments have been performed based in response to reviewers' suggestions, we have included these data, well aware that the quantification of distribution patterns of lipid-binding-domain-based fluorescent reporters has its caveats. In response to this comment, we have

therefore now included a discussion of the situation described by the reviewer (that the "shape" of the cell plate might influence quantification in the mutant) and have included additional references where the caveats of lipid reporter use have been defined in more detail. With regard to the data shown, the analysis has been performed to the best of our abilities and appears technically sound. We respectfully disagree with the reviewer that the reporter data "convey a strong message for this story", as we quite carefully try to present all data with equal weight, not to introduce bias according to a desired outcome. Instead, the reporter data are but one more piece of information contributing to the overall assessment of the roles of PI4P and PI4Kbs in the Arabidopsis root. Based in the reviewer's comment, we have added a brief note of caution to the description of the reporter data, stating that the slightly more diffuse reporter fluorescence at the cell plate of the double mutant might interfere with the quantification (please also see our response to the respective comment by reviewer 1, referring to the biological significance of the observed differences). Despite of this caveat, we would nonetheless choose to retain the reporter quantification as part of our data set, so the readers get a complete picture of what was done.

Comment:

3) Does endocytosis defect exert the fusion defect in cell plates as shown in figure 3?
The authors claimed defect in endocytosis is one of the reasons to block normal cell plate formation. I agree the balance of endocytosis and exocytosis is absolutely important to form the cell plate but why would defects in endocytosis, which removes or trims excess material, block the fusion of vesicles in the double mutant as shown in figure3? This is not easy to understand.

Response:

We thank the reviewer for pointing out this difficile aspect of membrane trafficking. From our understanding, the cell plate expands based on the addition of material delivered by targeted secretion at the leading edges. This process requires both the steady delivery of vesicles to and the recycling of "empty" vesicles from the leading edges to enable continuous progression. In other words, the endocytotic aspect would firstly be required to maintain the continuous delivery of material to the leading edges of the cell plate, and only as a second function contribute to the "trimming" and removal of excess material from the cell plate. Therefore, we think that the endocytosis aspect might be essential for the continuous growth of the cell plate. We have added a brief description of this notion in the discussion to clarify this point.

Comment:

4) The stage description is not clear
In Figure 5 e, the authors presented PIN2-GFP localization on the CP in WT and the double mutant of pik4β. It is unclear to me how the authors distinguish the PM and newly developed PM from CP. Is there some definition?

Response:

In Fig. 5 e, we show that the intensity of the PIN2-GFP marker at the cell plate of wild type cells is weaker than the intensity measured at the cell plate of pi4kb1 pi4kb2 double mutant cells. In both cases, we are using the PIN2-GFP fluorescence at the apical plasma membrane as a reference and report the ratio of intensities at the cell plate vs. plasma membrane , as is indicated by the dashed lines in the respective image panels. The cells were imaged at equivalent time points of cytokinesis, based on the FM 4-64 stain showing that the cell plate has just touched the parental plasma membrane. This procedure for quantification has previously been used by other groups to obtain equivalent quantifications in the contexts of their respective studies. We apologize for not describing this well enough. In response to this comment, we have now included a better description of this and also of other equivalent experiments, so the experimental procedure is explained better in the revised manuscript. We are not certain to what aspect the reviewer is referring by "newly developed PM".

Comment:

5) Abnormal localization of MAP65-3
As beautifully shown in Figure 8, GFP-MAP65-3 is over-stabilized in the double mutant. But,

mechanistically, this phenomena is not satisfactorily explained, particularly the link from PI4Kβ. I guess the link between MPK4 and MAP65-3 is of high interest for readers, especially those who are working on plant cytokinesis. Furthermore, the behavior of PI4Kβ and MAP65-3 in the mpk4 mutant should be compared. I agree this is a novel finding, but I think the authors should clarify and provide additional data to establish the link for example using synchronized suspension cells like in Sasabe et al. (2006).

Response:

As suggested by the editor, we are responding only briefly to this comment. We agree with the reviewer(s) that the mechanistic links between PI4Kbs, MAP65-3 and MPK4 are not currently well established. However, we have performed a number of experiments on the behavior of PI4Kb1 in the mpk4 mutant background, including an analysis of the subcellular distribution of mCherry-PI4Kb1 and an analysis of the distribution of the PI4P reporter FAPP1-PH. These data, which are included in the manuscript, seem to indicate no major differences to the patterns in wild type controls. Therefore, to explain the rather dramatic consequences of the lesions in the pi4kb1/pi4kb2 or the mpk4 mutants, a much more thorough analysis will have to be performed. While we do plan to pursue the issue further in the future, we feel that it will be beyond the scope of this manuscript to address these points at the requested depth.

Minor points

Comment:

In the abstract, most of the mutant name are not in Italic. Please change them into Italic eg. mpk4-2, pi4kβ1,2.

Response:

Done.

Comment:
In the figure legends Fig 1(c) Data are mean {plus minus} SD. (bars?)

Response:

We are not certain as to what change the reviewer is suggesting by noting "(bars?)". So, in response to this comment we have changed the phrasing of the legend for Fig. 1 c to now read "Bars indicate means plus/minus SD.".

Comment:

For Figure 2, captions are lacking in the figure. The top panels on the left should be annotated as "a" and the right panel should include "c" somewhere in the figure.

Response:
We are not certain what the reviewer is referring to, as in our version of the merged pdf the panels in Fig. 2 are correctly labeled. The right panels are magnifications of the areas of interest indicated by boxes in the left panels. The boxes are labelled with Roman I and II in the left panels, and these labels are also present in the magnified panels at the right. As we find the labeling correct, we would like to retain the labels as they are.

Comment:

In figure 10B I am wondering if the molecular weight marker labeling is correct because the predicted molecular weight of MPK4-myc is much less. I agree some protein shows different molecular weight from expected size but the difference in the panel is quite big. Is it possible to check also by anti-MPK4 (like A6979 from sigma)?

Response:

We agree with the reviewer's observation that the apparent size of the MPK4-myc fusion is substantially larger than that of the MPK4 protein proper. This effect is due to the use of a tagged MPK4 fusion previously described in Berrini et al. (Plant Cell 2012), which includes 9x cMyc tags, a polyhistidine tag and additional linker sequences. These tags add more than 18 kDa to the 43 kDa

of MPK4, amounting to a total size of 61 kDa. As the polyhistidine tag might additionally retard electronophoretic mobility, the protein migrated just below the 70 kDa marker. Judgement of the size is furthermore complicatied by the somewhat smeared appearance of the bands detected in the co-immunoprecipitation from non-purified complex plant extracts containing numerous highly abundant proteins in this size range. Importantly, the anti-cMyc antibody only detected the signal in transgenic lines expressing the cMyc-tagged MPK4 fusions and not in material from wild type controls, so we are confident that the detected bands reflect the MPK4 fusion protein, as labeled, and at its expected size. We apologize if all this was not sufficiently described in the manuscript. We have now included a better description of the MPK4 fusion protein, so the apparent size should no longer irritate.

Comment:

In supplemental Fig9, corresponding CBB stained gel image is absolutely necessary as a protein loading control.

Response:

We agree with the reviewer that a loading control would be desirable. Unfortunately, the expression of the PI4Kb protein is quite inefficient in our hands, and purified recombinant protein was severely limiting. Instead of a CBB-stained gel, we have therefore used equal volumes of the homogeneous solution of the recombinant PI4Kb1 protein solution in the assays and opted to perform the necessary controls with GST, MPK4 and MKK6DE without another loading control. As these experiments are merely confirmatory of the data presented in a previous study by Latrasse et al. (Genome Biology 18: 131, 2017) , and the analysis is strictly qualitative, we are not providing the requested loading control. Furthermore, the very similar catalytic activities detected for the individual assays also suggest equal distribution of the recombinant PI4Kb1 protein. On a different note, the first author of this study has graduated and left our laboratory for China, and we currently have no means to perform the experiment. If this information is not sufficient, we can alternatively remove the data and just refer to the published data by Latrasse et al. (2017).

Comment:

Line 690 pi4kb1 pi4kb2 should be written "pi4kβ1 pi4kβ2."

Response:

Done.

Comment:

Most of the cases, the authors mentioned two authors when the authors have citations in the text. I do not think this style is common. Is it EMBO journal style?

Response:

We used a current style file and EndNote software to forma the literature list. We think, this point might be an issue to be addressed with the copy editors. Copy editors: Please advise whether or not the EndNote style is correct.

Comment:
Line 975 I assume the authors meant degree not temperature, C should be removed.

Response:
Done.

3rd Editorial Decision 23rd November 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by two of the original referees whose comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

REFEREE REPORTS

Referee #1:

This is the re-review of a manuscript I had reviewed previously. The authors have more than satisfactorily addressed all my suggestions and comments for experimental and textual revisions. I have no further comments except that this has become a very fine manuscript that will be of great interest to many plant scientists as well as some cell and developmental biologists working in other areas such as MAP kinase and phospholipid signalling during cytokinesis in other systems. I am looking forward to seeing this work in press.

Referee #2:

I am satisfied with this revised version of the manuscript.

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: Ingo Heilmann
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2018-100303

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 χ2 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?	Within the limitations of the available biological material, sample sizes were chosen large enough/as large as possible to detect the effects of interest in each experiment, as closely as possible following the suggestions by Krzywinski and Altmann (Nature Methods 10, 12, 2013).
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.	NA
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.	A PASW Statistics (SPASS) 18 was used to evaluate the homogeneity of variance by independent t-tests. If the data meet the normal distribution, then independent two-tailed t-tests or one way ANOVA was used. Otherwise, a nonparametric test- Mann-Whitney U-test was used.
Is there an estimate of variation within each group of data?	Yes

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

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).	Anti-ARF1 (AS08325); Anti-PI4Kβ1 (custom raised, Eurogentec, Liège, Belgium); rabbit anti-mCherry antibodies (ab167453, Abcam); Rat monoclonal anti-α tubulin (clone YOL 1/34, EMD Millipore or Abcam); rabbit polyclonal anti-KNOLLE (provided by Prof. Gerd Jürgens, Tübingen, Germany); rabbit polyclonal anti-GFP (A11122, Invitrogen); Alexa Fluor 555 Goat anti-rat IgG (H+L) (Abcam or Invitrogen); Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (Invitrogen); goat anti-rabbit monovalent F(ab) fragments (Jackson ImmunoResearch, Cat# 111-007-003); Alexa Fluor 568 donkey anti-goat IgG (H+L).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	No sequence data were generated in this study. The new (reraised) antibody is available upon request.
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
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA