EMBO reports

Disentangling the molecular determinants for Cenp-F localization to nuclear pores and kinetochores

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Transaction Report:

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

1st Editorial Decision 2 August 2017

Thank you for the submission of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, referees #1 and #2 support the publication of your paper in EMBO reports. Nevertheless, they have a number of concerns and/or suggestions to improve the manuscript, which need to be addressed. In particular, we ask you to show experimentally that the Nup133-interacting region is indeed a coiled-coil element (point 1, referee #1).

Referee #3 is more critical and states that the conceptual advance of the study is limited, and that the manuscript in its present form should not be published in EMBO reports. However, after cross-commenting this referee pointed out that to warrant publication in EMBO Reports the effect/phenotype of Bub1/Nup133 binding-disrupting mutants of CENP-F (such as chromosome segregation defects) should be analyzed and shown (even without going into the molecular mechanisms of these phenotypes). I think this is a good suggestion, and I would ask you to address this in the revised manuscript, in addition to the other points of referee #3.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in a point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

Please add scale bars to all microscopic images.

Supplementary/additional data: The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

This manuscript provides a wealth of information about the interactions between Cenp-F and two of its partners, Nup133 and Bub1, and relates these interactions to the function of Cenp-F at both nuclear pores and kinetochores. A powerful feature of this work is the ability of the authors to engineer Cenp-F mutants that impair the binding to one partner while retaining binding to the other, thus providing strong evidence that the mutants have not simply altered the overall conformation of Cenp-F. However, mutations in Nup133 do not have these robust internal controls and so are not quite so convincing. Similarly, although it is likely that the mCenp-F SID region being investigated forms a coiled-coil, direct experimental evidence for this (and for the disruption of the coiled-coil by Gly mutants) is lacking. Irrespective of these minor criticisms, the authors do employ their convincing mCenp-F mutants to probe function in vivo and provide compelling evidence to indicate that the interactions with kinetochores and with nuclear bodies are separable and that Bub1 functions to tether Cenp-F to kinetochores.

1. Although sequence analysis indicates very strongly that regions of Cenp-F will form coiled-coils, there does not appear to be direct experimental evidence to support this hypothesis (molecular dynamics is not experimental evidence). The references given (refs 9 and 10) again simply rely on sequence analysis. It should not be difficult to show that the mCenp-F region does form a coiled coil (eg using crosslinking, CD, on dynamic light scattering) and this information would bolster the manuscript. Similarly, the L2688G+L269G mutations may well disrupt the coiled-coil but direct experimental evidence is not provided. This should be easily obtained and should be added.

2. Although the ability to engineer mCenp-F mutants that impair binding to one partner while retaining binding to the other is an extremely powerful control to eliminate the possibility that the mutation has introduced a conformational change in Cenp-F, the same cannot be said about all of the Nup133 mutants constructed. It would be helpful to have a control for the Nup133 mutants that showed that some other interaction or function was retained.

3. The generation of compensating mutations in mCenp-F SID and Nup133 involving the salt bridge between them is very powerful. It might also be possible to use a water-soluble carbodiimide (eg EDC) to cross-link these residues (and confirm using mass spec).
Referee #2:

Berto et al. report on a study of Cenp-F, a ~300 kDa protein with multiple functions in different cellular processes. In particular, the study is about the recruitment of Cenp-F to the kinetochore and the nuclear pore complex (NPC), respectively. The authors can convincingly show that two distinct areas of the protein are required for each function. The NPC interaction with Nup133 is mapped down to the residue level, on both interaction partners, using structure modeling and mutational analysis. The interaction with the kinetochore, is mediated via Bub1, and the distinct region of Cenp-F that establishes the contact is also revealed in this study.
The experiments are carefully planned, executed, and evaluated.
As a result of this study, one can now target the individual functions of Cenp-F in kinetochore and NPC biology.

I have only two minor suggestions:

1.) While I understand that mNup133 can only be modeled, the human structure is so close in sequence that one can safely assume that the helices 1 and 2/3 will be the same. Therefore, these regions should not be referred to as 'loops', but rather as helices or 'predicted' helices.
2.) To include a sequence alignment in Figure EV3 would be helpful to find the conserved residues that are part of the Cenp-F interface, i.e. E93.

Referee #3:

This manuscript by Berto et., reports the dissection of intermolecular interactions responsible for the Nuclear Pore/Nuclear Envelope and kinetochore localization of CENP-F, a multifaceted protein implicated in cancers. While CENP-F Nuclear Envelope (NE) localisation (mediated by Nup133) is required for dynein/dynactin recruitment to the NE, its kinetochore localization (dependent on several kinetochore associated proteins Bub1, CENP-I on Zwint1) is suggested to be involved in microtubule binding. However, the precise cellular function of CENP-F is still debated.

Here, the authors have used in silico modelling and yeast-two-hybrid assays to delineate the interaction surfaces involved in the intermolecular recognition of Nup133 and Bub1 by CENP-F. Their results suggest that CENP-F binds an alpha helical segment of Nup133 via one of the two leucine zipper modules present in its C-terminus. Mutations within this leucine zipper selectively perturbed its binding to Nup133. Likewise, mutation of a conserved cysteine within a neighbouring leucine zipper selectively perturbed CENP-F binding to Bub1. These observations suggest the involvement of two distinct CENP-F surfaces for its NE and kinetochore localization. Analysis of the cellular localization of various modelling-guided mutants of CENP-F strengthen this notion, thus providing insights into the molecular determinants of NE and kinetochore localization of CENP-F.

In my opinion, this work with some additional biochemistry (see below) will be suitable for a specialised journal. Unfortunately, due to its limited conceptual advance I am not convinced that this work warrants publication is the EMBO Reports.

Suggestions:
Overall, many conclusions drawn in this manuscript could be further strengthened by validating the key interactions using recombinant proteins

- CENP-F CID domain (aa 2663-2706) was modelled as parallel coiled-coil: it would be good to show that this domain can indeed form a dimer in vitro using Size Exclusion Chromatography analysis or/and SEC-MALS (by determining the molecular weight).
- Likewise, purified recombinant CENP-F CID domain with L2668G/L2696G and L2681E/L2683E mutations should be analysed separately in SEC and/or SEC-MALS. This will demonstrate if these mutations disrupt the coiled-coil structure of CENP-F CID domain.
- Biochemical validation (affinity pull down or/and SEC analysis) of wt and mutant CENP-F

interactions with Nup133 and Bub1 will significantly strengthen this manuscript.
- The fact that the R2687E mutation in full length CENP-F did not affect its ability to interact with Nup133 necessitates a more thorough analysis - also worth evaluating the ability of Nup133 C-terminal domain to bind CENP-F.

1st Revision - authors' response 31 January 2018

Referee #1:

1. Although sequence analysis indicates very strongly that regions of Cenp-F will form coiled-coils, there does not appear to be direct experimental evidence to support this hypothesis (molecular dynamics is not experimental evidence). The references given (refs 9 and 10) again simply rely on sequence analysis. It should not be difficult to show that the mCenp-F region does form a coiled coil (eg using crosslinking, , CD, with recombinant peptides or dynamic light scattering) and this information would bolster the manuscript. Similarly, the L2688G+L269G mutations may well disrupt the coiled-coil but direct experimental evidence is not provided. This should be easily obtained and should be added.

To answer to this key point, also raised by reviewer 3, we have used two complementary approaches;
- A crosslinking method, using HeLa cells transiently transfected with HA-mCenp-F-Ct2 [aa 2655-2860], either WT or bearing the L2688G/L269G mutations, or as additional control, the L2681E/L2683E mutations. **As now shown in Fig EV2C**, *in vivo* crosslinking with DSS induced the appearance of a band migrating at about 60 kDa (consistent with the size of a HA-mCenp-F-Ct2 dimer) in the HA-mCenp-F-Ct2-WT and -LE/LE-expressing cells, but not in HA-mCenp-F-Ct2-LG/LG expressing cells. Note that we initially reached similar conclusions upon crosslinking of cells expressing the GFP-mCenp-F-Ct2 vectors. However, in that case a diffuse band appearing in all conditions following crosslinking somehow hindered the result, and we thus have subcloned the WT and mutant form in the HA-tag-containing vector, that gave cleaner results.
- In parallel, we have performed SEC-MALS experiments (as suggested by reviewer 3) using synthetic peptides restricted to the mCenp-F-miniSID sequence that was used for modelling [aa 2663-2706]. As now shown in **Fig EV2D**, this study revealed the propensity of these short WT and L2681E/L2683E mutant peptides to dimerize. We observed, notably for the WT peptides, that the dimeric forms were strongly stabilized at higher salt concentration (Figure **EV2D,b**). This trend is consistent with the high isoelectric point of the studied WT peptide (pI=9) that features a majority of positively charged residues, which induce repulsive electrostatic forces counteracting the stability of the coiled-coil. Note that in the larger mCenpF-SID fragment, there are already two more acidic residues (pI=8.1) likely to reduce the electrostatic repulsion, as would be the case in the context of the full-length Cenp-F protein. The introduction of two acidic residues in the L2681E/L2683E probably stabilizes the dimeric form by reducing the strength of this electrostatic repulsion. In contrast, the molar mass of the L2668G/L2696G mutant is consistent with a monomeric form only, whatever salt concentration, indicating that this mutant lost its ability to dimerize.

2. Although the ability to engineer mCenp-F mutants that impair binding to one partner while retaining binding to the other is an extremely powerful control to eliminate the possibility that the mutation has introduced a conformational change in Cenp-F, the same cannot be said about all of the Nup133 mutants constructed. It would be helpful to have a control for the Nup133 mutants that showed that some other interaction or function was retained.

We agree that some mutations or deletions within a ß-propeller may lead to major conformational changes. Indeed, as mentioned in the manuscript, the replacement of helices a-1 and a-2/3 by short Gly-Ser-linkers (GGSG and GSGSG, respectively) likely impaired the folding of the mutant proteins, ultimately resulting in their instability explaining their very low expression levels in yeast (Figure EV3B,b). The fact that in contrast the other Nup133 mutant used, notably the a-1 mut and the E93R that show an impaired interaction with Cenp-F, are well-expressed as LexA fusions in yeast (Figure EV3B,b and Appendix Figure S2A,b) suggests that the overall folding is not entirely inhibited. **This point is now further strengthened in the legend to Figure EV3B**.

In the case of the E93R mutant, the fact that its interaction is impaired with mCenp-F-SID-WT, but is specifically rescued when combined with the compensatory R2687E mutation within mCenp-F-SID (Figure 2B) indicates that this mutant unlikely induces a major conformational change within Nup133-NTD.

Finally, we have tried to obtain more direct evidence of the proper function of the a-1 mut that carries 3 mutations within Nup133-NTD domain.
On one hand, when introduced **in the context of full-length mNup133 the a-1 mutation (V89D, M92D, T96D) does not impair the expression or targeting of Nup133 when expressed in HeLa cells** (Figure R1 for reviewers). However, since the N-terminal ß-propeller of Nup133 is not mandatory for Nup133 targeting to the NPC, this experiments only indicates that this mutation does not affect the folding of the whole Nup133 protein or its interaction with its main NPC tether, Nup107.

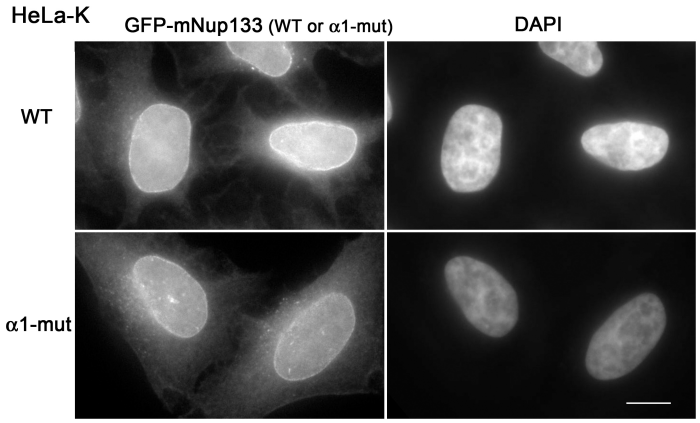

Figure R1: GFP-mNup133 a1-mut is properly targeted to the nuclear envelope.
HeLa-K cells expressing GFP-mNup133 either -WT or -a1-mut were fixed and stained with DAPI.

As a complementary approach, we have combined the a1 mutation with another mutation within Nup133 (L972E/L975E). Indeed, despite affecting Nup133 interaction with Nup107 its key targeting determinant to the NPCs), the Nup133$^{L972E/L975E}$ mutant reportedly displays a residual accumulation at the nuclear envelope that likely relies on its N-terminal domain (Boehmer et al., Mol Cell. 2008, 30: 721–731). Thereby, we aimed to visualize the reported contribution of Nup133-NTD to Nup133 targeting to the NE (Berke et al. - J Cell Biol, 2004; 167, 591-597).
However, possibly because of the competition with endogenous Nup133, it was extremely difficult to detect the NE localization of this construct, when expressed in HeLa cells. We thus performed these experiments in a CRIPSPR/Cas9-engeenered *Nup133*$^{-/-}$ mouse embryonic stem cell (mESC) line (Souquet et al., unpublished data). The data are provided in Figure R2.
Although the NPC targeting of the GFP-mNup133$^{L972E/L975E}$ fusion is largely impaired as compared to GFP-mNup133, a slight accumulation at the nuclear envelope can be observed if the WT form of Nup133 N-terminal domain (NTD) is present (WT), but not when this construct further carries a deletion of the entire NTD (DN) (Figure R2). This result is consistent with the study from Berke et al. (J Cell Biol, 2004) that suggested the existence at the NE of (yet unknown) Nup133-NTD binding sites of low affinity. The fact that in contrast**, the GFP-mNup133$^{L972E/L975E}$ further mutated within its NTD (V89D, M92D, T96D = a1-mut) is also slightly enriched at the NE** indicates that these mutations do not prevent the interaction of Nup133-NTD with its binding partner at the NE.

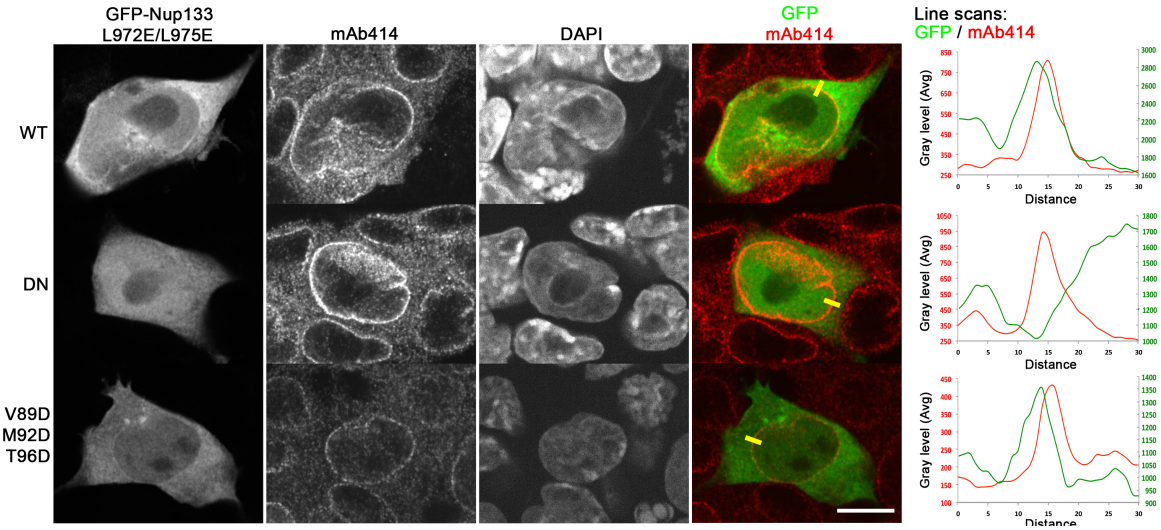

Figure R2. **The a1-mutation within mNup133-NTD does not prevent the residual targeting of Nup133 to the NE when interaction with Nup107 is impaired.**
Nup133$^{-/-}$ mESCs were transiently transfected with various plasmids, all carrying a mutant form of GFP-mNup133 (L972E/L975E) that inhibits Nup133 interaction with Nup107, its direct binding partner within the Y-complex. These constructs further bear the WT form of Nup133 N-terminal domain (WT), a deletion of its entire NTD (DN), or the a1-mutation within this NTD (V89D, M92D, T96D = a1-mut). The cells were fixed two days after transfection and immuno-labeled with mAb414 (a well-established NPC marker antibody) and DAPI. Scale bar is 10µm. The line scans (yellow lines on images, plotted from the cytoplasm towards the nucleoplasm, distances in pixels) measures the intensity of GFP-Nup133 (green lines) and mAb414 (red lines) signals at the NE.

While we are therefore confident that the Nup133 a1-mut allele retains at least some other interaction or function, we think that these data are not critical for the message of our study. Moreover, these analyses would require a lot of introduction as they were done in a different cellular model and this would rather bring confusion. We therefore prefer to keep these data for reviewers only.

3. The generation of compensating mutations in mCenp-F SID and Nup133 involving the salt bridge between them is very powerful. It might also be possible to use a water-soluble carbodiimide (eg EDC) to cross-link these residues (and confirm using mass spec).
We agree that besides the compensating mutation, the suggested crosslinking approach would have been a valuable alternative to validate our model.
However, this methodology might not be so straightforward to set up. Indeed, beyond issues with false negative due to the limited reactivity of the cross-link, we expected intramolecular cross-link within each Nup133-ß-propeller and Cenp-F dimeric peptides. Our model suggests for instance a close proximity between mCenp-F E2692 and K2693; a cross-link in this region may also perturb the interface with Nup133. In addition, this strategy would have first implied to set up conditions for the *in vitro* interaction between recombinant Nup133-ß-propeller and the synthetic Cenp-F SID dimeric peptide. As mentioned above, the SEC-MALS approach was already challenging as significant dimerization for the WT Cenp-F-miniSID peptide only occurred at 0.5 or 1M NaCl, a salt concentration that would likely not be compatible with the interaction of this Cenp-F-miniSID dimer with recombinant Nup133-NTD.
Due to time constraints and the request from the editor and reviewer 3 to provide more functional data regarding the Bub1-Cenp-F interaction, we have thus not pursued this approach.

Referee #2:

1.) While I understand that mNup133 can only be modeled, the human structure is so close in sequence that one can safely assume that the helices 1 and 2/3 will be the same. Therefore, these regions should not be referred to as 'loops', but rather as helices or 'predicted' helices.
As suggested, we have replaced and modified the nomenclature. We now refer to helices a1 and a2/3 instead of loops 1 or 3 in the text and on the corresponding figures

2.) To include a sequence alignment in Figure EV3 would be helpful to find the conserved residues that are part of the Cenp-F interface, i.e. E93.
The sequence alignment used to generate the colours in the model presented in Figure EV3-A is now provided as Appendix Figure S1.

Referee #3:

Following your discussion with the editor and your joint advices, we have performed additional studies to functionally characterize the Cenp-F mutants. This has revealed the redundant contribution of Bub1 (that directly interacts with Cenp-F KT-core) and Cenp-E to the kinetochore targeting of full-length Cenp-F. These data are now presented in Figure 5 and are described in the first page of the letter to the editor.

Suggestions
Overall, many conclusions drawn in this manuscript could be further strengthened by validating the key interactions using recombinant proteins

1. CENP-F CID domain (aa 2663-2706) was modelled as parallel coiled-coil: it would be good to show that this domain can indeed form a dimer in vitro using Size Exclusion Chromatography analysis or/and SEC-MALS (by determining the molecular weight). Likewise, purified recombinant CENP-F CID domain with L2668G/L2696G and L2681E/L2683E mutations should be analysed separately in SEC and/or SEC-MALS. This will demonstrate if these mutations disrupt the coiled-coil structure of CENP-F CID domain.
As mentioned above (answer to reviewer 1, point 1), and following your advices, we have performed SEC-MALS experiments on these 3 synthetic peptides. This analysis demonstrated that, consistent with our model, the L2668G/L2696G, but not the L2681E/L2683E mutation, disrupts the coiled-coil structure of Cenp-F SID domain.

2 Biochemical validation (affinity pull down or/and SEC analysis) of wt and mutant CENP-F interactions with Nup133 and Bub1 will significantly strengthen this manuscript.
As indicated in the answer to point 3 of reviewer #1, our SEC-MALS studies of the WT and mutant Cenp-F peptides has revealed that *in vitro,* the synthetic Cenp-F-miniSID WT dimer is rather unstable under physiological conditions and was therefore unlikely to be worth testing in combination with recombinant Nup133-NTD.
In respect to the interaction between Cenp-F and Bub1, as mentioned to the editor, Andrea Musacchio contacted us following the presentation of these data at an international meeting. His team has spent time to set up biochemical assays to validate *in vitro* the interaction between Cenp-F and Bub1 and was planning to submit a manuscript reporting this dataset. To avoid repeating already performed experiments, we have therefore decided not to develop this biochemical approach and rather focus on the functional experiments requested by the editor following your suggestions.

3. The fact that the R2687E mutation in** full length **CENP-F did not affect its ability to interact with** full length **Nup133 necessitates a more thorough analysis - also worth evaluating the ability of Nup133 C-terminal domain to bind CENP-F.
To evaluate the ability of mNup133 C-terminal domain [aa 501-1155] to bind to the WT or R2678E mutant form of mCenp-F SID, we performed Y2H assays using, in addition to full-length mNup133 [aa 1-1155], a novel mNup133 C-terminal domain vector [aa 501-1155]. However, this construct was transactivating when used in -LWH medium. Under conditions required to prevent this transactivation (i.e., addition of 1 mM 3-Amino-Triazole), the interactions of Nup133-Cterm with its established C-terminal partner, Nup107, was preserved while no interactions was detected between Nup133-Cterm and mCenp-F SID whether WT or R2687E. This experiment, now provided as **Figure EV4B**, is consistent with our previous studies using human Nup133 constructs that indicated that the C-terminal domain of Nup133 does not interact with Cenp-F (Bolhy et al., 2011). Note also that unlike WT mCenp-F SID, the interaction of the R2687E mutant with full-length mNup133 was no longer observed upon addition of 1 mM 3-Amino-Triazole, indicating that the $^{mCenp\text{-}F}$R2687E mutant may interact with the full-length protein in a rather weak manner. This interaction is however sufficient to allow the GFP-mCenp-F-Ct2-R2687E fusion construct to interact with

Nup133 *in vivo*, as revealed by its accumulation in GLFG bodies (the latter result, previously integrated in Fig 3, has have now been combined with the Y2H data in Figure EV4, C).

We have therefore modified the manuscript (page 9), to clarify the fact that this mutation within mCenp-F C-terminal domain likely leads in fact to a non-specific (and thus, not worth studying) interaction with mNup133 "Note however that a likely non-specific interaction was observed when the $^{mCenp\text{-}F}$R2687E mutant was assayed against full-length mNup133 (Fig EV4)". In addition, to clarify the main message, we have combined these non-critical data into Fig EV4.

2nd Editorial Decision 20 February 2018

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the referees that were asked to re-evaluate your study (you will find enclosed below). As you will see, referees #1 and #2 now support the publication of your manuscript in EMBO reports. Referee #3 is still critical, and feels that the manuscript lacks the level of conceptual advance required for EMBO reports, although he also sates that the manuscript improved significantly in terms of the technical quality. After further correspondence with the referees, we think, however, that the manuscript in its present form is suitable for publication in our journal.

Before we can proceed with formal acceptance, I have the following editorial requests:

As indicated by referee #1, it would be important to cite the work by Andrea Musacchio and the results related to the present work (mentioned in your point-by-point response) in your manuscript. You indicated in our correspondence that the manuscript will be uploaded to BioRxiv. If this is the case, or will happen, please cite this accordingly and include it into the reference list. See:

http://embor.embopress.org/authorguide#referencesformat

The format should be: 1. Author NAME1, Author NAME2, (YEAR) article title. bioRxiv doi.

Further, in several figures you introduced sub-labels (i.e. Fig. 2, 5, EV2, EV3, EV5), e.g. Fig. 2Aa. This is rather confusing, and we ask you to change this, e.g. using (i), (ii) and (iii). Please change this in the figures, the legends, and in all the call-outs related to these panels in the manuscript text.

Fig. EV3B is called out before EV3A, please change the order in the figure, and also change this in the legend and the related call-outs in the manuscript text.

Appendix Fig S2 is only called-out in the figure legends. Can a call-out be added to the main text?

Finally, please find attached a word file of the manuscript text with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address.

We now strongly encourage the publication of original source data, in particular of Western blots, with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure. of about 400 pixels) that can be used as visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The authors have made a good attempt to address my crticisms. Although technical problems have frustrated some aspects, I think that overall the work is now suitable for publication in EMBO Reports. It might be helpful to other readers if Dr Musacchio could perhaps provide a personal communication regarding his work or, if it has now been published, a reference could be included.

Referee #2:

The authors have adequately addressed the concerns by the reviewers. I support publication without further revisions.

Referee #3:

The revised manuscript is improved significantly in terms of the technical quality (with the addition of additional biochemistry). Unfortunately however this reviewer feels that the manuscript still lacks the level of conceptual advance required to warrant publication in EMBO reports. The additional cell-based experiments included in the manuscript only suggest that Bub1 is responsible for kinetochore targeting (although they still rely on Y2H - they could have performed IPs to show the mutant CENP-F indeed fails to interact with Bub1) but do not try to evaluate the functional consequence of disrupting the interactions involving CENP-F (which was my main suggestion for the revision). Hence unfortunately this reviewer still feels less enthusiastic in supporting the publication of this manuscript in EMBOr. I have no doubt that this work in its current form will be solid contribution in a more specialised journal.

2nd Revision - authors' response 2 March 2018

As indicated by referee #1, it would be important to cite the work by Andrea Musacchio and the results related to the present work (mentioned in your point-by-point response) in your manuscript. You indicated in our correspondence that the manuscript will be uploaded to BioRxiv. If this is the case, or will happen, please cite this accordingly and include it into the reference list
A line has been included on page 11. Since Andrea Musacchio told me that his manuscript will be uploaded on BioRXiv in a few days and provided title and author list, I included this reference in the ref list [ref 53]. The doi will be included at the proof stage. *"Note that a direct interaction between the Bub1 kinase domain and a dimeric coiled-coil in Cenp-F C-terminal domain has been meanwhile demonstrated through biochemical reconstitution [53]. "*

We also included one line to mention the study from the Medema's lab that came out after we resubmitted the ms and that was also relevant (ref [54]).
This result was also unexpected since depletion of Bub1 or the lack of its C-*terminal tail were reported to cause an efficiently mislocalization of Cenp-F from kinetochores [30, 53, 54].*

Further, in several figures you introduced sub-labels (i.e. Fig. 2, 5, EV2, EV3, EV5), e.g. Fig. 2Aa. This is rather confusing, and we ask you to change this, e.g. using (i), (ii) and (iii). Please change this in the figures, the legends, and in all the call-outs related to these panels in the manuscript text.
This has been modified

Fig. EV3B is called out before EV3A, please change the order in the figure, and also change this in the legend and the related call-outs in the manuscript text.
We instead now call this figure earlier (page 7, line 3)

Appendix Fig S2 is only called-out in the figure legends. Can a call-out be added to the main text?
(now cited on page 6 and 16)

Finally, please find attached a word file of the manuscript text with changes we ask you to include in your final manuscript text, and some queries (comments), we ask you to address.
All the queries have been taken in account
Page 22: lines have been included to describe the Kaleidagraph plots
Page 23: we have changed the color of the CCAX box and included it in the legend. The part that was black uppercase has been corrected.

We now strongly encourage the publication of original source data, in particular of Western blots, with the aim of making primary data more accessible and transparent to the reader.
Source data have been provided for the western blots for which only part of the gel was presented (4 pdf files have been provided corresponding to panels presented in figures EV2, EV3, and Appendix figures S2 and S3).

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: Valérie DOYE
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2017-44742V1

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	No statistical methods were used to predetermine sample size; (n) values were chosen in accordance with standard practices in kinetochores analysis in mammals and are indicated in the corresponding figure legend of the main figure or in appendix table S1 reporting the whole dataset used in the statistic.
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?	The inclusion / exclusion criteria used for the experiment in figure 4B are described in appendix table XX reporting the whole dataset used in the statistic.
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.	NO
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.	The investigators were not blinded to allocation during experiments and outcome assesment
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, (see the field Below)
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	To do the statistic of the figure 4B we used the Wilcoxon test provided by the KaleidaGraph software.
Is there an estimate of variation within each group of data?	NO
Is the variance similar between the groups that are being statistically compared?	The test used in this situation (Wilcoxon) allows to compare groups without make any assumption about the distribution.

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://jjj.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).	All antibodies used in this study are listed in the "Western blot analyses" and "Immunofluorescence microscopy" sections of the Materials and Methods chapter. For commercial antibodies, catalog numbers are provided. For the other antibodies, the publication in which they were first described and characterized is provided. For the rat monoclonal anti-mouse
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The sources of the cell lines used in this study is indicated in the Method section.

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

D- Animal Models

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

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	All plasmids used in this study are listed in Table S2 where it is indicated that their sequences are available upon request. -- RAPHAEL --
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).	RAPHAEL ??
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	Fiji IJ1 macro code for kinetochore quantification is provided in appendix figure S5. The modelling of the proteins is described in method section. RAPHAEL ??

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No