THE EMBO JOURNAL

Manuscript EMBO-2016-94462

# Quantitative analysis of human centrosome architecture by targeted proteomics and fluorescence imaging

Manuel Bauer, Fabien Cubizolles, Alexander Schmidt and Erich A. Nigg

*Corresponding author: Erich A. Nigg, University of Basel*

**Review timeline:**

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|---|---|
| Submission date: | 01 April 2016 |
| Editorial Decision: | 09 May 2016 |
| Revision received: | 08 July 2016 |
| Accepted: | 25 July 2016 |

*Editor: Hartmut Vodermaier*

## Transaction Report:

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

1st Editorial Decision 09 May 2016

Thank you for submitting your manuscript on quantitative analyses of human centrosome to The EMBO Journal. We have now received comments from three expert referees, in light of which we would be happy to consider a revised manuscript further for publication as a Resource Article in our pages. However, it will be essential to satisfactorily address a number of important issues raised by the reviewers before acceptance would be warranted. In particular, a key concern shared by all three referees concerns the potential confounding effects of cell type and cell cycle stage, and this would need to be addressed with decisive additional data. The second major issue is more presentational in nature, and relates to a clearer discussion of the usefulness and the biological insights drawn from the presented data. In addition, there are a number of well-taken requests for specific control experiments, which should also be taken into account.

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

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REFEREE COMMENTS

Referee #1:

I have now read the manuscript "Quantitative analysis of human centrosome architecture by targeted proteomics and fluorescent tagging of endogenous proteins" by Erich Nigg and colleagues. In this manuscript, the authors used two complementary approaches, targeted proteomics and EGFP-tagging of centrosomal proteins at endogenous loci, to measure protein abundance in cultured human cells and purified centrosomes.

 

The data provided in the manuscript is important for use by many other scientists when studying centrosomes. I have however several concerns that should be addressed prior publication:
a) There should be an initial figure summarizing all the experiments performed in the manuscript and how they relate to each other (cell type, cell cycle stage, type of data). It should be clear which data is cell cycle relevant, and what data can be compared with each other. For which proteins can the ratio of protein at cytoplasm vs centrosome be calculated and in which cycle stage?
b) The authors should show growth curves for cell lines expressing tagged-centrosome proteins to demonstrate this tagging did not affect protein function and therefore their viability. Is the centrosome number in these cells normal? Given the cell lines only have one allele tagged, how are the authors sure that both WT and tagged allele localize equally well at the centrosome?
c) The authors show enrichment of the endogenously-tagged proteins at the centrosome; their last point is mitotic- what do they mean as mitosis? Perhaps early and late mitosis should be separated as they are biochemically very distinct.
d) The authors should explain in all figure legends what cells and protocols they are using.

Referee #2:

This manuscript by Bauer et al. offers an interesting quantitative insight into the general stoichiometry of components that make up human centrosomes. Here, the authors combine SRM mediated targeted proteomics of total extract and centrosome preparations with endogenous EGFP tagging method to measure the absolute and relative abundance of key centrosome proteins, and use this information to make predictions/modeling of human centrosome assembly and dynamics. Overall this is a technically clever study that addresses an issue of significant interest to the centrosome filed. While the technical aspect is the undeniable strength of this study, a weakness of the manuscript in its current sate is the minimal biological insight it seems to provide on centrosome biology. This aspect of the manuscript should be improved before publication.

1. Most conclusions are reasonably well supported by the data, but I am concerned about the experimental methodology used for quantitative analysis of protein abundance in Figure1, Figure2 and Figure3. The authors observe that the expression levels, relative to SAS-6 vary considerably. I'm wondering if this is related to cell cycle regulation. For example, SAS-6 is cell cycle regulated so the baseline number used for the relative quantification takes this into account, providing a convoluted view of things. How can we then compare relative levels of other proteins whose levels are not regulated in a cell cycle dependent manner? Additionally, different cell types spend a different amount of time in different stages of cell cycle. How do the authors account for this? It seems therefore necessary for the authors perform SRM analysis to attest the protein abundance data from synchronized cells to examine their differences in G1/S/G2/M. This would allow for unambiguous insight into the dynamic stoichiometry of centrosome components during the cell cycle and would increase the impact of this study and will help to establish a more accurate and specific centrosome protein abundance map.

2. In Figure 2 and 3, the authors can perhaps try to perform the SRM analysis of centrosome protein abundance with the cell cytosols isolated from centrosome purification in order to make more conclusive comparisons. Similarly to the correlative MS profiling this laboratory performed initially with centrosome preparations? At least, it may help to determine the quality of centrosome purification from an SRM perspective.

3. In both Figure4 and Figure5, the authors need to examine their cell synchronization efficiency with specific cell cycle markers by either IF staining or WB.

4. In FigureS3, S4 and S5, it will be essential to quantify the expression level of EGFP-tagged proteins in WB experiments. Besides, the result of STIL WB experiment shows that the expression of EGFP-STIL is much lower than its endogenous level, which is a little bit at odds with the IF measurements. The author may also want to repeat the WB experiments with different STIL antibodies to determine whether this GFP tag affects the STIL protein stability.

 

5. The discussion should be shortened. The authors may want to discuss more about the application and limitation of their quantitative information instead of summarizing data. I like the innovative aspects of the paper but I am left asking myself: "What have we learned here?". It's important to improve this aspect of the paper to ensure others will want to take full advantage of this interesting dataset.

6. Do the authors know if the AAV-mediated GFP fusion proteins are functional? It is argued that they are incorporated at ~50% of the WT allele but it seems of importance to know if the allele is functional or not. Can both alleles be tagged?

Referee #3:

Quantitative analysis of human centrosome architecture by targeted proteomics and fluorescent tagging of endogenous proteins by Bauer et al. described the (absolute) quantification of several centrosome proteins by SRM and EGFP tagging of endogenous proteins. The authors perform SRM analysis using aqua peptides for absolute quantification and perform fractionation to be able to analyze all proteins of interest. They also perform purification of centrosomes to increase the number of proteins analyzed, using a label free quantification approach in combination with iBAQ. As a second quantification technique they use EGFP tagging which combines spatial with quantitative information.

Overall the manuscript provides robust quantitative data on the investigated centrosome associated proteins, however it remains unclear what this information means or how it affects the associated biology. The accumulation of quantitative data might be of interest to the broader community and the work to get there is impressive. Unfortunately the authors do not provide any example of the usefulness of the information.

Specific comments:
On page 8. The authors start with the statement; "Having established an efficient SRM workflow, the approach was applied to..." This established method is described but there is no data presented to support the statement. The manuscript would be strengthened if this 'establishment' of the efficient SRM method is supported by data, e.g. as supplementary figure showing the quality of the MS, transitions, peak shapes, reproducibility etc.

On page 7. The authors comment they need to fractionate the cell lysates to be able to confidently quantify the selected proteins by SRM. How does this fractionation influence the SRM assays? Are all fractions measured for every transition, are heavy peptides spiked in, etc.? Combining fractionation with SRM is not trivial and should be described better. Moreover, in the discussion the increase in measurement time of SRM is mentioned as limiting factor with respect to time resolution of the technique. This is not clear from the results section.

On page 8-9 the authors make several statements on the relative ratios of the monitored proteins in their relation to their function. However, for Figure 1B it becomes clear that when analyzing another cell line these ratios can change dramatically. The authors should explain what this means for the observed ratios in relation to the proteins functions and moreover, for their earlier rationale of the study, that protein concentrations are crucial to describe the biology.

For example on page 8 the authors state "From the results of this analysis several conclusions can be drawn: first, of all proteins analyzed, Plk4 is the least abundant", However, in the next cell line the authors choose to focus on KE37 Plk4 is one of the more abundant proteins and STIL has the lowest abundance. So what does this mean and how relevant is the observed data? Also relating copy numbers (Sas-6 and STIL) to structure of the cartwheel, will differ per cell type.

Page 10: "Second, it is interesting that Cep135 is much more abundant at purified centrosomes than either STIL or Sas-6". Why is this not observed in the whole cell analysis?

 

On page 11-12, the authors state SRM is important for accurate protein quantification, especially for low abundant proteins. However, most striking differences are observed for the higher abundant proteins in Fig 3.

The fluorescence panels in suppl Fig 3C-5C are very difficult to read. Zoom in (like in 3C) are required

1st Revision - authors' response 08 July 2016

Point-by-point reply:

Referee #1:

The data provided in the manuscript is important for use by many other scientists when studying centrosomes.

***We thank this referee for his/her appreciation of our work***

I have however several concerns that should be addressed prior publication:
a) There should be an initial figure summarizing all the experiments performed in the manuscript and how they relate to each other (cell type, cell cycle stage, type of data). It should be clear which data is cell cycle relevant, and what data can be compared with each other. For which proteins can the ratio of protein at cytoplasm vs centrosome be calculated and in which cycle stage?

***We are grateful for this suggestion. We now provide a schematic that provides the requested information and illustrates the connections between various experiments (new Figure 1). Moreover, we describe the flowchart illustrating our approach in a new opening paragraph of the Results section. We trust that this will greatly help our readers to "navigate" the paper.***

b) The authors should show growth curves for cell lines expressing tagged-centrosome proteins to demonstrate this tagging did not affect protein function and therefore their viability. Is the centrosome number in these cells normal? Given the cell lines only have one allele tagged, how are the authors sure that both WT and tagged allele localize equally well at the centrosome?

***We now provide growth curves, showing that there are no detectable differences between WT and tagged cell lines (see new Fig EV2, panel A). We also document that there are no detectable differences in centriole numbers (based on staining of cells with antibodies against the centriolar marker CP110; see new Fig EV2, panel B). Together, these data strongly argue that the tagged alleles are fully functional. Additionally, we emphasize that evidence from the literature also supports this conclusion (for Sas-6: Keller et al., 2014, op.cit); for g-tubulin: Khodjakov and Rieder 1999, op.cit; for STIL: Arquint et al., 2015, Curr. Biol.). Finally, we note that equal association of WT and tagged alleles of g-tubulin with centrosomes was already documented in the original manuscript (former Fig. S3D, now quantified in FigEV3, panel D); in the case of Sas-6 and STIL, sensitivity of Western blotting was insufficient to detect these proteins in fractionated samples and this is explained in the manuscript.***

c) The authors show enrichment of the endogenously-tagged proteins at the centrosome; their last point is mitotic- what do they mean as mitosis? Perhaps early and late mitosis should be separated as they are biochemically very distinct.

*** As we had explained in the Figure legend, the term 'mitotic' referred to cells that were enriched by nocodazole, as these mostly represent cells in 'early mitosis' (prometaphase-like), we now use the term "early mitosis" in the corresponding Figure panels.***

d) The authors should explain in all figure legends what cells and protocols they are using.

***Much information about cell types and procedures is now summarized in a new Figure 1. We are confident that this Figure will contribute to clarify these points. In addition, we have carefully re-

 

checked all Figure legends and we are confident that these confer all the requested information.***

Referee #2:

This manuscript by Bauer et al. offers an interesting quantitative insight into the general stoichiometry of components that make up human centrosomes. Here, the authors combine SRM mediated targeted proteomics of total extract and centrosome preparations with endogenous EGFP tagging method to measure the absolute and relative abundance of key centrosome proteins, and use this information to make predictions/modeling of human centrosome assembly and dynamics. Overall this is a technically clever study that addresses an issue of significant interest to the centrosome filed.

***We thank this referee for these positive comments***

While the technical aspect is the undeniable strength of this study, a weakness of the manuscript in its current state is the minimal biological insight it seems to provide on centrosome biology. This aspect of the manuscript should be improved before publication.

***We are most grateful for this comment - it has encouraged us to better "distill" the key messages of biological relevance. We have re-written several passages and are confident that the revised manuscript is much improved with regard to this critical point. Without repeating these passages here, we believe that our data allow us to draw several intriguing conclusions of biological relevance, as exemplified most strikingly by the predicted 1:2 ratio of STIL and Sas-6 in centriolar cartwheels and the prediction that human centrioles harbor cartwheels with 15-16 stacks. (As we explain in the manuscript, cartwheels have so far eluded visualization by electron microscopy or cryo-EM tomography in human cells; hence their structure is completely unknown). In addition, we discuss in the revised manuscript that quantitative information may be particularly important also in the context of the increasing realization that centrosomes display properties of liquid droplets, whose formation critically depends on protein concentration (Hyman et al., 2014 op. cit.; Brangwynne 2013 op. cit).This being said, we emphasize that the main merit of our paper consists in the fact that it addresses the considerable challenges associated with extracting accurate quantitative information on key centrosomal proteins. We believe that our data provide a benchmark for validation of future biochemical and structural studies. From this perspective, we agree with the Editor that a "Resource article" is the appropriate format for publication of our study.***

1. Most conclusions are reasonably well supported by the data, but I am concerned about the experimental methodology used for quantitative analysis of protein abundance in Figure1, Figure2 and Figure3. The authors observe that the expression levels, relative to SAS-6 vary considerably. I'm wondering if this is related to cell cycle regulation. For example, SAS-6 is cell cycle regulated so the baseline number used for the relative quantification takes this into account, providing a convoluted view of things. How can we then compare relative levels of other proteins whose levels are not regulated in a cell cycle dependent manner? Additionally, different cell types spend a different amount of time in different stages of cell cycle. How do the authors account for this? It seems therefore necessary for the authors perform SRM analysis to attest the protein abundance data from synchronized cells to examine their differences in G1/S/G2/M. This would allow for unambiguous insight into the dynamic stoichiometry of centrosome components during the cell cycle and would increase the impact of this study and will help to establish a more accurate and specific centrosome protein abundance map.

***We agree that the referee puts the finger on a number of important points and are grateful for these comments.
- With regard to former Figure 1 (now Figure 2A), we note that no calibration was used for quantitative analysis of the corresponding data. Instead, this Figure shows absolute values (average copy number/cell), as determined by SRM applied to total cell lysates prepared from asynchronously growing cells (as explained in text and legend). However, with regard to former Figures 2 and 3 (now Figure 2C and 3), we agree with the referee that use of Sas-6 for

standardization of all other proteins was problematic. Thus, we have recalculated the data in Figures 2C and 3 using g-tubulin for calibration. As we explain in the text, g-tubulin is the one protein for which we can deduce absolute numbers (average copy/centrosome) and, moreover, g-tubulin shows nearly constant expression throughout most of the cell cycle (except for very late G2 and mitosis). To the best of our knowledge, most centrosomal proteins studied here show comparatively little variation in expression during the cell cycle. The one striking exception concerns Sas-6 and STIL, both of which are degraded during mitosis and nearly absent from most G1 phase cells. Because the two proteins show nearly identical cell cycle profiles, the relative ratio between the proteins is unaffected by any correction factor used to compensate for cell cycle effects – thus, our conclusion of a 2:1 ratio for Sas-6:STIL is based on solid foundation.
- Related to the above point, we agree that different cell types may spend different amounts of time at different cell cycle stages, at least in principle. However, while such differences may be marked in a living multicellular organism, most cells in culture show very similar cell cycle distribution profiles (at least when grown under optimal conditions) and this is certainly the case for the cell lines analyzed here. So, we are confident that any differences related to cell cycle profiles would be extremely minor.
- Finally, this referee suggests that we carry out SRM analyses for all proteins and all cell lines at different cell cycle stages (after synchronization). We agree that, in theory, such data would be desirable. In practice, however, we feel that the acquisition of such data is not a realistic option (and for this reason have focused on the acquisition of cell cycle data by analysis of fluorescence emanating from endogenously GFP-tagged proteins). In the revised manuscript we now explain more clearly why it was impractical to use SRM for cell cycle analyses: although SRM measurements are powerful, they are also technically challenging, costly and very time consuming. Our focus on low abundance proteins required extensive sample fractionation by off gel electrophoresis prior to all quantitative analyses. Analysis of cells at different cell cycle stages would thus require large amounts of cells, and the need to analyze large numbers of samples for each time point would put huge demands on time and resources. Finally, we note that while SRM is the technique of choice for obtaining information about *absolute* amounts of proteins, information about relative amounts can much more easily obtained by Western blots (assuming that suitable antibodies are available). For many of the proteins analyzed in our study, Western blot cell cycle profiles have in fact been reported in the literature. What had hitherto been missing, but is provided in the present study, is information about *absolute* amounts of proteins (copy numbers per cell or centrosome).***

2. In Figure 2 and 3, the authors can perhaps try to perform the SRM analysis of centrosome protein abundance with the cell cytosols isolated from centrosome purification in order to make more conclusive comparisons. Similarly to the correlative MS profiling this laboratory performed initially with centrosome preparations? At least, it may help to determine the quality of centrosome purification from an SRM perspective.

***Former Figures 2 and 3 reported on protein abundance at purified centrosomes, while former Figure 1 reported on total cell lysates. Because the centrosome purification procedure is not a one-step fractionation process, we cannot directly compare protein abundance in cytosol and organelle. We do not consider it justified or informative to repeat correlative MS profiling (as done in Anderson et al., 2003), because the questions being asked in the two studies are rather different: in the 2003 study, the "quality" of centrosome purifications was critical for demonstrating the centrosome-association of newly identified proteins. In contrast, all proteins studied here are genuine, extensively validated centrosome components. Minor contamination of our centrosome preparations by cytoplasm would not be a major concern, as it would not affect relative quantitative values and only slightly affect absolute values.***

3. In both Figure4 and Figure5, the authors need to examine their cell synchronization efficiency with specific cell cycle markers by either IF staining or WB.

*** To document efficiency of our cell synchronization protocols used in Figures 4 and 5, we have now carried out Western blots for the following cell cycle markers: cyclin A, cyclin B1 and phospho-histone H3 (Serine 10), and a-tubulin as loading control. Results confirm efficacy of our cell synchronization protocols; they are shown in Fig EV5, panel D.***

4. In Figure S3, S4 and S5, it will be essential to quantify the expression level of EGFP-tagged

 

proteins in WB experiments. Besides, the result of STIL WB experiment shows that the expression of EGFP-STIL is much lower than its endogenous level, which is a little bit at odds with the IF measurements. The author may also want to repeat the WB experiments with different STIL antibodies to determine whether this GFP tag affects the STIL protein stability.

***Following this suggestion, we have quantified all relevant Western blot bands for the cell lines harboring tagged Sas-6, STIL and g-tubulin. The results show that EGFP tagged alleles (in +/EGFP cells) are always expressed at ca. 50% of the total protein levels seen in (+/+) cells (see Fig EV2, 3 and 5, panels B). Concerning the STIL Western blot (Fig EV4, panel B), we believe that there was a misunderstanding: the referee states that expression of EGFP-STIL is “much lower than its endogenous level”, but this is not correct. Instead, the referee was apparently misled by a background band (which is now marked by asterisk and explained in the legend). As we had stated in the original manuscript, our anti-STIL antibody does not recognize GFP-tagged STIL, presumably because the fusion with the tag interferes the epitope. During revisions, we have tried to detect GFP-STIL using a commercial antibody (from Bethyl laboratories), but, unfortunately, got no signal at all - not even for endogenous STIL.***

5. The discussion should be shortened. The authors may want to discuss more about the application and limitation of their quantitative information instead of summarizing data. I like the innovative aspects of the paper but I am left asking myself: "What have we learned here?". It's important to improve this aspect of the paper to ensure others will want to take full advantage of this interesting dataset.

***As suggested, we have shortened and re-written parts of the Discussion (see also our reply to a similar comment by referee 1). We are confident that our revised text better illustrates the usefulness as well as limitations of quantitative information.***

6. Do the authors know if the AAV-mediated GFP fusion proteins are functional? It is argued that they are incorporated at ~50% of the WT allele but it seems of importance to know if the allele is functional or not. Can both alleles be tagged?

*** We are confident that GFP-tagged proteins are functional. First, there are no detectable differences in growth between WT and tagged cell lines (see new Fig EV2, panel A). Second, there are no detectable differences in centriole numbers (based on staining of cells with antibodies against the centriolar marker CP110; see new Fig EV2, panel B). Together, these data strongly argue that the tagged alleles are fully functional, and evidence from the literature also supports this conclusion (for Sas-6: Keller et al., 2014, op.cit); for g-tubulin: Khodjakov and Rieder 1999, op.cit; for STIL: Arquint et al., 2015, Curr. Biol.). In the case of g-tubulin, we further show WT and tagged versions are recovered in similar proportions from centrosomes (former Fig. S3D, now quantified in Fig EV3, panel D); in the case of Sas-6 and STIL, sensitivity of Western blotting was insufficient to detect these proteins in fractionated samples, as explained in the manuscript. Finally, tagging of both alleles by AAV-mediated fusion would be rather impractical; presumably, this could in future be attempted using CRISPR/Cas9 gene editing technology.***

Referee #3:

Overall the manuscript provides robust quantitative data on the investigated centrosome associated proteins, however it remains unclear what this information means or how it affects the associated biology. The accumulation of quantitative data might be of interest to the broader community and the work to get there is impressive. Unfortunately the authors do not provide any example of the usefulness of the information.

***We are grateful that this referee recognizes the amount of effort that was required for the accumulation of the quantitative data reported in our study, and that he/she acknowledges that these data will be of interest to a broad community. We feel that the statement “unfortunately, the authors do not provide any example of the usefulness of this information” is slightly unfair, but we agree that we did not do a particularly good job illustrating the biological usefullness of our data. We have thus re-written several passages and are confident that the revised manuscript is much improved with regard to this critical point. Without repeating these passages here, we believe that our data

 

allow us to draw several intriguing conclusions of biological relevance, as exemplified most strikingly by the predicted 1:2 ratio of STIL and Sas-6 in centriolar cartwheels and the prediction that human centrioles harbor cartwheels with 15-16 stacks. (As we explain in the manuscript, cartwheels have so far eluded visualization by electron microscopy or cryo-EM tomography in human cells; hence their structure is completely unknown). In addition, we discuss in the revised manuscript that quantitative information may be particularly important also in the context of the increasing realization that centrosomes display properties of liquid droplets, whose formation critically depends on protein concentration (Hyman et al., 2014 op. cit.; Brangwynne 2013 op. cit).This being said, we emphasize that the main merit of our paper consists in the fact that it addresses the considerable challenges associated with extracting accurate quantitative information on key centrosomal proteins. We believe that our data provide a benchmark for validation of future biochemical and structural studies. From this perspective, we agree with the Editor that a "Resource article" is the appropriate format for publication of our study.***

Specific comments:
On page 8. The authors start with the statement; "Having established an efficient SRM workflow, the approach was applied to..." This established method is described but there is no data presented to support the statement. The manuscript would be strengthened if this 'establishment' of the efficient SRM method is supported by data, e.g. as supplementary figure showing the quality of the MS, transitions, peak shapes, reproducibility etc.

***We agree that our rather cursory description of the elaboration of an efficient SRM workflow did not do justice to the extensive work associated with this key preparatory step. The corresponding data are of a rather technical nature, but we agree that they are important and likely to be of interest to readers with competence in proteomics; hence in the revised manuscript we now include a schematic of the workflow used for development of the SRM assays, as well as representative data-sets for one particular low abundance protein, CPAP (see new Appendix Figure S2). We also describe the workflow in the legend to Appendix Figure S2, and we emphasize that further detailed information on all selected SRM-assays (regarding detection and quantification limits, reproducibility and performance) can be found in a recent publication (Bauer et al.,2014 op. cit.).

On page 7. The authors comment they need to fractionate the cell lysates to be able to confidently quantify the selected proteins by SRM. How does this fractionation influence the SRM assays? Are all fractions measured for every transition, are heavy peptides spiked in, etc.? Combining fractionation with SRM is not trivial and should be described better. Moreover, in the discussion the increase in measurement time of SRM is mentioned as limiting factor with respect to time resolution of the technique. This is not clear from the results section.

***Indeed, sample fractionation (on the peptide level) was required to increase the sensitivity of the SRM assays to a level that allowed confident quantification of the least abundant proteins (including STIL and Plk4). Without fractionation, we were not able to confidently identify endogenous peptides derived from these proteins within cell lysates. To better explain the combination of fractionation with SRM, we now describe the workflow used for sample preparation in a new Appendix Figure S3. We also emphasize that heavy reference peptides were spiked into peptide samples at the end of tryptic digestion, i.e. *before* C18 purification and fractionation. Therefore, fractionation steps have no impact on quantification accuracy, as peptide ratios remain constant across the different fractions. The referee is correct in assuming that we measured transition intensities individually for all fractions. However, due to the focusing nature of the off gel-fractionation employed, most peptides were only found in one single fraction (occasionally two fractions). For the few peptides that were found in two fractions, transition intensities were summed up before ratio calculation.
- Regarding our remark on "temporal resolution", we think there has been a misunderstanding. We did not mean to imply that measurement time is a limiting factor, but rather that the amount of time required to process large numbers of fractionated samples made it impractical to use SRM for monitoring protein changes through the cell cycle. To avoid future misunderstanding, we have deleted the term "temporal resolution" and re-written the corresponding passage in the Discussion.
***

On page 8-9 the authors make several statements on the relative ratios of the monitored proteins in

their relation to their function. However, for Figure 1B it becomes clear that when analyzing another cell line these ratios can change dramatically. The authors should explain what this means for the observed ratios in relation to the proteins functions and moreover, for their earlier rationale of the study, that protein concentrations are crucial to describe the biology.

***We agree with this point. Although all statements on pages 8 and 9 were correct with regard to RPE-1 cells (i.e. in the stated context of the analysis), it is true that the situation changes when other cell lines are considered. Thus, in the Results section of the revised manuscript we have extensively re-written the corresponding paragraphs. We have deleted most of the specific statements that are limited in their validity to RPE-1 and we have also deleted former Figure 1 (RPE-1 data). Instead, we now discuss all data in the context of Figure 2A (which summarizes data for all cell lines, including RPE-1). We trust that this re-organization of material is clearer than our original description.***

For example on page 8 the authors state "From the results of this analysis several conclusions can be drawn: first, of all proteins analyzed, Plk4 is the least abundant", However, in the next cell line the authors choose to focus on KE37 Plk4 is one of the more abundant proteins and STIL has the lowest abundance. So what does this mean and how relevant is the observed data? Also relating copy numbers (Sas-6 and STIL) to structure of the cartwheel, will differ per cell type.

***We are grateful to the referee for drawing our attention to this inconsistency. In fact, while the numerical data in former Supplementary Table 1 had been correct (now Appendix Table S1), we had made an error in depicting the height of the Plk4 bar in the KE37 sample (former Figure 1B); this mistake has now been corrected in the revised Figure 2A. Moreover, we agree that some statements were problematic because the ranking of proteins by relative abundance is not the same in all cell lines. We have thus re-written this passage of the Results section. We also agree that cartwheel structure (number of stacks) may differ between cell lines, but emphasize that in the context of the passages criticized here (pages 8-10) this is not a concern. In these passages (now re-written) we had merely used the term “cartwheel” for sub-categorization of the respective proteins; we did not intend to “relate copy numbers to cartwheel structure”. This was done later in the manuscript, where we analyzed protein abundance at purified centrosomes.***

Page 10: "Second, it is interesting that Cep135 is much more abundant at purified centrosomes than either STIL or Sas-6". Why is this not observed in the whole cell analysis?

***As we point out repeatedly in the manuscript, there is no straightforward correlation between abundance in whole cell lysates and abundance at purified centrosomes. This underscores the importance of posttranslational mechanisms to control centrosomal recruitment of individual proteins.***

On page 11-12, the authors state SRM is important for accurate protein quantification, especially for low abundant proteins. However, most striking differences are observed for the higher abundant proteins in Fig 3.

*** We agree that our statement was unjustified and accordingly have deleted it. Also, considering that SRM data were used for calibration of iBAQ data (Fig EV1, panel E), we have redrawn Figure 3 and now show SMR data (red bars) side-by-side with iBAQ data (grey bars).***

The fluorescence panels in suppl Fig 3C-5C are very difficult to read. Zoom in (like in 3C) are required

***We note that the inset in former Figure S3C (now Fig EV3, panel C) showed a mitotic cell (not merely a blow up) and this had been explained in the legend. However, we understand that the fluorescence panels in former Figures S4C and S5C have been difficult to see; hence, for better visibility we now provide brighter images as well as insets (higher magnifications) for these panels (see Fig EV4 and 5, panels C).***

 

2nd Editorial Decision 25 July 2016

Thank you for submitting the revised version of your Resource Article for our consideration. It has now been assessed once more by the original referees, and I am pleased to inform you that all three reviewers are satisfied with the revision and have no more reservations regarding publication in The EMBO Journal. As you will see from the comments below, referee 2 mentions only one remaining minor point, which in my opinion would however not warrant additional revision work at this stage.

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

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REFEREE COMMENTS

Referee #1:

I have now looked at the revised version and the rebuttal, I think the authors have address most comments and I think it is a nice resource paper.

Referee #2:

The authors have addressed most of my concerns with strong experimental support and detailed explanations. It was a good idea to recalculate the data with gamma-tubulin and the newly normalized quantitative dataset will be interesting resources to the centrosome field. So I fully support the publication of this manuscript with one minor revision.

(1) I am still concerned about the STIL WB experiment. The STIL (A302-441A, Bethyl Laboratories) antibody has been reported in many papers that it works for western blotting of endogenous STIL. For example, in the recent Holland JCB paper, this antibody could detect both GFP-STIL and endogenous STIL in WB. They should try this antibody.

Referee #3:

The authors have addressed all concerns and the manuscript can be published.

 

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: Erich Nigg |
| Journal Submitted to: Embo Journal |
| Manuscript Number: EMBOJ-2016-94462 |

**Reporting Checklist For Life Sciences Articles (Rev. July 2015)**

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

## A- Figures

### 1. Data

**The data shown in figures should satisfy the following conditions:**

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

### 2. Captions

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

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

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

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

**Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.**

**In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).**

## 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? | NA |
| 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. | NA |
| Is there an estimate of variation within each group of data? | NA |
| Is the variance similar between the groups that are being statistically compared? | NA |

## C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

| | |
|---|---|
| 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). | see section material and method P23 on the manuscript |
| 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | This is specified on manuscript P22 |

* 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 accession codes for deposited data. See author guidelines, under 'Data Deposition'.<br><br>Data deposition in a public repository is mandatory for:<br>a. Protein, DNA and RNA sequences<br>b. Macromolecular structures<br>c. Crystallographic data for small molecules<br>d. Functional genomics data<br>e. Proteomics and molecular interactions | The raw mass spectrometric data used in this study and the Mascot analysis files are available via ProteomeXchange: accession code PXD003927 |
| 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). | See point 18 above. |
| 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. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.<br><br>Examples:<br>**Primary Data**<br>Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462<br>**Referenced Data**<br>Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26<br>AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208 | NA |
| 22. 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

| | |
|---|---|
| 23. 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 |