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Annexin A2 is required for the early steps of cytokinesis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Nonia Pariente

1st Editorial Decision 13 March 2014

Thank you for your patience while your study has been under peer-review at EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, all referees highlight the interest of the findings and, although referee 3 is more negative about the overall advance, referees 1 and 2 are more supportive of the study. Nevertheless, all three raise serious concerns that question the conclusiveness and physiological relevance of your study and would need to be addressed.

Given that all three referees provide constructive suggestions on how to strengthen the study, I am happy to invite its revision. As the reports are below, I will not detail them here. However, it is clear that a more thorough quantification and statistical analyses (obtained from at least three independent experiments in every case) are required throughout the manuscript, as well as more details on how the data was analyzed and represented. Another serious concern is that of possible off-target effects of the siRNA used, and additional siRNAs would need to be used and rescue experiments performed (ideally with a stable rescue system). In addition, the physiological relevance of your results was questioned by two of the referees, given that Annexin A2 knockout mice are viable, and extending your study to additional cell lines, as well as further discussion of this issue, would be required.

All additional technical issues raised would need to be addressed, including the need for time-lapse analysis of actin dynamics and strengthening the chromosome segregation analyses.

I would encourage you to address at least one of the other two main point brought up by referee 3 (physical associations of Annexin A2 with cytokinetic machinery or use of Annexin A2 mutants to dissect which of its activities are required), which would clearly make for a more insightful, stronger study with more impact in the field, although this might not be required for publication.

Do not hesitate to get in touch with me if I can be of any help during the revision process. I look forward to receiving your revised study.

REFEREE REPORTS:

Referee #1:

Annexin A2 is a calcium-dependent anionic lipid binding protein that has been implicated in a series of cellular functions including endocytosis, exocytosis, actin regulation etc. The manuscript by Benaud et al. now describes the consequences of Annexin A2 depletion for cleavage furrow formation during cytokinesis. The authors observe strong defects in cytokinesis following transfection of an Annexin A2 siRNA into HeLa cells. Furthermore, the authors provide good evidence that the furrow defect is caused by the failure to correctly assemble the contractile ring and to concentrate RhoA at the cell equator during anaphase.

The paper is clearly written and the experiments/analyses generally of high quality. Despite the pleiotropic nature of Annexin A2, demonstrating a strong requirement for cytokinesis in human cells would be an important and very interesting contribution to the field, even if the exact molecular role of the protein in the process remains elusive in this manuscript. In my opinion, the manuscript could be appropriate for publication in EMBO Reports. There are two important concerns regarding the observed phenotypes of Annexin A2 siRNAs and the proposed important role of the protein in cytokinesis (see below). These concerns can be addressed by the authors.

(1) First, the observed cytokinesis phenotype is very dramatic and novel for Annexin A2. Despite dozens of papers published on the protein, none has reported a cytokinesis defect upon interference with Annexin A2 function. Therefore, the authors should make sure that the reported defect is indeed caused by loss of Annexin A2 and no other protein. To address this, additional data are required. The authors could use additional siRNAs targeting Annexin A2 and provide the % of bi or multi nucleation for each together with protein depletion data. The rescue experiment in Fig1 is not clear at all to me. In the micrograph many HA negative cells are mono-nucleate but in the graph HA neg cells are shown as 100% multinucleate. This discrepancy is of concern. Furthermore, rescue experiments using transient expression are inherently unreliable and sensitive to effects of transgene expression on proliferation (if terminal phenotypes are assessed). Generating a stable (or inducible) RNAi resistant cell line model for Annexin A2 in combination with terminal phenotype analysis and live cell imaging would be far superior.

(2) Second, Annexin A2 knockout mouse is viable and fertile (e.g.: 14702107) somewhat questioning the relevance of the findings presented here. How do the authors reconcile this fact with their work. Is redundancy within the Annexin family in mouse really sufficient to explain this discrepancy? The authors should test the effect of Annexin A2 depletion on cytokinesis/multinucleation in other human cell backgrounds with multiple siRNAs.

Lastly, the authors' interpretation of the function of Annexin A2 in linking the central spindle to the contractile ring is possible and worth discussing. However, a possibly simpler explanation for the observed phenotypes would be that Annexin A2 is required for Ect2 and RhoA recruitment to the cell periphery. The resulting ingression defect could also generate a situation where the central spindle appears dislodged from the cell envelope.

Referee #2:

Overall it is n interesting and well-written manuscript that demonstrate a putative involvement of

annexin A2 in regulating early stages of cytokinesis. Furthermore, that manuscript demonstrates that annexin A2 may actually work by regulating the connection between midzone Ect2 and furrow plasma membrane, thus regulating RhoA activation and actin dynamics during cytokinesis. These findings are novel and would be interesting to wide scientific audience. However, manuscript has multiple issues (listed below) that needs to be resolved before manuscript can be published. Especially, much of presented data are not statistically analyzed. The off-target effects of Annexin A2 siRNA are still a concern. While authors did do rescue analysis of overall cytokinesis phenotype, they did not do any rescues in their subsequent data. I do realize that to do rescues in all experiments would be too much. However, the key findings, such as RhoA localization and Ect2 localization should have a rescue experiments.

Figure 1. Authors state in the text that Anx2-1 leads to 64% binucleation. Yet, in their rescue experiments (panel D), the Anx2-HA negative cells appear to have 100% binucleation. The discrepancy needs to be explained. Statistical analysis needs to be done on data regarding binucleation induced by Anx2-1 and Anx2-8 (as
compared to control). Was that experiment repeated more then once?

Figure 2. Time-lapse images of cells with asymmetric furrow formation and no furrow formation needs to be shown.

Figure 2. It would be good to see the localization of endogenous annexin A2.

Figure 3. Quantitation of action and myosin II localization needs to be performed. Statistical analysis (from at least three independent experiments) needs to be performed on anillin localization. Finally, data really needs to be supported by time-lapse analysis of actin dynamics, since that is the major conclusion of the manuscript. With various filamentous actin biosensors (such as LifeAct) widely available, that should not be a difficult experiment to do.

Figure 5. Quantification and statistical analysis of Ect2 localization phenotype need to be performed. Since this is the most interesting and novel finding, the rescues with HA-tagged Anx2 needs to be done and quantified.

Referee #3:

Benaud et al submit a manuscript that proposes the role of Annexin A2 in cytokinesis. In particular, the authors argue that Annexin A2 is directly involved in the assembly of the cytokinetic furrow. These are potentially interesting observations but the manuscript is mostly descriptive and there is a very limited insight into the potential role of Annexin A2 in cytokinesis. For example, there are no physical associations between Annexin A2 and the cytokinetic machinery to rationalise the phenotypes observed. Moreover, the manuscript would be far more informative if the contribution of known activities in Annexin A2 (actin binding, phospholipid binding) were determined for cytokinesis. Lastly the literature on the knockout mice for Annexin A2 shows that these animals are completely viable in the absence of this gene, suggesting that the cytokinetic phenotypes described by the authors might be cell type-specific.

Experimental issues:

There is a worrying inconsistency in the depletion of Annexin A2 throughout the manuscript, as reflected in the complete depletion of Anx2 by the siRNA in figure 1A while figures 1C and 4B show a much more modest depletion of the endogenous protein.

The quantification of the rescue in figure 1D is confusing, the graph in this panel should show the quantification of binucleated cells for the whole set of controls included in panel 1C.

The authors claim that "Cells depleted for annexin A2 displayed the same dynamic of chromosome segregation as the control cells". However, the phase-contrast experiments do not allow a clear analysis of chromosome segregation. Other strategies should be used to draw this kind of conclusion, such as using fluorescent H2B labeling of chromosomes.

Microscopy data in figures 3A and 5 requires some kind of quantification.

1st Revision - authors' response 26 June 2014

Point by point response to the referee:

Referee#1:

(1) The authors should make sure that the reported defect is indeed caused by loss of Annexin A2 and no other protein. To address this, additional data are required.

We have now provided additional data to address this concern:
1- Data and quantification with two independent siRNA targeting strictly different regions of AnxA2 RNA are provided. In addition, the siRNA data is now coupled to rescue experiments for all the major phenotypes described.
2- In addition, we have generated a new annexin A2 mutant that both validate the specificity of the rescue and provides interesting functional information. We have generated a two single point mutant I7L8/EE previously published that we have validated in fig E2A. This mutation on the S100A10 binding site of annexin A2 abrogates the formation of the heterodimer $AnxA2_2S100A10_2$. Our results indicate that whereas WT annexin A2 rescues the Anx2 siRNA phenotype, the mutant version is unable to perform the same rescue. Our results therefore suggest that the Anx2S100A10 dimer and not the annexin A2 monomer is involved in cytokinesis progression (addressed in the discussion)
3- Rescue experiments with both WT si resistant AnxA2HA and I7L8/EE si resistant AnxA2HA are now provided for the binucleation (Fig1C), Rho A localization phenotype (Fig.3E) and Ect 2 localization phenotype (Fig 4C). Statistical analysis for each set of experiments is now provided.
The rescue experiment in Fig1 is not clear at all to me. In the micrograph many HA negative cells are mono-nucleate but in the graph HA neg cells are shown as 100% multinucleate. This discrepancy is of concern.
The presentation of the graph for the rescue experiment in figure 1 was confusing since it was relative numbers and not actual 100% (previously fig1D). In order to clarify the data we have made a new graph with a new presentation and rewrote the explanation in the figure legend (now figure 1C). Statistical analysis for Anx2 siRNA in figure 1E has been calculated and indicated in the figure legend. We have also indicated that the result corresponds to three independent experiments.

Generating a stable (or inducible) RNAiresistant cell line model for Annexin A2 in combination with terminal phenotype analysis and live cell imaging would be far superior.
We have tried, but failed to generate a stable cell line expressing si RNA resistant Anx2. This is most likely due to the pleiotropic function of annexin A2 (included in interphase), which makes over expression of Annexin A2 not viable to the cells. We thus couldn't establish a stable rescue system. All the rescue experiments were therefore performed as co-transfection.

(2) Second, Annexin A2 knockout mouse is viable and fertile (e.g.: 14702107) somewhat questioning the relevance of the findings presented here. How do the authors reconcile this fact with their work. Is redundancy within the Annexin family in mouse really sufficient to explain this discrepancy?
We have now discussed the viability of the knockout mice in the first paragraph of the discussion and proposed another member of the annexin family that could compensate the loss of annexin A2. In addition, in order to show that the phenotype described here is not cell type specific, we have performed our study in an additional cell line, the U2OS cells, where we obtained results similar to those observed in Hela cells.

Lastly, the authors' interpretation of the function of Annexin A2 in linking the central spindle to the contractile ring is possible and worth discussing. However, a possibly simpler explanation for the observed phenotypes would be that Annexin A2 is required for Ect2 and RhoA recruitment to the cell periphery. The resulting ingression defect could also generate a situation where the central spindle appears dislodged from the cell envelope.

We agree with the reviewer that one possible explanation for the observed phenotype is that annexin A2 is directly required for RhoA and Ect 2 recruitment. I had discussed this issue in the third paragraph of the discussion. I made some changes to clarify this.

Refeere#2:

Much of presented data are not statistically analyzed:
We have now provided statistical analysis for Figure 1C, 1E, 2B, 2D, 3A, 3E, 4B, 4C.

The key findings, such as RhoA localization and Ect2 localization should have a rescue experiments.
Rescue experiments with both WT si resistant AnxA2HA and I7L8/EE si resistant AnxA2HA are now provided for the binucleation (Fig1C), Rho A localization phenotype (Fig.3E) and Ect 2 localization phenotype (Fig 4C). Statistical analysis for each set of experiments is now provided.

Figure 1. Authors state in the text that Anx2-1 leads to 64% binucleation. Yet, in their rescue experiments (panel D), the Anx2-HA negative cells appear to have 100% binucleation. The discrepancy needs to be explained. Statistical analysis needs to be done on data regarding binucleation induced by Anx2-1 and Anx2-8 (as compared to control). Was that experiment repeated more then once?
The presentation of the graph for the rescue experiment in figure 1 was confusing since it was relative numbers and not actual 100% (previously fig1D). In order to clarify the data we have made a new graph with a new presentation and rewrote the explanation in the figure legend (now figure 1C). Statistical analysis for Anx2 siRNA in figure 1E has been calculated and indicated in the figure legend. We have also indicated that the result corresponds to three independent experiments.

Figure 2. Time-lapse images of cells with asymmetric furrow formation and no furrow formation needs to be shown.
Time lapse illustrating the four cytokinetic phenotypes quantified in figure 1E are now added as supplementary movies E1, E2, E3, E4.

Figure 2. It would be good to see the localization of endogenous annexin A2.
Endogenous localization of Annexin A2 in mitotic cells has been challenging. The commercial antibody that we are using works only under MetOH cell fixation (we have tested TCA and PFA with cytoskeleton stabilization buffer), which is not optimal for membrane and cortical associated proteins. Note RhoA staining at the equatorial cortex can only been observed in condition of TCA fixation and live imaging. Our various experiment show high variability, and we do not believe they are representative of the true endogenous localization in live cells. We have, therefore, decided to present only the live exogenous annexin A2 localization.

Figure 3. Quantitation of action and myosin II localization needs to be performed. Statistical analysis (from at least three independent experiments) needs to be performed on anillin localization. Finally, data really needs to be supported by time-lapse analysis of actin dynamics, since that is the major conclusion of the manuscript. With various filamentous actin biosensors (such as LifeAct) widely available, that should not be a difficult experiment to do.
Quantification and statistical analysis for actin and anillin localization has now been performed and presented on fig 2B and 2D respectively. We now provide the time-lapse analysis of filamentous actin with the LifeAct biosensor figure 2A and supplemental movie E6 (control siRNA) and S7 (ANX2 siRNA).

Figure 5. Quantification and statistical analysis of Ect2 localization phenotype need to be performed. Since this is the most interesting and novel finding, the rescues with HA-tagged Anx2 needs to be done and quantified.
Quantification and statistical analysis for Ect2 localization phenotype is now provided in figure 4-B. The rescue of the Ect2 phenotype has been performed and quantified in Figure 4C.

Referee #3:

The manuscript would be far more informative if the contribution of known activities in Annexin A2 (actin binding, phospholipid binding) were determined for cytokinesis.
In order to try to better understand the contribution of annexin A2, we have generated several annexin mutants. We have first generated a two single point mutant I7L8/EE previously published that we have validated in fig E2A. This mutation on the S100A10 binding site of annexin A2 abrogates the formation of the heterodimer $AnxA2_2S100A10_2$. Our results indicate that whereas WT annexin A2 rescues the Anx2 siRNA phenotype, the mutant version is unable to perform the same rescue. (Results presented thru out the manuscript). Our results therefore suggest that the Anx2S100A10 dimer and not the annexin A2 monomer is involved in cytokinesis progression (addressed in the discussion)
We have also generated the annexin mutant with defective calcium binding site that does not bind phospholipids. This mutant is commonly referred as a dominant negative in various publications. However, Hela cells expressing this mutant in transient transfection did not seem to proceed through cytokinesis under our experimental conditions, again illustrating the various function of Annexin A2 in interphase cells and possibly in earlier phases of mitosis. Supporting this theory, we have also noticed that a too severe down regulation of annexin A2 with our siRNA had the same consequence.

Lastly the literature on the knockout mice for Annexin A2 shows that these animals are completely viable in the absence of this gene, suggesting that the cytokinetic phenotypes described by the authors might be cell type-specific.
1- We have now performed our study in an additional cell line, the U2OS cells, where we obtained results similar to those observed in Hela cells (fig E1). Downregulation of annexin A2 (fig E1A) induces the formation of binucleated cells (fig E 1B and 1C) and reduces the recruitment of RhoA at the constriction ring (fig E1D). Clearly our results are not cell type specific.
2- We have now discussed the viability of the knockout mice in the first paragraph of the discussion and proposed another member of the annexin family that could compensate the loss of annexin A2.

There is a worrying inconsistency in the depletion of Annexin A2 throughout the manuscript, as reflected in the complete depletion of Anx2 by the siRNA in figure 1A while figures 1C and 4B show a much more modest depletion of the endogenous protein
The discrepancies in the level of Annexin A2 down regulation shown thru out the manuscript comes from the variation in experimental procedure used in the three figure. In figure 1A we are 48hrs post siRNA transfection whereas in figure 3B we are 36hrs post transfection. For the rescue experiment (now Fig E2B) we are in condition of co-transfection and have used a lower concentration of siRNA. Theses differences are now clearly explained in the material and method section “transfection”.

The quantification of the rescue in figure 1D is confusing, the graph in this panel should show the quantification of binucleated cells for the whole set of controls included in panel 1C.
The presentation of the graph for the rescue experiment in figure 1 was confusing since it was relative numbers and not actual 100% (previously fig1D). In order to clarify the data we have made a new graph with a new presentation and rewrote the explanation in the figure legend (now figure 1C). Statistical analysis for Anx2 siRNA in figure 1E has been calculated and indicated in the figure legend. We have also indicated that the result corresponds to three independent experiments.

The authors claim that "Cells depleted for annexin A2 displayed the same dynamic of chromosome segregation as the control cells". However, the phase-contrast experiments do not allow a clear analysis of chromosome segregation. Other strategies should be used to draw this kind of conclusion, such as using fluorescent H2B labeling of chromosomes.
In order to better illustrate the dynamic of chromosome segregation we have repeated the phase contrast experiments while staining the DNA with Hoechst. The data is presented in movies E1, E2, E3, E4.

Microscopy data in figures 3A and 5 requires some kind of quantification.
Quantification is now provided for former figure 3A and 5 now respectively figure 2 B and 4B.

2nd Editorial Decision 18 July 2014

Thank you for the submission of your revised study to EMBO reports, and for your patience during its peer-review. We have now received reports from the three initial referees and, I am sorry to say that although referee 2 is now supportive of publication, both referees 1 and 3 are not satisfied with the revision and have crucial concerns regarding the conclusiveness of the study.

Given these crucial issues and the fact that EMBO reports only allows one round of major revision, we have decided to reject your study at this stage. In view of the potential interest of the findings, however, we would be open to considering the resubmission of a considerably strengthened manuscript, that addresses the remaining concerns in full. This would include the generation and analysis of stable rescue lines, and achieving consistent and significant levels of Annexin A2 depletion throughout the study, both of which will be instrumental in demonstrating a causal link between protein loss and phenotype.

Please note that if you are interested in this option, resubmissions are treated as new submissions rather than revisions and are editorially assessed afresh, especially with respect to novelty at the time of resubmission. If no novelty concerns arise, we would aim to engage referees 1 and 3 in its assessment, although this would depend on their availability.

I am sorry to disappoint you this time, and hope that the referee comments are helpful in your continued work in this area.

REFEREE REPORTS:

Referee #1:

The authors have included a number of additional experiments and dataset to address the reviewers' concerns, which has improved the work.

While the phenotypic analysis is well executed, the provided rescue data are not decisive enough to demonstrate that loss of AnxA2 is causing the observed cytokinesis defect. The transient rescue experiment and its unorthodox analysis provided in Figure 1C is prone to the impact of proliferation and other effects (negative selection of toxic proteins). E.g. if expression of wild-type but not mutant AnxA2 reduces cell proliferation, rescue data similar to the ones shown in the figure may be obtained and mistaken for positive rescue. Therefore, a stable or inducible siRNA rescue system would be critical as it would allow solid terminal and dynamic time-lapse data to be recorded. The authors state in their rebuttal that generating cells stably expressing siRNA resistant AnxA2 was not possible. Yet, in Figure 1F of their manuscript, the authors show localization of AnxA2-GFP in stably expressing cells. I am confused.

This is an interesting manuscript that should be published in EMBO Reports. However, it seems critical to this reviewer to firmly establish a link between the cytokinetic phenotype observed and the loss of AnxA2.

The causal relationship between protein loss and phenotype is especially important here since mouse experiments suggest that deletion of the gene may not be lethal. As discussed by that authors, this may of course be due to functional redundancy with other Anx proteins. However, other explanations such as off target effects remain a possibility in the absence of solid rescue data.

Referee #2:

Authors have addressed all my concerns. Thus, I find this manuscript now suitable for publication in EMBOR.

Referee #3:

The authors submit a revised version of their manuscript that addresses some of the questions raised before. However, there are still some key issues that need to be resolved.

The quantification of the cytokinetic phenotypes in figure 1C is still confusing. The authors should stick to the percentage of binucleate cells over the total number of cells, as they do in figure E1C. A more serious issue with this rescue experiment is the fact that depletion of endogenous Anx2 is very modest, thus precluding meaningful conclusions. This problem is highlighted by the western blot in figure E2 B, which shows that cells transfected with wt ANX2 and siRNA against ANX2 still express a considerable amount of the endogenous Anx2, whereas depletion of endogenous Anx2 in cells expressing the I7/L8EE mutant seems more efficient. In this context, the rescue activity by these constructs seems to correlate with the efficiency of depletion of the endogenous protein.

Related to the issue above, the authors argue in their response that the inconsistency of the Anx2 depletion through the paper is due to experimental differences in terms of transfection timing and dose of RNAi. Although this explanation helps to understand the issue, it still leaves the manuscript with worrying depletion inconsistencies.

Resubmission - authors' response 17 December 2014

We believe that in this new version of the manuscript we have strengthened the data we present and we have now addressed the last concerns of the reviewers.

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Please find the original referees' comments here and the authors' responses attached at the end:

Referee #1:

The authors have included a number of additional experiments and dataset to address the reviewers' concerns, which has improved the work.

While the phenotypic analysis is well executed, the provided rescue data are not decisive enough to demonstrate that loss of AnxA2 is causing the observed cytokinesis defect. The transient rescue experiment and its unorthodox analysis provided in Figure 1C is prone to the impact of proliferation and other effects (negative selection of toxic proteins). E.g. if expression of wild-type but not mutant AnxA2 reduces cell proliferation, rescue data similar to the ones shown in the figure may be obtained and mistaken for positive rescue. Therefore, a stable or inducible siRNA rescue system would be critical as it would allow solid terminal and dynamic time-lapse data to be recorded. The authors state in their rebuttal that generating cells stably expressing siRNA resistant AnxA2 was not possible. Yet, in Figure 1F of their manuscript, the authors show localization of AnxA2-GFP in stably expressing cells. I am confused.

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Referee #2:

Authors have addressed all my concerns. Thus, I find this manuscript now suitable for publication in EMBOR.

Referee #3:

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Authors' response:

Referee1:

Referee 1 was not convinced by the transient rescue experiment and its analysis presented in figure 1C and requested that the rescue experiment be performed with a stable cell line to acquire final or time-lapse data.
We have now removed figure 1C and performed an all-new set of experiments illustrated in figure 1E and figure E2A. We have generated Hela cells stably expressing si resistant GFP tagged annexin A2 to perform the rescue. The cytokinetic defect in parental and annexin A2 GFP Hela cells following Anx2 siRNA was analyzed by live cell imaging. The percentage of bi-nucleated cells and the statistical analysis of the rescue are now presented in Figure 1.

As we are explaining in the material and method section, no stable clones expressing the I7/L8EE annexin A2 mutant at a level equivalent to the endogenous annexin were obtained. This most likely reflects a partial dominant negative effect of the mutant. Under those conditions it can be argued that the lack of rescue of the mutant is a consequence of the low level of expression. Therefore, for the rescue with mutant annexinA2, transient transfection type of experiments, achieving level of expression similar to the endogenous protein is more appropriate. Since the use of mutant forms of annexin A2 was a previous request of referee #3 and since in the previous revised version the phenotypic analysis and its rescue was satisfactory to the referees, we have decided to keep the transient rescue with the HA versions of annexin A2 (figure 3and 4) as a complement of the stable rescue presented in figure 1.

Referee2:

Referee 2 stated that we had addressed all its concerns in the previous revision of the manuscript and that the manuscript was suitable for publication.

Referee3:

Point1: Referre3 found the quantification of figure 1C confusing and requested quantification as percentage of binucleated cells.
We have now removed figure 1C and performed an all-new set of experiments illustrated in figure 1E and figure E2A. We have generated Hela cells stably expressing si resistant GFP tagged annexin A2 to perform the rescue. The cytokinetic defect in dividing parental and annexin A2 GFP Hela cells following Anx2 siRNA was analyzed by live cell imaging. The percentage of bi-nucleated cells and the statistical analysis of the rescue are now presented in Figure 1.
Referee 3 found the depletion of annexin A2 too modest in the previous rescue experiment.
Figure E2A present the western blot illustrating the efficient depletion of the endogenous protein achieved in the new stable rescue experiment.

As we are explaining in the material and method section, no stable clones expressing the I7/L8EE annexin A2 mutant at a level equivalent to the endogenous annexin were obtained. This most likely reflects a partial dominant negative effect of the mutant. Under those conditions it can be argued that the lack of rescue of the mutant is a consequence of the low level of expression. Therefore, for the rescue with mutant annexinA2, transient transfection type of experiments, achieving level of expression similar to the endogenous protein is more appropriate. Since the use of mutant forms of annexin A2 was a previous request of referee #3 and since in the previous revised version the phenotypic analysis and its rescue was satisfactory to the referees, we have decided to keep the transient rescue with the HA versions of annexin A2 (figure 3and 4) as a complement of the stable rescue presented in figure 1.

Point2: referee 3 found the depletion of annexin A2 inconsistent thru out the manuscript.
We are now presenting consistent and significant level of annexin A2 depletion through out the manuscript, which is representative of the depletion achieved for the phenotypic analysis. Western blots of figure 3B, E1A and E2A have been replaced.

3rd Editorial Decision 13 January 2015

Thank you for your patience while we have reviewed your resubmitted study. It was assessed by referees 1 and 3 of your related submission, who are now referees 2 and 1, respectively. As you will see in their reports below, both now support the publication of your study in EMBO reports, although referee 2 (ex-1) raises some minor issues that should be taken care of. Regarding his/her last point, the use of the word "suggest" is already sufficiently cautious, but it would make sense to include in the text the alternative scenario brought up in this report.

Given the overall support, I am writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once the minor issues raised by referee 2 have been addressed, in addition to a few others, as follows.

- the Materials and Methods section in the main text is excesively auccinct. As you manuscript is overall not excessively long, and the expanded material section is in any case rather short, please include all Materials&Methods in the main text.

- Please indicate the statistical test applied to the data in the legend to all relevant figures, and the type of error bars used in figure 3D.

After all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports!

REFEREE REPORTS:

Referee #1:

the authors have addressed my previous concerns

Referee #2:

The new version of the MS by Benaud has suitably addressed my remaining concern regarding the rescue experiment following Anx2 siRNA depletion. The work describes a new and early cytokinetic role of Anx2 related to RhoA action and is generally of high quality. I recommend publication of the work in EMBO Reports. Prior to publication this reviewer recommends that the following minor points and corrections should be implemented by the authors.

Minor points:
- Introduction: "... enriched in phosphatidyl inositols (PIPs)" should be "phosphatidylinositols"
- New rescue experiment in Figure 1E: the data shown in the Figure panel illustrates fixed cells. The legend mentions live cell imaging. It would be useful to clarify this and clearly state which method of analysis was used for deriving the data shown in the graph in Figure 1E.
- In addition, it would be helpful for being able to better interpret the western blot in Fig E2A to state how many cells in the transgenic Anx2-GFP cell line population express the transgene.
- Figure 2A: In contrast to Fig2B phalloidin staining, the difference in equatorial LifeAct-mCherry accumulation between Ctl and Anx2 siRNA is not obvious. The authors shown therefore either quantify the signal or down tune the statement related to 2A in the main text.
- Results: " ... nor the interaction between Ect 2 and MgcRacGAP ..." should be "Ect2"
- "These results suggest that annexin A2 is necessary for the localization of Ect2 at the equatorial plasma membrane and to maintain a connection between the central spindle and the cortex." This statement is going beyond the data shown in the MS. The furrow ingression defect of Anx2 depleted cells may account to a large extent for the failure of the midzone markers to appear close to the equatorial cortex. Thus, the role of Anx2 could be only to mediate RhoA concentration/action whereas the effect on spindle-cortex coupling may be indirect. Thus, this reviewer recommends rephrasing of this sentence and the sections in the discussion related to it. Without further biochemical evidence of Anx2 acting as a linker between midzone and equatorial membrane/cortex elements, these statements remain very speculative.

3rd Revision - authors' response 19 January 2015

As you requested I've performed the following minor revision to the manuscript:

1- Regarding the reviewer last point, the sentence "annexin A2 is necessary ..... to maintain the connection between the central spindle and the cortex" was replaced by "These results suggest that annexin A2 is necessary for the localization of Ect2 at the equatorial plasma membrane and for the maintenance of a connection between the central spindle and the cortex." Since I am not stating that it is direct effect of annexin A2 down regulation, I thus believe I can keep this statement in the text. However I agree that many scenarios can explain this observation and I am now clearly mentioning the one suggested by the reviewer. (See below point 5 of the reviewer)

2- The Materials and Method section is now entirely included in the main text.

3- The statistical test used is now specified in all the relevant figures legends. In figure 3D, there was an error in the plotting of the error bars, which are classic ±SD. I have replaced the graph in the figure with a new one displaying the correct error bars. The type of error bar is now specified in the legend.

In addition, the following changes in response to referee#2 minor points were made:

1- - *Introduction: "... enriched in phosphatidyl inositols (PIPs)" should be "phosphatidylinositols" and Results: " ... nor the interaction between Ect 2 and MgcRacGAP ..." should be "Ect2"*

These two typos in the text have been corrected

2- - *New rescue experiment in Figure 1E: the data shown in the Figure panel illustrates fixed cells. The legend mentions live cell imaging. It would be useful to clarify this and clearly state which method of analysis was used for deriving the data shown in the graph in Figure 1E.*
To clarify the experiment performed and illustrated in figure 1E we have replaced the figure legend by the following: " E- Dividing Hela and si resistant annexinA2-GFP Hela cells were monitored by videomicroscopy 36hrs post transfection with control or Anx2-1 si RNA. Quantification of binucleation is presented in percentage of number of cells undergoing mitosis during the time lapse (graph). Error bars, s.d. of three experiments (n≥100). *p= 0.005. Cells were fixed and labeled for tubulin, DNA and GFP (right panel). Immunoblot characterizing the cells is shown in Fig E2".

3- - *In addition, it would be helpful for being able to better interpret the western blot in Fig E2A to state how many cells in the transgenic Anx2-GFP cell line population express the transgene.*

The sentence " About 90% percent of the stable Anx2-GFP stable cell line population expressed the transgene." was added to the expression constructs and stable cell lines section of the Materials and Methods.

4-- *Figure 2A: In contrast to Fig2B phalloidin staining, the difference in equatorial LifeActmCherry accumulation between Ctl and Anx2 siRNA is not obvious. The authors shown therefore either quantify the signal or down tune the statement related to 2A in the main text.*

I am now more specific in the description and down tuned the statement related to 2A in the main text. The text "In cells lacking annexin A2, actin poorly accumulated at the presumptive furrow, or accumulated on a single side of the equatorial cortex." was changed for" In cells lacking annexin A2, actin poorly accumulated at the presumptive furrow or accumulated on a single side of the equatorial cortex (Fig.2B). Furthermore, in asymmetrically contracting cells, actin also distributed to the poles of the cell (Fig. 2A and 2B)."

5-- *"These results suggest that annexin A2 is necessary for the localization of Ect2 at the equatorial plasma membrane and to maintain a connection between the central spindle and the cortex." This statement is going beyond the data shown in the MS. The furrow ingression defect of Anx2 depleted cells may account to a large extent for the failure of the midzone markers to appear close to the equatorial cortex. Thus, the role of Anx2 could be only to mediate RhoA concentration/action whereas the effect on spindle-cortex coupling may be indirect. Thus, this reviewer recommends rephrasing of this sentence and the sections in the discussion related to it. Without further biochemical evidence of Anx2 acting as a linker between midzone and equatorial membrane/cortex elements, these statements remain very speculative.*

In the discussion related to the spindle-equatorial cortex coupling, the sentence "The uncoupling of the central furrow and the equatorial cortex constriction observed in absence of annexin A2 points out annexin A2 as a new molecular link between the central spindle and the contractile ring" was replaced by "Although we cannot exclude that the uncoupling of the

central furrow and the equatorial cortex constriction observed in absence of annexin A2 is only a downstream consequence of the alteration of RhoA function, a role for annexin A2 as a new molecular link between the central spindle and the contractile ring needs to be now investigated"

I hope I have addressed all the points.

4th Editorial Decision 20 January 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

