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Proteomic and functional analysis of the mitotic Drosophila centrosome

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

1st Editorial Decision 09 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three expert reviewers, whose comments are copied below. As you will see, the referees appreciate the comprehensive nature of the work as well as the large amount and overall quality of the experimental data. At the same time, they however also raise a number of substantive concerns, in some cases about too limited follow-up mechanistic analysis of newly identified candidate proteins, but also more direct issues with the current analysis in its present state.

Taking these various points into consideration, we conclude that given the comprehensiveness and timeliness of the study, we should be able to consider a revised version for publication even in the absence of additional downstream mechanistic analyses (such as those requested by referees 1 and 3). What would however be essential for such a revised manuscript to be successfully considered is a consolidation of the experimental data, their mining and their presentation/discussion as requested by the specific points of referees 2 and 3. In this respect, it will be essential to provide the raw mass spectrometry data, as well as to properly compare the data with previously reported datasets. Related to this, it is apparent that some results would also need to be better discussed in the context of already available evidence in the literature. It will also be important to streamline the presentation and to better outline the rationale behind the design of the various follow-up validations of proteomically identified candidate proteins. On the other hand, we would not consider the TAP tagging/purification of identified candidate proteins (referee 3) as crucial within the scope of this revision.

I would therefore like to invite you to prepare a revised version of the manuscript, taking into

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account the main points raised by the referees as outlined above (in this respect, please note that it will be important to diligently respond to all the points raised at this stage, as it is EMBO Journal policy to allow only a single round of major revision). When preparing your letter of response, please also bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

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

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This ms presents a mass spectrometric analysis of drosophila centrosomes immune-isolated from a centrosome enriched fraction of embryo homogenate. This has led to the identification of a number of candidate centrosomal proteins in addition to already known centrosome-associated proteins (260 altogether). Only a subset of these candidate proteins were tagged with GFP and a fraction of them shown to localize at the centrosome in cultured fly cells.
All of them were subjected to RNAi in cultured cells and the effect monitored by IF. Centrosome loss, as judge by gamma-tubulin labeling, was observed for 11 proteins (not validated at the EM level however), and few others gave either 'small' or 'fuzzy' centrosomes. In the first group, unexpected, like translation factors for example, were found, casting some doubt on the specificity of these effects.
Another group of proteins gave a depletion phenotype compatible with an effect on either duplication or segregation of centrosomes. Some of them could be expected to have such an effect - gamma-TuRC components, motor proteins..), others once again were less expected, like ribosomal proteins, the effect being not apparently mimicked by cycloheximide treatment, or DNA-binding proteins that regulate DNA replication, whose effect could correspond to the known deregulation of centrosome duplication in S-arrested cells.
A last group of proteins has a depletion phenotype on cell cycle progression which in all cases requires a specific study for their interpretation. Here too, unexpected candidates were found.
Altogether, there is a considerable amount of work, involving a great number of authors. The paper however looks more like a progress report on a whole series of candidate proteins, which all will require a specific analysis to reach firm conclusions. It mainly deals with a crude classification of phenotypes, grouping gene products that are a priori functionally unrelated, thus imposing the authors to deal with a broad range of aspects, instead of focusing on specific functions on the centrosome. The main message is that such a biochemical approach has inherent risks indeed, linked to the real enrichment of the fraction analyzed, to lead to too many different directions, whose relevance to centrosome functions has then to be demonstrated.

Referee #2 (Remarks to the Author):

This work describes the proteome of the Drosophila mitotic centrosome, subsequent RNAi analysis of the candidate proteins, and siRNA of human homologues of those Drosophila genes implicated in centrosome function through the RNAi.

The results incorporated in this paper have been widely anticipated by the Drosophila cell cycle field and are thus of general interest and importance and should be published.

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However, there are a number of points that need to be addressed, including a substantial re-write of the text. I think it is imperative that studies such as this should effectively combine Results and Discussion to both avoid repetition, and to allow the authors to fully discuss their proteomic / RNAi screen results within the context of the general article. If this means resubmission as a different type of article for EMBO, then that is what should be done.

Results

1. The authors convincingly demonstrate purification and enrichment of Drosophila centrosomes from the syncytial blastoderm embryo, validating their methodology.

2. There is a very good level of detail in the Supplementary Methods, regarding the MS analysis. However, I was unable to find the cut off point for false positives. As far as I could see, there was no Supplementary table of the raw data (i.e the peptides identified that allowed verification of protein identity). It may be readers just need pointing to the data, given that, in paragraph 1 of the Discussion the authors say: "This takes also into account that the cut-off level at the level of MS identification (Suppl Info) was set high".

Action: If the raw peptide MS data is not there, it needs to be incorporated into another Supplementary Table (in the style of Nousiainan et al., 2006 or Hughes et al., 2008).

3. A true reflection of the "validity and robustness" of the proteomic approach is confused by the addition of an extra 71 proteins not identified by MS, when the RNAi was carried out. (i.e. the authors chose an additional 96 proteins that had previously been described as "centrosomal" in Drosophila via UNIPROT). Firstly, they do not discuss why these 71 proteins were not identified by MS (presumably low abundance etc., but should still be discussed), and secondly, by then discussing phenotypes of RNAi without distinguishing those identified by proteomics and those added afterwards, it muddies their analysis (for example, of the 71 not identified via MS, 30 had phenotypes in their RNAi. So when discussing the fact that 95 proteins have a "centrosome related function", they should make clear that only 65 of these came from their MS - i.e.65/260).

Action: The authors should ideally separate the proteomic data from their additional UNIPROT-based data, and the discussion surrounding them, to truly reflect the points they are trying to make about their proteomic approach.

4. An additional confusion comes from those gene products for which they decided to study localization of, and the manner in which this is written and represented. They chose 36 proteins for GFP-tagging, and 10 of these for antibody productio, resulting in 30 proteins for which they could investigate localization, to validate their proteomics. However, these 36 were not randomly chosen, but were biased for those with coiled-coil domains and those that gave centrosomal phenotypes when knocked-down using RNAi (i.e. the 11 that gave an 'O' phenotype + 25 others with coiled-coils, 14 of which also showed phenotypes in their RNAi). Although clearly demonstrating previously undescribed centrosomal localizations for some proteins, the biased nature of their choices cannot be used to accurately reflect the robustness of their MS. Additionally, localization to the mitotic spindle cannot be used to reflect a localization to the centrosome. They are different functional sub-cellular structures (although related). The authors make it clear when an antibody localizes to both centrosomes and spindle. Those that localize to spindles, but not centrosomes (4/30) cannot be considered to validate their MS.

Action: The authors should address the above points clearly in their text.

5. The examples the authors choose to focus on are perhaps not taken as far, or clear-cut, as they could be. Their work on eIF4a complements that of Somma et al., 2008, whose RNAi based screen for mitotic defects identified many components of the eIF3 complex to be required for mitotic spindle assembly. This complementary study should at least be mentioned in the text in this section. In addition, the comparison between the phenotype of eIF4a and eIF4e (as a way to fully inhibit translation) is confused by the fact that a phenotype (all be it somewhat different) was found for depletion of eIF4e, and that eIF4e has previously been shown to localize to both centrosomes and the mitotic spindle in Drosophila S2 cells (Hughes et al., 2008).

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Their choice of taking a closer look at Feo (Prc-1) and Lat (which is involved in DNA repair) also produces less than straight forward results: The lat RNAi phenotype is described as being the same as total inhibition of DNA replication, so the authors cannot conclude anything about the relationship between Lat and the centrosome, while Feo has been previously shown to be a MAP required for cytokinesis - the defects in centrosomal size and number could therefore reflect a pleiotropic effect on cell cycle regulation due to previously failed cytokineses, or additional roles of Feo on the spindle.

Action: Either replace some of these examples with clear cut ones that move those fields of research on further, or cut the section considerably. The previously published observations on the proteins of interest should be included.

5. The section on the human homologues of the Drosophila centrosomal proteome is generally well written and interesting!. However, we are not told how many of the 95 Drosophila genes that gave centrosomal phenotypes have human orthologues in the text and, importantly, how the homologue searching was carried out. Also, I was confused by some of the data in the table: In the human/fly comparison table (part of Supplementary Table 1), some genes (such as CG1962, grp, lok, CKIIalpha, tankyrase etc.) have phenotypes when knocked-down in human cells, but there appears to be no phenotype for the corresponding Drosophila genes? This suggests the list of homologues were not totally chosen on the basis of a phenotype in Drosophila RNAi (or maybe this reviewer has not been able to follow the text). To my mind, it suggests that the authors may well have carried out RNAi on the human homologues of ALL 260 genes (not just those that gave phenotypes in S2 cells). If this is so, they need to say! If not, we need to understand what exactly was done.

Action: Address the above points

Minor points:

1. Authors should cite the Raff lab, Stephens et al., 2009 and 2010 JCB papers on Ana3 and Ana2 respectively, at top of page 2 of intro.

2. At the top of page 3, it should be made clear that "Aur" is specifically the kinase Aurora A, not e.g. Aurora B.

3. 14-3-3-e has a shared cell cycle progression phenotype between flies and humans in Supplementary Table 1, but is not highlighted in green in the right hand panel of the table.

Referee #3 (Remarks to the Author):

In this manuscript Muller and colleagues present a functional proteomic screen towards the characterization of the Drosophila centrosome. By performing mass spectrometry (MS) on immunopurified centrosomes, the authors identified about 260 proteins that are enriched in centrosome preparations. Using an RNAi interference approach in conjunction with GFP-tagging and localization they identify 27 centrosome/spindle proteins with potentially interesting functions in the regulation of centrosome biogenesis and mitotic spindle assembly. This is a nice paper and a tremendous amount of work. Less impressive however, is the rather meager downstream characterization of the most promising candidates. The inclusion of such data would greatly increase the impact of this manuscript.

- Mass spectrometry was obviously at the origin of this work. Surprisingly there's no mass spectrometry data included in the manuscript as far as I can tell. It is therefore difficult to understand what was considered specific or background during the purification/MS process.

- Andersen and colleagues used a similar procedure 7 years ago to define the human centrosome proteome. Although this is not a problem by itself, I'm a little surprised the two datasets were not compared further. RNAi data from different screens have been compared but not the proteomic data.

- An interesting aspect of this work could have been to build on their exciting immunopurified

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centrosome MS data and perform tandem-affinity purification on the top candidates. This would potentially provide some mechanistic insights on the likely role of the proteins indentified and localized to specific cellular locales.

- It is very difficult to appreciate the localization of new centrosome proteins on centrosomes based on the data presented in Figure 2. For example I can't see any Nup153 or Lam co-localizing with gamma-tubulin. The other 6 you can barely discern colocalization with centrosomes. Insets would be required in all cases to better visualize things. The same is true for the TAP data presented in Figure S2 and Abs in S3.

- I was under the impression work from the Raff lab suggested that Dm-Spd2 was not required for centriole duplication. The data in Fig 3 suggests it has the same phenotype in SL2 cells and that it is required for duplication... This should be discussed.

- In Figure 4, the authors show that gamma-tubulin is not recruited to centrosomes. This data is potentially interesting but it would be nice to see if any centrioles are present in these cells. If so it would suggest that PCM recruitment is impaired in eIF-4a RNAi treated cell. Levels of gamma-tubulin should also be tested to rule out localization versus protein stability/overall levels.

- Out of the 260 initially tested, 12 novels proteins with confirmed centrosome/spindle localization were identified. This seems like a relatively minor fraction. Is the conclusion from this data that the immunopurified centrosome are heavily contaminated by non-centrosomal/spindle proteins, are there problems with GFP/TAP localization or both?

1st Revision - authors' response 10 June 2010

Referee #1 (Remarks to the Author):

This ms presents a mass spectrometric analysis of drosophila centrosomes immune-isolated from a centrosome enriched fraction of embryo homogenate. This has led to the identification of a number of candidate centrosomal proteins in addition to already known centrosome-associated proteins (260 altogether). Only a subset of these candidate proteins were tagged with GFP and a fraction of them shown to localize at the centrosome in cultured fly cells. All of them were subjected to RNAi in cultured cells and the effect monitored by IF. Centrosome loss, as judge by gamma-tubulin labeling, was observed for 11 proteins (not validated at the EM level however), and few others gave either 'small' or 'fuzzy' centrosomes. In the first group, unexpected, like translation factors for example, were found, casting some doubt on the specificity of these effects. Another group of proteins gave a depletion phenotype compatible with an effect on either duplication or segregation of centrosomes. Some of them could be expected to have such an effect -gamma-TuRC components, motor proteins..), others once again were less expected, like ribosomal proteins, the effect being not apparently mimicked by cycloheximide treatment, or DNA-binding proteins that regulate DNA replication, whose effect could correspond to the known deregulation of centrosome duplication in S-arrested cells. A last group of proteins has a depletion phenotype on cell cycle progression which in all cases requires a specific study for their interpretation. Here too, unexpected candidates were found. Altogether, there is a considerable amount of work, involving a great number of authors. The paper however looks more like a progress report on a whole series of candidate proteins, which all will require a specific analysis to reach firm conclusions. It mainly deals with a crude classification of phenotypes, grouping gene products that are a priori functionally unrelated, thus imposing the authors to deal with a broad range of aspects, instead of focusing on specific functions on the centrosome. The main message is that such a biochemical approach has inherent risks indeed, linked to the real enrichment of the fraction analyzed, to lead to too many different directions, whose relevance to centrosome functions has then to be demonstrated.

Authors' response:

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This referee raises a number of points, some specific to this manuscript and other which also touch on the methodology, merit and publication of high throughput proteomic and high throughput RNAi functional studies.

First, concerning the revision of the points specific to this manuscript:

(A) We have now addressed the point of specificity of the structural centrosome defects in more detail. In total 5 experiments address this point.

1. Inhibition of protein translation by cycloheximide, which results in a distinct phenotype (centrosome number defects) to elF-4a depletion (PCM loss).
2. Knockdown of a core component of translation, eIF-4e, which also results in a distinct phenotype (centrosome number defects) to elF-4a depletion (PCM loss).
3. (new in the revision) Total -tubulin level does not change, following eIF-4a depletion, although this treatment abolishes centrosomal localisation of -tubulin.
4. (new in the revision) Labelling of eIF-4a depleted cells with a number of core centriolar markers suggests that localization of all tested centriole markers is unaffected, while -tubulin is displaced from the centriole.
5. Cell cycle analysis of eIF-4a depleted cells revealed a massive accumulation in prophase. This phenotype was much less pronounced in eIF-4e depleted cells.

These new data are incorporated in Fig. S4 & S5. Please see also response to referees 2 & 3.

Taken together, these results indicate that PCM loss through eIF-4a depletion is mechanistically distinct from the inhibition of global protein translation.

(B) The term "centrosome loss" in the manuscript refers to elimination of gamma-tubulin from the amorphous PCM, but not to defects in the centriole structure. Therefore, the analysis has been performed by immunofluorescence microscopy rather than electron microscopy. Furthermore, immunofluorescence microscopy allowed for quantification of large number of cells, for localisation or misclocalisation of different proteins at the same time, as opposed to electron microscopy-based analysis.

(C) We have addressed the point of the referee that the manuscript resembled a "progress report" in two ways:
1. By complete reorganization and re-writing of the entire manuscript (see also response to referees 2 & 3).
2. We carried out a more detailed comparison to previously published data, to set our results in the context of what is new and what does this manuscript contribute to the field. This is now incorporated in the new Supplemental Table S2.

(D) Second, concerning the amount of information and depth of analysis presented in such a (high throughput proteomic and RNAi) study:

There is absolutely no doubt that much more work is required, by us and others, for the detailed characterisation of the new molecules identified

The present study goes well beyond a biochemical and proteomic approach alone. All identified molecules are functionally characterised in a Drosophila cell system, with quantification of 10 parameters; in addition human homologues were further characterised in HaCaT cells.

That "a priori functionally unrelated" proteins get grouped together is indeed one of the important messages of the paper. This is not so surprising, for two reasons. First, diverse disease phenotypes have being described, resulting from centrosome dysfunction, but with no obvious link to the "classic" centrosome functions. In most cases, the connection to the centrosome has been revealed by the discovery of centrosome associated proteins mutated in these diseases. Second, a number of proteins with well-established non-centrosomal localisation and function have been found to associate with the mitotic centrosome where they fulfill regulatory functions. We have substantially re-written the introduction and discussion parts, to put our findings in the context of multiple roles of unconventional centrosomal components already described in the literature.

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Therefore, the publication of this study is important at this point because
- it will stimulate / initiate studies on the 12 new reported molecules, that have been for the first time localized to the centrosome and/or spindle
- it provides a resource of information (plus access to clones, cell lines and other reagents) to the research community for further studies
- it provides data to drive further studies on the multiple roles of unconventional centrosomal components

Referee #2 (Remarks to the Author):

This work describes the proteome of the Drosophila mitotic centrosome, subsequent RNAi analysis of the candidate proteins, and siRNA of human homologues of those Drosophila genes implicated in centrosome function through the RNAi. The results incorporated in this paper have been widely anticipated by the Drosophila cell cycle field and are thus of general interest and importance and should be published. However, there are a number of points that need to be addressed, including a substantial re-write of the text. I think it is imperative that studies such as this should effectively combine Results and Discussion to both avoid repetition, and to allow the authors to fully discuss their proteomic / RNAi screen results within the context of the general article. If this means resubmission as a different type of article for EMBO, then that is what should be done.

Authors' response:
We implemented this suggestion and have now combined the Results and Discussion sections into one.

Results:
1. The authors convincingly demonstrate purification and enrichment of Drosophila centrosomes from the syncytial blastoderm embryo, validating their methodology.

2. There is a very good level of detail in the Supplementary Methods, regarding the MS analysis. However, I was unable to find the cut off point for false positives. As far as I could see, there was no Supplementary table of the raw data (i.e the peptides identified that allowed verification of protein identity). It may be readers just need pointing to the data, given that, in paragraph 1 of the Discussion the authors say: "This takes also into account that the cut-off level at the level of MS identification (Suppl Info) was set high".
Action: If the raw peptide MS data is not there, it needs to be incorporated into another Supplementary Table (in the style of Nousiainan et al., 2006 or Hughes et al., 2008).

Authors' response:
We have now supplied the peptide data, individual Mascot Scores and other relevant MS data, in the new Supplementary Table S1, according to the format suggested by the referee.

3. A true reflection of the "validity and robustness" of the proteomic approach is confused by the addition of an extra 71 proteins not identified by MS, when the RNAi was carried out. (i.e. the authors chose an additional 96 proteins that had previously been described as "centrosomal" in Drosophila via UNIPROT). Firstly, they do not discuss why these 71 proteins were not identified by MS (presumably low abundance etc., but should still be discussed), and secondly, by then discussing phenotypes of RNAi without distinguishing those identified by proteomics and those added afterwards, it muddies their analysis (for example, of the 71 not identified via MS, 30 had phenotypes in their RNAi. So when discussing the fact that 95 proteins have a "centrosome related function", they should make clear that only 65 of these came from their MS - i.e.65/260).
Action: The authors should ideally separate the proteomic data from their additional UNIPROT-based data, and the discussion surrounding them, to truly reflect the points they are trying to make about their proteomic approach.

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Authors' response:
The controls are clearly marked in the new Supplementary Table S3 in columns E, F and G.

Concerning the selection of controls:
We had originally selected 96 proteins from the UniProt database, via the search terms centrosome and Drosophila, to be employed as controls in the RNAi functional assays. Comparison of those 96 to our MS-identified set, resulted in 35 overlaps. Those 35 were removed from the control list of 96. We had therefore left 61 control proteins, from the original 96.

These "historical" selection and subtraction steps have been now removed from the text, as they obviously created confusion. We now simply state that 61 controls were selected through UniProt. This permitted us now also to make a more clear distinction between the non-MS-identified control proteins and the MS-identified proteins.

4. An additional confusion comes from those gene products for which they decided to study localization of, and the manner in which this is written and represented. They chose 36 proteins for GFP-tagging, and 10 of these for antibody productio, resulting in 30 proteins for which they could investigate localization, to validate their proteomics. However, these 36 were not randomly chosen, but were biased for those with coiled-coil domains and those that gave centrosomal phenotypes when knocked-down using RNAi (i.e. the 11 that gave an 'O' phenotype + 25 others with coiled-coils, 14 of which also showed phenotypes in their RNAi). Although clearly demonstrating previously undescribed centrosomal localizations for some proteins, the biased nature of their choices cannot be used to accurately reflect the robustness of their MS. Additionally, localization to the mitotic spindle cannot be used to reflect a localization to the centrosome. They are different functional sub-cellular structures (although related). The authors make it clear when an antibody localizes to both centrosomes and spindle. Those that localize to spindles, but not centrosomes (4/30) cannot be considered to validate their MS.
Action: The authors should address the above points clearly in their text.

Authors' response:
We have now, more clearly, explained in the text the two criteria of selection of the proteins employed in localisation studies.

Second, we have re-written the relevant passages, to remove any implication that the selected targets localisation would be used to validate the overall success rate of the immunoaffinity and MS approach.

In addition, there likely was a misunderstanding because number "12" appeared twice in the text, concerning localisation: Of the analysed proteins, 12 localised at the centrosome and 5 at the spindle (Table S5). From these 17 proteins, we identified 12 new localisations, (8 at the centrosome and 4 at the spindle) (Table II and Table S5). This point has now been clarified in the text.

Finally, we removed the statement about the validation of the approach in the context of spindle localisation, according to the suggestion of the referee.

5. The examples the authors choose to focus on are perhaps not taken as far, or clear-cut, as they could be. Their work on eIF4a complements that of Somma et al., 2008, whose RNAi based screen for mitotic defects identified many components of the eIF3 complex to be required for mitotic spindle assembly. This complementary study should at least be mentioned in the text in this section. In addition, the comparison between the phenotype of eIF4a and eIF4e (as a way to fully inhibit translation) is confused by the fact that a phenotype (all be it somewhat different) was found for depletion of eIF4e, and that eIF4e has previously been shown to localize to both centrosomes and the mitotic spindle in Drosophila S2 cells (Hughes et al., 2008). Their choice of taking a closer look at Feo (Prc-1) and Lat (which is involved in DNA repair) also produces less than straight forward results: The lat RNAi phenotype is described as being the same as total inhibition of DNA replication, so the authors cannot conclude anything about the relationship between Lat and the centrosome, while Feo has been previously shown to be a MAP required for cytokinesis - the defects in centrosomal size and number could therefore reflect a pleiotropic effect on cell cycle regulation due to previously failed cytokineses, or additional roles of Feo on the spindle.

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Action: Either replace some of these examples with clear cut ones that move those fields of research on further, or cut the section considerably. The previously published observations on the proteins of interest should be included.

Authors' response:
We have now streamlined this section in the following way:

(a) We have removed most of the functional characterisation Feo from the Results and Supplemental Information.

(b) We had previously included Lat as a protein that we localised to the centrosome but whose function we could not separate from a general inhibition of DNA replication. We think that this negative result is worth mentioning, nonetheless we have now significantly condensed this section.

(c) We have discussed in the text the results by Hughes et al. 2008 relating to eIF-4e and by Somma et al. 2008 relating to the eIF3 complex.

(d) We have performed additional experiments to further characterize the function of eIF-4a at the centrosome. We show that total -tubulin level does not change, following eIF-4a depletion, although this treatment abolishes centrosomal localisation of -tubulin. Labelling of eIF-4a depleted cells with a number of core centriolar markers suggests that localization of all tested centriole markers is unaffected, while -tubulin is displaced from the centriole.

These new data are incorporated in Figure 4 & Figure S5. Please see also response to referees 1 & 3.

5. The section on the human homologues of the Drosophila centrosomal proteome is generally well written and interesting!. However, we are not told how many of the 95 Drosophila genes that gave centrosomal phenotypes have human orthologues in the text and, importantly, how the homologue searching was carried out. Also, I was confused by some of the data in the table: In the human/fly comparison table (part of Supplementary Table 1), some genes (such as CG1962, grp, lok, CKIIalpha, tankyrase etc.) have phenotypes when knocked-down in human cells, but there appears to be no phenotype for the corresponding Drosophila genes? This suggests the list of homologues were not totally chosen on the basis of a phenotype in Drosophila RNAi (or maybe this reviewer has not been able to follow the text). To my mind, it suggests that the authors may well have carried out RNAi on the human homologues of ALL 260 genes (not just those that gave phenotypes in S2 cells). If this is so, they need to say! If not, we need to understand what exactly was done.
Action: Address the above points

Authors' response:
A new section has now been added in the Supplementary Information, detailing our Bioinformatics approach for the identification of human orthologues.

It is important to note here that the number of human orthologues has increased at the time of this manuscript's final revision, compared to the time when we selected the targets for the RNAi in human cells. Therefore not all presently known orthologues have been screened. Concerning the selection of human homologues that were functionally analysed by RNAi in HaCaT cells: 75 proteins were selected for having a phenotype in Drosophila and annotated human orthologues at the time of the experiments. In addition 17 positive controls for human cells (based on centrosome localisation and/or centrosome-related function in Uniprot) were included.

The confusion by Table S1 stems from the fact that the function of some of the identified proteins is distinct between Drosophila and human (as expected judging from other comparative studies between Drosophila and human cells, employing RNAi based approaches).

We have now expressed more clearly that the proteins selected for siRNA in human cells indeed were selected (a) on the basis of phenotype in Drosophila (75 targets) and (b) additionally 17 positive controls for the human system were included.

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Minor points:
1. Authors should cite the Raff lab, Stephens et al., 2009 and 2010 JCB papers on Ana3 and Ana2 respectively, at top of page 2 of intro.
2. At the top of page 3, it should be made clear that "Aur" is specifically the kinase Aurora A, not e.g. Aurora B.
3. 14-3-3-e has a shared cell cycle progression phenotype between flies and humans in Supplementary Table 1, but is not highlighted in green in the right hand panel of the table.

Authors' response:
All three "Minor points" suggestions have now been implemented in the manuscript.
Point (3): The overlap is recorded as a shared cell cycle progression phenotype in the left hand table of the new Supplementary Table S3 in the data sheet "phenotypes comparison". Within the class of cell cycle progression SL2 have a "sub-G1" phenotype, HaCaT cells an "over G2" phenotype after 14-3-3-e knock down.

Referee #3 (Remarks to the Author):

In this manuscript Muller and colleagues present a functional proteomic screen towards the characterization of the Drosophila centrosome. By performing mass spectrometry (MS) on immunopurified centrosomes, the authors identified about 260 proteins that are enriched in centrosome preparations. Using an RNAi interference approach in conjunction with GFP-tagging and localization they identify 27 centrosome/spindle proteins with potentially interesting functions in the regulation of centrosome biogenesis and mitotic spindle assembly. This is a nice paper and a tremendous amount of work. Less impressive however, is the rather meager downstream characterization of the most promising candidates. The inclusion of such data would greatly increase the impact of this manuscript.

Authors' response:
It is indeed "the tremendous amount of work involved" in the primary functional characterisation of all identified Drosophila proteins and selected human homologues (altogether over 400 proteins), that precluded even further characterisation of all interesting molecules before submission of this manuscript. There is absolutely no doubt that much more work will be required, by us and others, in further analysis.

We have now expanded the downstream characterisation for eIF-4a, one of the most interesting findings, as outlined below. Two of the new experiments are performed, in fact, in response to specific suggestions of this referee (see also our response to the penultimate referee point, below).

1. (new in the revision) Total -tubulin level does not change, following eIF-4a depletion, although this treatment completely abolishes centrosomal localisation of -tubulin.
2. (new in the revision) Antibody labelling of eIF-4a depleted cells with a number of core centriolar markers suggests that localization of all tested centriole markers is unaffected, while -tubulin is displaced from the centriole.
3. (new in the revision) Expression of GFP-fusions of centriolar proteins for which we had no antibodies available was employed to analyse the effect of eIF-4a depletion in SL2 cells for an additional number of core centriolar proteins. This also supports the fact that eIF4-a inactivation results in PCM depletion leaving centriolar markers unchanged.

These new data are incorporated in Fig. S4 & S5. Please see also response to referees 1 & 2.

Mass spectrometry was obviously at the origin of this work. Surprisingly there's no mass spectrometry data included in the manuscript as far as I can tell. It is therefore difficult to understand what was considered specific or background during the purification/MS process.

Authors' response:
The MS data are now included in the new Supplementary Table S1. The background (identified in the preimmune antibody mock purification control) is clearly indicated in the MS list of Supplementary Table S1 ("MS data mock isolation" sheet) and, additionally, in Supplementary

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Table S3 ("Drosophila SL2 phenotypes" sheet, column E). We took this opportunity to update and completely reanalyse the obtained MS data leading to the detection of 4 more previously known (localized) centrosomal proteins while removing 9 less relevant proteins.

Andersen and colleagues used a similar procedure 7 years ago to define the human centrosome proteome. Although this is not a problem by itself, I'm a little surprised the two datasets were not compared further. RNAi data from different screens have been compared but not the proteomic data.

Authors' response:
A direct comparison between the two datasets is presented in Supplementary Table S2. Moreover, we have now carried out a detailed comparison, for additional clarity, which is presented in Supplementary Table S2. Finally, a detailed discussion of the two datasets in two sections of Results & Discussion has been added (please see under headings: "Identification of Ö" and "Highest functional conservationÖ").

An interesting aspect of this work could have been to build on their exciting immunopurified centrosome MS data and perform tandem-affinity purification on the top candidates. This would potentially provide some mechanistic insights on the likely role of the proteins indentified and localized to specific cellular locales.

Authors' response:
We do agree that such TAP experiments would provide significant mechanistic insight on the centrosomal protein networks. But, similarly to the response to the first point, such experiments would constitute a whole new project (and a manuscript to describe them) in itself; work that is really beyond the scope of this manuscript.

It is very difficult to appreciate the localization of new centrosome proteins on centrosomes based on the data presented in Figure 2. For example I can't see any Nup153 or Lam co-localizing with gamma-tubulin. The other 6 you can barely discern colocalization with centrosomes. Insets would be required in all cases to better visualize things. The same is true for the TAP data presented in Figure S2 and Abs in S3.

Authors' response:
We have now corrected this, by incorporating higher magnification inserts in Figure 2, including Nup153 and Lam that localise to both centrosome and spindle.

I was under the impression work from the Raff lab suggested that Dm-Spd2 was not required for centriole duplication. The data in Fig 3 suggests it has the same phenotype in SL2 cells and that it is required for duplication... This should be discussed.

Authors' response:
We have now taken up this point in the text, discussing the results from the Raff and Gatti labs and comparing them in more detail to the results presented here.

In Figure 4, the authors show that gamma-tubulin is not recruited to centrosomes. This data is potentially interesting but it would be nice to see if any centrioles are present in these cells. If so it would suggest that PCM recruitment is impaired in eIF-4a RNAi treated cell. Levels of gamma-tubulin should also be tested to rule out localization versus protein stability/overall levels.

Authors' response:
We have now performed both parts of this important experiment. Total -tubulin level does not change, following eIF-4a depletion, although this treatment abolishes centrosomal localisation of -tubulin (Figure 4). Second, centrioles are indeed present in these cells. Labelling of eIF-4a depleted

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cells with several centriolar markers suggests that localization of all tested centriole markers is unaffected, while -tubulin is displaced from the centriole (Figure S5).

Out of the 260 initially tested, 12 novels proteins with confirmed centrosome/spindle localization were identified. This seems like a relatively minor fraction. Is the conclusion from this data that the immunopurified centrosome are heavily contaminated by non-centrosomal/spindle proteins, are there problems with GFP/TAP localization or both?

Authors' response:
We have already tried to address this issue by using different tagging and antibody localisation approaches. From the 35 proteins tested, all GFP localisations (17 out of 17) could be reproduced with the TAP tag or with alternative N- or C- terminal tag fusion (Table S5).

We therefore suggest that differences between MS identification and localisation are due to inherent differences between the embryonic syncytical system, from which the centrosomes have been isolated, and the Drosophila SL2 cellular system, in which the fusion proteins were expressed.

Ultimately we will only reach a conclusion to this question when all of the components have been localised in the Drosophila preblastoderm embryo. Once more, this is a very important experiment, however it is beyond the scope of this manuscript.

2nd Editorial Decision 21 June 2010

Thank you for submitting your revised manuscript for our consideration. It has now been assessed once more by the original reviewer 2. While this referee acknowledges that the manuscript has been technically improved in response to the original set of comments, s/he still points out a substantial number of significant concerns, relating mainly to the presentation and interpretation of the results, and to the overall accessibility and flow of the manuscript. As I agree that these should be addressed before eventual publication, I am therefore returning the manuscript to you once more, for a final round of revision according to the referees' criticisms and suggestions. I am hoping you will be able to get a carefully re-revised and edited version back to us as soon as possible, and that we will then be able to eventually proceed with acceptance and publication of the study. Please let me know if you should require any further clarification in this respect.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The changes made by the authors since the initial submission have improved the manuscript, and dealt with many of the scientific comments I made in the initial review, but I still encountered many ambiguities within the text that preclude me from recommending its publication at this time.

These include:
(i) p8 and 9 - the section on GFP-tagging still does not get across the motivation for tagging. Is it to verify centrosomal localization of identified proteins (in which case, why not randomly pick?), or is

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it to get information regarding the localization of those that gave RNAi phenotypes (in which case it should be moved to after the RNAi section).
(ii) The new section on "hit-rates" (p10) is not logically presented. The hit rate between different screens should be compared against the SAME moment in time. To explain, the Hughes and Goshima papers were directly comparable: both used RNAi to identify novel genes involved in mitotic spindle formation - but one used genome-wide RNAi, while the other used RNAi againsr 83 novel microtubule binding proteins. However, they were both describing "novel genes" at the same moment of time (i.e. using what was known in databases in 2007). If the authors want to compare how effective their proteomic approach was at identifying novel centrosomal proteins, to the Goshima screen, they should include in their hit rate, those proteins that have been identified as being centrosomal since 2007 (i.e. up to the Goshima screen). This will actually increase the hit rate (probably by quite a lot). But the way it's currently described does not make sense.
(iii) I could not make sense of the numbers on p11, top paragraph. It says "RNAi-mediated depletion of 49 MS identified proteins resulted in centrosome aberrations in the form of single and/or abnormally large centrosomes, indicating malfunction of centrosome duplication and/or segregation (Table 1)". But Table 1 (and Fig 1) show 56 proteins in this category. At first, I thought this was because some of the 56 might fall into a category where they affect BOTH duplication and structure maintenance, but this turns out to include g-tub, Grip128, Grip75, ncd, Qm, Rpl14, Rpl14, Rpl27, Rpl3, Rpl,6, Rpl7A and RplS4 - so I don't know what the 49 are.
(iv) I could not find a section talking about the 28/251 which were involved in centrosome structure (logically, following Table1, this should come before those affecting centrosome duplication).
(v) The phenotype of CG7033 RNAi should be discussed in light of it encoding a predicted component of the TCP tubulin chaperone complex (Hughes et al). And it should be recognized that, contrary to the authors suggestions, it was not shown in that paper to localize to the spindle in that paper (p11 bottom).
(vi) The section on CG7033 mentions the Cycloheximide experiments which are described later in the paper. At this point, the readers should be pointed to that section, instead of repeating the experimental results twice.
(vii) The actual section on translation (p16-17) needs to be tightened up - it's really quite long.
(viii) I could not follow the second half of p18, where the comparisons of human and Drosophila orthologues are discussed. The authors say: "we analysed the knockdown effect on centrosome and cell cycle for 75 of these proteins" (i.e. human/drosophila orthologues). "We included 17 controls" (which would total 75+17 = 92). Then it says: "in total, we analysed orthologues of 94 Drosophila proteins, of which 72 were identified by mass spectrometry".

2nd Revision - authors' response 03 August 2010

Please find attached our manuscript, revised according to the referee's and your suggestions. The revisions are detailed in my point-by-point response to the referee, below.

I would like to thank you very much for the highly constructive comments and suggestions made.

Referee #2 (Remarks to the Author):

The changes made by the authors since the initial submission have improved the manuscript, and dealt with many of the scientific comments I made in the initial review, but I still encountered many ambiguities within the text that preclude me from recommending its publication at this time.

These include:
(i) p8 and 9 - the section on GFP-tagging still does not get across the motivation for tagging. Is it to verify centrosomal localization of identified proteins (in which case, why not randomly pick?), or is it to get information regarding the localization of those that gave RNAi phenotypes (in which case it should be moved to after the RNAi section).

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We have now moved the protein localisation section within the first paragraph of the RNAi section according to the referee's suggestion and in line with the fact that the major motivation of the tagging was to obtain information on the proteins identified to have a relevant RNAi phenotype.

Furthermore, we have rewritten part of the text to clarify the connection between these two experiments.

(ii) The new section on "hit-rates" (p10) is not logically presented. The hit rate between different screens should be compared against the SAME moment in time. To explain, the Hughes and Goshima papers were directly comparable: both used RNAi to identify novel genes involved in mitotic spindle formation - but one used genome-wide RNAi, while the other used RNAi againsr 83 novel microtubule binding proteins. However, they were both describing "novel genes" at the same moment of time (i.e. using what was known in databases in 2007). If the authors want to compare how effective their proteomic approach was at identifying novel centrosomal proteins, to the Goshima screen, they should include in their hit rate, those proteins that have been identified as being centrosomal since 2007 (i.e. up to the Goshima screen). This will actually increase the hit rate (probably by quite a lot). But the way it's currently described does not make sense.

We have implemented the suggestion of the referee. We have explained in the text the motivation for referring to the status of 2007 for comparative reasons and we have changed the comparison to reflect the numbers from the databases and literature of the year 2007.

(iii) I could not make sense of the numbers on p11, top paragraph. It says "RNAi-mediated depletion of 49 MS identified proteins resulted in centrosome aberrations in the form of single and/or abnormally large centrosomes, indicating malfunction of centrosome duplication and/or segregation (Table 1)". But Table 1 (and Fig 1) show 56 proteins in this category. At first, I thought this was because some of the 56 might fall into a category where they affect BOTH duplication and structure maintenance, but this turns out to include g-tub, Grip128, Grip75, ncd, Qm, Rpl14, Rpl14, Rpl27, Rpl3, Rpl,6, Rpl7A and RplS4 - so I don't know what the 49 are.

The number 49 in the text is correct, as it only refers to a subgroup of centrosome duplication/segregation phenotypes that display large and/or one centrosome. In contrast, Table 1 refers to all centrosome segregation phenotypes including overreplication phenotypes and mixed phenotypes. For clarity, we have now modified the main text to refer to the 56 targets, as listed in Table 1, explaining that they include the additional classes of segregation/duplication phenotypes.

(iv) I could not find a section talking about the 28/251 which were involved in centrosome structure (logically, following Table1, this should come before those affecting centrosome duplication).

The section on centrosome structural aberrations was the penultimate one of the Results. According to the referee's suggestion, we have moved this section up; it has now become the 3rd section of Results. In this way, the 28/251 putative structural proteins are introduced; this is immediately followed by the section on different classes of structural phenotypes. This re-structuring indeed provides a more logical flow of the text.

(v) The phenotype of CG7033 RNAi should be discussed in light of it encoding a predicted component of the TCP tubulin chaperone complex (Hughes et al). And it should be recognized that, contrary to the authors suggestions, it was not shown in that paper to localize to the spindle in that paper (p11 bottom).

We have now extended the discussion on CG7033 as a TCP chaperonin component, in the context of the relevant literature (Hughes et al. 2008, Monzo et al. 2010). We have corrected the reference on CG7033 localisation and we have now annotated CG7033 as a new spindle-localised component, also in the respective annotations of Figure 1, Table 2 and Table S3.

(*vi) The section on CG7033 mentions the Cycloheximide experiments which are described later in the paper. At this point, the readers should be pointed to that section, instead of repeating the experimental results twice.*

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It is not possible to avoid referring to the cycloheximide experiment twice as we are discussing the effect of proteins of the protein translational machinery twice: in the section of centrosome structure and in the section of centrosome segregation. However, we have now reduced the text in the second section, to avoid redundancy.

(vii) The actual section on translation (p16-17) needs to be tightened up - it's really quite long.

We have shortened the section. Moreover, we have now explained more clearly the potential relevance of mitotic regulation of translation in the context of centrosome phenotypes.

(viii) I could not follow the second half of p18, where the comparisons of human and Drosophila orthologues are discussed. The authors say: "we analysed the knockdown effect on centrosome and cell cycle for 75 of these proteins" (i.e. human/drosophila orthologues). "We included 17 controls" (which would total 75+17 = 92). Then it says: "in total, we analysed orthologues of 94 Drosophila proteins, of which 72 were identified by mass spectrometry".

The numbers 92 and 94 are both correct but the associated description was obviously confusing. The relevant text has been reorganised and rewritten. The numbers are explained below:

(1) Of the 94 human orthologues of Drosophila proteins, 92 were analysed in HaCaT, while 2 additional ones (spd-2 & PERQ1) were analysed only in U2OS cells. This information can be found in Supplementary Table 3.

Therefore, the sentence "In total we analyzed the human orthologues of 94 Drosophila proteins".

(2) The 92 analysed in HaCaT are
- 75 orthologues of Drosophila proteins that had a phenotype in SL2 cells and
- 17 orthologues of Drosophila proteins that had no phenotype in SL2 cells

For the purposes of the RNAi analysis in human cells, true controls were considered those that showed no phenotype in SL2 cells. Therefore, the sentence "… by short interfering RNA (siRNA) mediated silencing in human HaCaT cells. We analyzed the knockdown effect on centrosome and cell cycle for 75 of these proteins. In addition, we included 17 controls."

(3) We analysed in human cells the orthologues of
- 76 Drosophila proteins that had a phenotype in SL2 cells, of which 62 were MS-identified and 14 were controls
- 18 Drosophila proteins that had no phenotype in SL2 cells, of which 9 were MS-identified and 9 were controls

For clarity, we have now removed this classification, as confusing and superfluous, given that the corresponding information is included in Supplementary Table 3. We have completely re-written the text to include only 3 main numbers that are derived as follows:

(i) 94 orthologues of Drosophila proteins were analysed (76 +18, see point 3), of which
(ii) 71 were MS-identified (62+9, see point 3) and
(iii) 23 were SL2 cell controls (14+9, see point 3).

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