EMBO *reports*

FBXL13 directs the proteolysis of CEP192 to regulate centrosome homeostasis and cell migration

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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 4 August 2017

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings and consider in particular the biochemical experiments demonstrating that Cep192 is a target of Fbxl13 solid. However, all referees are also concerned that the functional link to cell migration is not very strong. While I appreciate that the main focus of the paper is on the identification of a novel substrate for Fbxl13, I think that the data on the functional role of Cep192 ubiquitination have to be strengthened to some extent before the study becomes suitable for publication in EMBO reports. Referee 2 suggests to perform rescue experiments by co-depleting Fbxl13 and Cep192 and along these lines, referee 1 proposes to test if the effect on cell migration depends on the catalytic activity of Fbxl13. Referee 3 indicates that microtubule regrowth assays under Fbxl13 overexpression conditions would strengthen the functional significance of the Fbxl13-Cep192 axis.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the 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, please ensure to specify also the test used to calculate p-values in the respective figure legends.

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.

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REFEREE REPORTS

Referee #1:

Here, Fung E and colleagues show that Fbxl13 associates with Centrin-2, Centrin-3, CEP152 and CEP192, and localizes to centrosomes. Furthermore, they show that Fbxl13 directly interacts with CEP192 at its N-term, and regulates its degradation via ubiquitylation and the proteasome. Through controlling the level of CEP192, the authors propose that Fbxl13 controls levels of centrosomal MT arrays and in turn regulates cell migration. Together, they concluded Fbxl13 as a novel regulator of MT nucleation activity and may have a potential role in cell migration. The biochemistry is the strongest aspect of the manuscript but the data supporting biological significance of Fbxl13-mediated ubiquitylation of CEP192 is not very strong and conflicts with previous work from other groups. I have the following concerns

Major concerns:

- The authors show that Fbxl13 depletion causes a decrease in cell migration in wound healing assays. The effect is rather small and is not consistent with published work from Sharp and Pellman linking higher levels of CEP192 to increase migration. This casts significant doubts on the biological significance of Fbxl13 mediated regulation of CEP192 levels. That aspect of the manuscript needs to be streghteened.

- The authors should demonstrate that Fbxl13 function in cell migration is dependent on catalytic activity in rescue experiments.

-The peptide count of CEP152 is low in both Fbxl13 isoform 1 and 3. The author need to include a more detailed, quantitative explanation about how they subtract false-positive pick their hits (in addition to filtering with the CRAPome).

-CEP192 was not a hit in the Fxbl13 LC-MS/MS, therefor there is a logical gap as to why CEP192 was chosen as a substrate of Fbxl13. For instance, CEP152 interacts with CEP63, CPAP or Plk4 as well, are these all potential substrates of Fxbl13? There's a stronger proteomic link between duplication and Fbxl13 yet there is a very slight defect on duplication.

-The author should show that depletion of CEP152 in cells does not alter Fbxl13 association with CEP192 to further prove that in vivo, Fbxl13 recruits CEP192 independently to CEP152.

- The authors should quantify the cycloheximide chase experiment (Fig 4D). The representative image shows a slower degradation of both CEP192 and Centrin 2 in the presence of exogenous Fbxl13.

- The authors concluded that RNAi-mediated Fbxl13 increased CEP192 (Fig EV3) and in turn slightly increased the proportion of U-2OS with more than two centrosomes from 2% to 4%. This is

rather a small increase, and could be due to indirect effect of knockdown E3 ligase function. More importantly, the author did not show if centrosomal pool of CEP192 increased (by IF) under Fbxl13 knockdown. This seems like an important experiment.

-Did author investigate g-tubulin recruitment during M-phase since CEP192 is required for centrosome maturation? Is there a mitotic role for Fbxl13?

Minor concerns:

Fig 1:
-Since the Flbx13 antibody is working well in WB, did author try to look the endogenous Fbx13l localization?

Fig 3:
-Soneen et al 2013a and Soneen et al 2013b are same paper.

Fig 4:
- The authors should discuss the decreased level of CEP192 isoform2 in Fbxl13 RNAi treated cells? (Fig 4E)

Fig 5:
-InFig5A, the input of CEP192 aa 1-630 (myc) in lane 4 and lane 5 are much lower than control. These two lanes are cell treated with siFbxl13, and one would in principle expect higher expresions level of CEP192 aa 1-630 when its E3 ligase is downregulated. Moreover, the much lower expression level of CEP192 aa 1-630 may influence IP efficiency making it more difficult to pull down Ubiquitin. This needs to be addressed.
- The authors show that the ΔF mutant of Fbxl13 binds to CEP192 but not Skp1. However, overexpression of this mutant causes a significant decrease of CEP192 aa 1-630 (Fig5E, input lane4), and ubiquitination of CEP192 (Fig5E, IP lane4). The authors concluded that a functional SCF complex is required for efficient CEP192-3 polyubiquitylation. I don't see much evidence for this based on the data shown and this issue needs to be addressed.

Fig 6:
-Since Fbxl13 binds to both CEP192 and CEP152, it will be important to investigate its effect on Plk4 if one would like to propose it functions in centrioles duplication. In Fig 6D, the difference between control and Fbxl13 treated cells are small. The author used unpaired T-test to compare two sets, however in this case the distribution of g-tubulin should be rather random. A nonparametric Mann-Whitney test may be more suitable in this case.
- The authors should comment on why only 25% of wild type cells have centrosomal MT arrays?
-Fig 6G, the difference looks rather small and likely is not biologically significant.

Referee #2:

This manuscript authored by Fung et al. titled Fbxl13 directs ubiquitin-mediated proteolysis of Cep192 to regulate centrosome homeostasis and cell migration is an well planned out and executed study elucidating Cep192 as one of the first substrates for Fbxl13 and sheds light on the role of this pathway in regulating centrosome duplication and cell migration, tying it to potential implications in tumorigenesis and metastasis. The authors performed a screen using two different isoforms of Fbxl13 in which they identified a number of factors that are involved in centrosome biology leading them to Cep192 (which didn't appear to be isolated in the initial screen) being a target of Fbxl13-mediated ubiquitination. The authors then went on to show that Fbxl13 functions to regulate cell migration that they suggest is due to regulation of microtubule nucleation/arrays and centrosome duplication through controlling Cep192.

While this reviewer thoroughly enjoyed the manuscript, and felt that it is well organized and the data that is presented is well controlled and convincing, providing novel insight into an F-Box protein that previously had no known target, there are a few shortcomings that should be addressed that would strengthen the conclusions before this manuscript would be acceptable for publication.

Major concerns:

1. The study is largely utilizing overexpressed proteins. The conclusions would be strengthen if the authors could put more emphasis on assessing endogenous interactions (rather than overexpressed or semi-endogenous) and regulation of the ubiquitination of endogenous Cep192 by Fbxl13 (as many proteins can be aberrantly ubiquitinated when overexpressed).

2. While the data demonstrating Fbxl13 targets Cep192 and thereby affects centrosome biology/duplication is straightforward and convincing (keeping in mind it is largely overexpression studies). The jump to connecting the regulation of Cep192 to cell motility is less convincing. One might expect, as is the case for most substrate recognition subunits of SCF complexes, that Fbxl13 recognizes and targets for degradation multiple proteins. For instance, to make the model stronger, the authors could assess if the reduced migration of cells following depletion of Fbxl13 is reversed by co-depletion of Cep192, which would provide further evidence that the change in migration due to depletion of Fbxl13 is largely through reducing Cep192 stability rather than another potential substrate.

Minor concerns:

1. The authors should provide a more extensive explanation why they chose the 2 isoforms of Fbxl13 for their interaction screen and did not choose or use the remaining 2. Would they anticipate that they might pull out a different set of factors with the other 2 isoforms?

2. The methods used by the authors for ubiquitination assays under denaturing conditions should be more extensively described or referenced.

3. While the manuscript is fairly well written, there are some grammatical and spelling/typos scattered throughout that should be cleaned up.

Referee #3:

Fung et al. characterized the orphan ubiquitin ligase SCFFBXL13. Starting from an unbiased proteomics screen for interactors of the F-box protein FBXL13, the authors identified different centrosomal proteins as interaction partners, including Centrin-2, Centrin-3, CEP152, and Cep192. Among these, only Cep192 is shown to be a specific ubiquitylation-substrate of FBXL13, while the interaction with Centrin-2 and Centrin-3 localizes FBXL13 to the centrosomes. Functional studies demonstrate a role of this new degradation pathway in the regulation of microtubule nucleation activity and cell motility.
Overall, this is a very interesting paper which reports important findings, including the first ubiquitin-ligase for CEP192 and a role for centrosomes in controlling cell migration. The experiments are performed in a technically sound manner and support the conclusions of the paper. Therefore, I support publication of this paper in EMBO Reports and I have only minor concerns which are outlined below:
1. Standard deviations should be included in Figure 6F.
2. Figure 6G reports differences only for shFbxl13 (87). Are the difference across the samples calculated using ANOVA? What do the numbers after "shFBXL13" refer to?
3. Microtubule regrowth assays under conditions of forced FBXO13 overexpression would strengthen the data on the role of FBXL13 in regulating microtubule nucleation activity.

1st Revision - authors' response 3 November 2017

Referee #1

This reviewer appreciates the biochemistry of the manuscript, but has some concerns regarding the biological significance of FBXL13-mediated ubiquitylation of CEP192. We would like to thank the reviewer for the insightful comments and constructive criticism. We have performed the suggested experiments and we believe the conclusions of the manuscript have been strengthened. She/he asked that we address the following specific issues (italicized):

1. "The authors show that Fbxl13 depletion causes a decrease in cell migration in wound healing assays. The effect is rather small and is not consistent with published work from Sharp and Pellman linking higher levels of CEP192 to increase migration. This casts significant doubts on the biological significance of Fbxl13 mediated regulation of CEP192 levels. That aspect of the manuscript needs to be streghteened."
First, please note that the effect of depleting FBXL13 on cell migration is significant and can be obtained upon depletion of FBXL13 by both siRNA (Figure 7A, B, C) and shRNA (Figure EV4D, E, F). This significant reduction in cell migration mediated by either removal of FBXL13 trough siRNA or shRNA can be rescued by expressing FBXL13. Moreover, we show in the updated version of the manuscript that the migration deficit elicited by siRNA of FBXL13 can be rescued by siRNA of CEP192 (Figure 7D, E, F), thus demonstrating that the migration deficit observed after FBXL13 depletion is indeed mediated by the accumulation of CEP192.

In addition, please note that the Sharp manuscript states:

"Because cell polarization is integrally linked to cell migration, we then examined whether Cep192 knockdown affected cell motility using a standard 2-D in vitro scratch assay. Our initial hypothesis was that the loss of Cep192 and resulting increase in cell polarization would increase the rate at which cells moved. However, we found that Cep192 knockdown actually significantly reduced the rate at which U2OS cells moved into the scratch zone by ~30% (Fig. 10)."

In our experimental settings siRNA of FBXL13 increases the levels of CEP192 and γ-tubulin at the centrosomes favoring the formation of centrosomal microtubules. This should correspond to less extra-centrosomal microtubules, according to Sharp findings. Less extra-centrosomal microtubules should prevent cell polarization and the ability of cells to migrate. Therefore, our findings are in accordance to the model of Sharp *et al.*, but not with their findings on the siRNA of CEP192 on cell migration. To better clarify this point, we have updated our model which is now represented in the new Figure 7G.
The discrepancy between our findings and the findings by Sharp *et al.* could be due to the fact that the authors are targeting all the isoforms of CEP192, therefore depleting the entire CEP192 pool. We, however, only depleted CEP192 containing the n-terminal extension. Depleting the entire pool of CEP192 could have a profound effect on cell survival since CEP192 is an essential gene. Of notice, to exclude effects on cell viability induced by CEP192 depletion, we performed wound-healing assays without the addition of serum.
In the manuscript by Godinho *et al.*, the authors report that CEP192 depletion prevents invasion induced by centrosome amplification. The cell lines and cell culture settings are substantially different than the ones reported here. It is difficult to compare the role of CEP192 in such different systems.
It is quite clear that CEP192 regulation is fundamental for cell migration. Our data create a framework from which to further investigate the role of CEP192 with regard to its impact on cell migration and invasion in normal and cancer cells. Our findings additionally provide the basis to alter CEP192 levels using 'physiological' modulation of CEP192 levels *via* FBXL13 dependent ubiquitylation.

2. "The authors should demonstrate that Fbxl13 function in cell migration is dependent on catalytic activity in rescue experiments."

As asked for by this reviewer, we performed rescue experiments of siFBXL13 mediated migration-decrease using either WT FBXL13 or a catalytically inactive form of FBXL13 [lacking the F-box domain and unable to bind to Skp1 (FBXL13 DF-Box)]. Indeed, we now demonstrate that FBXL13 DF-Box is unable to rescue the siFBXL13 mediated migration phenotype, while WT FBXL13 (competent to induce degradation of CEP192) readily abrogates the effect, thus demonstrating that FBXL13 function in cell migration is dependent on the catalytic activity of the SCF^{FBXL13} ligase (Figure 7A, B, C).

3. "The peptide count of CEP152 is low in both Fbxl13 isoform 1 and 3. The author need to include a more detailed, quantitative explanation about how they subtract false-positive pick their hits (in addition to filtering with the CRAPome)."

We have subtracted all the common hits present in four independent purifications from other F-box proteins such as FBXL7, FBXL17 and cyclin F as reported in Figure 1B.
All the hits reported in Table 1 are derived using the output provided by the MASCOT database. This takes into account the number of unique peptide and relative quantitation (through the emPAI The Exponentially Modified Protein Abundance Index
score). MASCOT output classifies the hits relative to their confidence of identification and abundance. Please note that CEP152 was the first centrosomal protein after Centrin-2 and Centrin-3 in the output list. For this reason, CEP152 was chosen as a promising candidate for further investigation. The low peptide numbers of CEP152 in the MS could be due to the low abundance of endogenous Cep152 compared to Centrin-2 or Centrin-3 [measured in (Bauer et al, 2016)].

4. "CEP192 was not a hit in the Fxbl13 LC-MS/MS, therefor there is a logical gap as to why CEP192 was chosen as a substrate of Fbxl13. For instance, CEP152 interacts with CEP63, CPAP or Plk4 as well, are these all potential substrates of Fxbl13? There's a stronger proteomic link between duplication and Fbxl13 yet there is a very slight defect on duplication."

Please note that there is strong evidence in the literature that demonstrates a direct interaction between CEP152 and CEP192 (Firat-Karalar et al, 2014; Sonnen et al, 2013). This is the reason why CEP192 was chosen for further investigation. We show that the interaction between FBXL13 and CEP192 is specific (Figure 2B, C, D) and direct (Figure 3E, F, G). In the same experimental conditions used to detect interaction between FBXL13 and CEP192 we did not detect interaction between FBXL13 and CPAP and, FBXL13 and PLK4 (please see Figure R1 below).

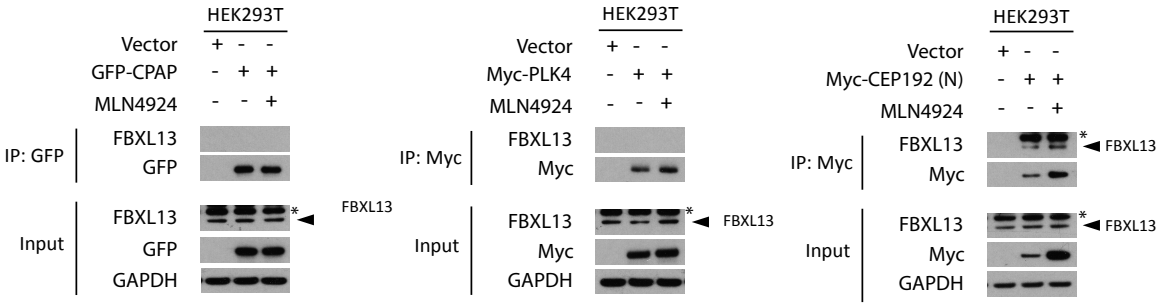

Fig. R1. Co-Immunoprecipitation studies of CPAP, PLK4 and CEP192. While CEP192 interacts with FBXL13, no interaction with CPAP and PLK4 was detectable. * denotes an unspecific band detected by the FBXL13 antibody.

We agree that there is a proteomic link between FBXL13 and centrosomal duplication. For this reason, we tested the effect of FBXL13 on centrosome duplication. However, the effect of FBXL13-alterations on centrosome overduplication was mild and was not followed up further. While it has been shown that siRNA of CEP152 and CEP192 block centrosome duplication (Sonnen et al, 2013), to our knowledge, there is no evidences that high expression of CEP152 and CEP192 induces centrosome overduplication. Indeed, centrosome overduplication and *de novo* centrosome biogenesis can be mediated by trans-auto-phosphorylation of Plk4 alone (Lopes et al, 2015).

5. "The author should show that depletion of CEP152 in cells does not alter Fbxl13 association with CEP192 to further prove that in vivo, Fbxl13 recruits CEP192 independently to CEP152."

We performed the requested experiment and now show that after depletion of CEP152 the interaction between FBXL13 and CEP192 is not affected (Fig. 3F), thus demonstrating that FBXL13 recruits CEP192 independently of CEP152. Further experiments in Figure 3 map the interaction between FBXL13 and CEP192 to a region of interaction not present in CEP152. In addition, we show interaction between FBXL13 and CEP192 *in vitro* using purified proteins, thereby further demonstrating direct interaction (Fig. 3G).

6. "The authors should quantify the cycloheximide chase experiment (Fig 4D). The representative image shows a slower degradation of both CEP192 and Centrin 2 in the presence of exogenous Fbxl13."

The expression of FBXL13 clearly induces proteolysis of CEP192, which can be rescued by treating cells with MG132, a proteasome inhibitor (Figure EV1B). The CHX experiment was started under conditions where levels of CEP192 are already reduced as a result of the high expression of FBXL13. Against this background, the reduction of CEP192 after 4 h of CHX treatment is elevated in the setting of FBXL13 overexpression (0.19 ->0.5) when compared to the control setting (1.0 -> 0.54). At the 8h and 12 h time-points, FBXL13 becomes massively destabilized under CHX conditions, which is typical for F-Box proteins as a result of auto-ubiquitylation and degradation. Thus, FBXL13 activity at these time points is reduced to nearly endogenous levels for which reason no further enhancement of degradation can be expected. As indicated by this reviewer, we now include quantification values for CEP192 in Fig. 4D.

7. "The authors concluded that RNAi-mediated Fbxl13 increased CEP192 (Fig EV3) and in turn slightly increased the proportion of U-2OS with more than two centrosomes from 2% to 4%. This is rather a small increase, and could be due to indirect effect of knockdown E3 ligase function. More importantly, the author did not show if centrosomal pool of CEP192 increased (by IF) under Fbxl13 knockdown. This seems like an important experiment."

Indeed, the effect of FBXL13-alterations on centrosome overduplication is mild and was therefore not considered as the main function of FBXL13 and the main focus of this paper. However, these mild effects are highly reproducible and not observed in the control siRNA samples nor in experiments in which another E3 ligase (FBXL17) was inactivated by siRNA.
We also tested the centrosomal levels of endogenous CEP192 by IF upon FBXL13 knockdown (Figure R2). In this experiment, CEP192 levels were unchanged, in contrast to the increased levels detected by immunoblotting. Here, the specific effect on isoform 3 of CEP192 is very likely masked by the presence of other more abundant isoforms of CEP192 at the centrosomes, which are detected by the antibody. This cross-reactivity can however not be overcome in the IF setting.

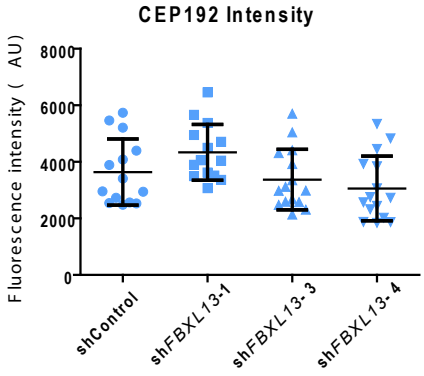

Fig. R2. Fluorescence intensity of CEP192 after shRNA of FBXL13. The fluorescence intensity of CEP192 is not affected by shRNA of FBXL13. P-value >0.05

8. "Did author investigate g-tubulin recruitment during M-phase since CEP192 is required for centrosome maturation? Is there a mitotic role for Fbxl13?"

According to our data FBXL13 suppresses CEP192. Thus FBXL13 should be reduced in mitosis to allow CEP192 recruitment. This possibility has been commented in the discussion part of the manuscript (page 14 line 3-7) and implies that FBXL13 could be cell cycle regulated. However, the investigation of the regulation of FBXL13 is not within the scope of the current manuscript that, instead, focuses on the characterization of FBXL13 as an E3 ubiquitin ligase and identifies the first ubiquitylation-substrate of FBXL13.

Minor concerns:

"Fig 1:

-Since the Flbx13 antibody is working well in WB, did author try to look the endogenous Fbx13l localization?"

Unfortunately, like with many other rabbit antibodies, the centrosomal signal generated by the FBXL13 antibody is not specific. The same signal was obtained with pre-immune serum. For this reason, we looked at the localization of Flag-tagged FBXL13, which is clearly enriched at centrosomes (Figure 2E).

"Fig 3:
-Soneen et al 2013a and Soneen et al 2013b are same paper."

We apologize for this oversight, which occurred during the generation of the reference list via endnote. This mistake has now been corrected.

"Fig 4:
- The authors should discuss the decreased level of CEP192 isoform2 in Fbxl13 RNAi treated cells? (Fig 4E)"

This effect of FBXL13 inactivation on the CEP192 isoform 2 was not repeatedly observed, making it difficult to discuss this observation with certainty. Given the function of FBXL13 as an ubiquitin E3 ligase, the effect, if any, is certainly not directly mediated by FBXL13. It is conceivable that there is a negative feedback loop caused by high levels of CEP192 isoform 3 which downregulates the expression of other isoforms.

"Fig 5:
In Fig5A, the input of CEP192 aa 1-630 (myc) in lane 4 and lane 5 are much lower than control. These two lanes are cell treated with siFbxl13, and one would in principle expect higher expresions level of CEP192 aa 1-630 when its E3 ligase is downregulated. Moreover, the much lower expression level of CEP192 aa 1-630 may influence IP efficiency making it more difficult to pull down Ubiquitin. This needs to be addressed."

Please note that these are co-expression experiments where we co-transfected cells with siRNA and cDNA. The transfection reagents used for this purpose are generally rather toxic. Under these co-transfection conditions, it is often observed that expression of proteins can vary. For this reason, we did not detect high levels of CEP192 fragments in the input. Since CEP192 is overexpressed, it is unlikely that FBXL13 siRNA has a substantial effect on CEP192 levels.
The reviewer states: "*…the much lower expression level of CEP192 aa 1-630 may influence IP efficiency making it more difficult to pull down Ubiquitin"*. We disagree with this statement since we are isolating CEP192 and not ubiquitin. Importantly, the immunoprecipitated levels of CEP192 are identical thus excluding the possibility that the difference in ubiquitylation are due to differences in CEP192 levels. If anything, low levels of CEP192 should be better ubiquitylated than high overexpressed levels.

"The authors show that the ΔF mutant of Fbxl13 binds to CEP192 but not Skp1. However, overexpression of this mutant causes a significant decrease of CEP192 aa 1-630 (Fig5E, input lane4), and ubiquitination of CEP192 (Fig5E, IP lane4). The authors concluded that a functional SCF complex is required for efficient CEP192-3 polyubiquitylation. I don't see much evidence for this based on the data shown and this issue needs to be addressed."

From this experiment, it is not possible to assess whether overexpression of FBXL13 ΔF cause a significant decrease of CEP192. It is likely that the two genes are expressed at different levels as a result of co-expression. The immunoprecipitated levels of CEP192 in the FBXL13 WT and FBXL13 ΔF lanes are nearly identical, yet the level of ubiquitylation of CEP192 is substantially decreased in the FBXL13 ΔF. Typically, some residual ubiquitylation activity can be observed when overexpressing ΔF-forms of F-Box proteins. We conclude that ubiquitylation of CEP192 is mediated by the SCF^{FBXL13} ligase.
In addition, as specified above, we also functionally demonstrate that FBXL13 ΔF-Box is unable to rescue the siFBXL13 mediated migration phenotype, while WT FBXL13 (competent to induce degradation of CEP192) readily abrogates the effect, thus demonstrating that FBXL13 function in cell migration is dependent on the catalytic activity of the SCF^{FBXL13} ligase (Figure 7A).

"Fig 6:
Since Fbxl13 binds to both CEP192 and CEP152, it will be important to investigate its effect on Plk4 if one would like to propose it functions in centrioles duplication. In Fig 6D, the difference between control and Fbxl13 treated cells are small. The author used unpaired T-test to compare two sets, however in this case the distribution of g-tubulin should be rather random. A nonparametric Mann-Whitney test may be more suitable in this case."

Although microscopy is a relatively poor quantitative method, the difference in γ-tubulin intensity is significant both using unpaired T-test and a nonparametric Mann-Whitney test as suggested. The expression of FBXL13 induces a significant decrease of γ-tubulin intensity (P<0.01) (Fig. 6D). For clarity Figure 6D results are reported below:

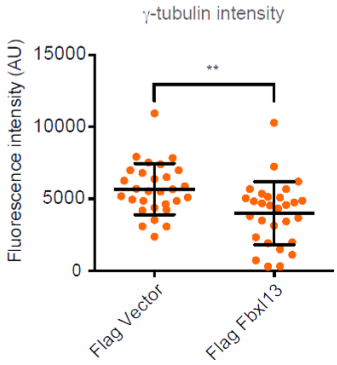

Fig. 6D. Fluorescence intensity of γ-tubulin after expression of Flag-FBXL13. The fluorescence intensity of γ-tubulin is significantly reduced using non-parametric Mann-Whitney test. U-value=220; Z-*score*=3.110;*p*-value=0.00188

"The authors should comment on why only 25% of wild type cells have centrosomal MT arrays?"

Please note that we scored only transfected cells. We have now corrected the results to quantify the differences between FBXL13 expressing cells and control cells.
We also show in a separate set of experiments that expression of FBXL13 prevents microtubule regrowth in regrowth assays (Figure EV3E, F).

"Fig 6G, the difference looks rather small and likely is not biologically significant."

The difference between control cells and cells where FBXL13 has been knocked down is clearly significant in the sample where most efficient FBXL13 knock-down is achieved, and a clear tendency is observed in the samples with lower efficiency of knock-down. g-tubulin is a central regulator of the microtubule cytoskeleton, thus also small differences are likely to be biological significant. Indeed, we show an important role of FBXL13 in modulating cellular migration trough the control of CEP192.

Referee #2

This reviewer is very positive about our study. He/she indicates: *"…this reviewer thoroughly enjoyed the manuscript, and felt that it is well organized and the data that is presented is well controlled and convincing, providing novel insight into an F-Box protein…"*. He/she asked us to address the following specific concerns:

Major concerns:

"1. The study is largely utilizing overexpressed proteins. The conclusions would be strengthen if the authors could put more emphasis on assessing endogenous interactions (rather than overexpressed or semi-endogenous) and regulation of the ubiquitination of endogenous Cep192 by Fbxl13 (as many proteins can be aberrantly ubiquitinated when overexpressed)."

As asked for by this reviewer, we have now confirmed interaction between endogenous FBXL13 and CEP192 after treating cells with MG132 to block degradation (Fig. 2D). The detection of

endogenous ubiquitylation of individual proteins is typically very difficult to assess, for which reason we used the setting of overexpressed proteins, as is customary in the field for such experiments. However, we show the effect of FBXL13 on endogenous CEP192 expression in different experiments (Fig. 4C, D, E, G, EV1B).

"2. While the data demonstrating Fbxl13 targets Cep192 and thereby affects centrosome biology/duplication is straightforward and convincing (keeping in mind it is largely overexpression studies). The jump to connecting the regulation of Cep192 to cell motility is less convincing. One might expect, as is the case for most substrate recognition subunits of SCF complexes, that Fbxl13 recognizes and targets for degradation multiple proteins. For instance, to make the model stronger, the authors could assess if the reduced migration of cells following depletion of Fbxl13 is reversed by co-depletion of Cep192, which would provide further evidence that the change in migration due to depletion of Fbxl13 is largely through reducing Cep192 stability rather than another potential substrate."

As suggested by the reviewer, we co-depleted FBXL13 and CEP192 and analyzed the effects on cell migration. Indeed, we now show that siRNA-mediated silencing of both FBXL13 and CEP192 rescues the migration defect observed after siRNA of FBXL13 alone (Fig. 7D, E, F). We thus present evidence that CEP192 is the biologically relevant substrate of FBXL13.

Minor concerns:

"1. The authors should provide a more extensive explanation why they chose the 2 isoforms of Fbxl13 for their interaction screen and did not choose or use the remaining 2. Would they anticipate that they might pull out a different set of factors with the other 2 isoforms?"

It may be possible that the other isoforms of FBXL13 might also interact with some different proteins since they are modified in the LRR, which we have shown to be the region of FBXL13 interaction with substrates. When we started the study in 2014, the only isoforms of FBXL13 associated to the Gene ID: 222235 were the two we have isolated.

"2. The methods used by the authors for ubiquitination assays under denaturing conditions should be more extensively described or referenced."

This has been corrected in the current version of the manuscript.

"3. While the manuscript is fairly well written, there are some grammatical and spelling/typos scattered throughout that should be cleaned up."

We apologize for this deficiency. We have corrected the typos and grammatical mistakes.

Referee #3

This reviewer appears very enthusiastic about our study and states: *"The experiments are performed in a technically sound manner and support the conclusions of the paper. Therefore, I support publication of this paper in EMBO Reports and I have only minor concerns which are outlined below:"*

"1. Standard deviations should be included in Figure 6F."

The SDs have now been included in the current version of the manuscript.

"2. Figure 6G reports differences only for shFbxl13 (87). Are the difference across the samples calculated using ANOVA? What do the numbers after "shFBXL13" refer to?"

According to the suggestion of reviewer one, we have recalculated the differences using both unpaired T-test and a nonparametric Mann-Whitney test. In both cases a significant increase of g-Tubulin is detectable in the sample with most efficient knock-down of FBXL13.

We apologize for the misleading numbering of the shFBXL13 constructs. These numbers refer to our internal reference for the shRNA oligos targeting FBXL13.
We have re-named these constructs to oligo # 1-2-3-4 and reported the sequences in the material and methods section of the manuscript.

"3. Microtubule regrowth assays under conditions of forced FBXO13 overexpression would strengthen the data on the role of FBXL13 in regulating microtubule nucleation activity."

As suggested, we performed microtubule regrowth assay upon forced FBXL13 expression. The results presented in Figure EV3E, F show a significant difference between control-transfected cells and cells transfected with FBXL13.

References:

Bauer M, Cubizolles F, Schmidt A, Nigg EA (2016) Quantitative analysis of human centrosome architecture by targeted proteomics and fluorescence imaging. *The EMBO journal* 35: 2152-2166

Firat-Karalar EN, Rauniyar N, Yates JR, 3rd, Stearns T (2014) Proximity interactions among centrosome components identify regulators of centriole duplication. *Current biology : CB* 24: 664-670

Lopes CA, Jana SC, Cunha-Ferreira I, Zitouni S, Bento I, Duarte P, Gilberto S, Freixo F, Guerrero A, Francia M, Lince-Faria M, Carneiro J, Bettencourt-Dias M (2015) PLK4 trans-Autoactivation Controls Centriole Biogenesis in Space. *Developmental cell* 35: 222-235

Sonnen KF, Gabryjonczyk AM, Anselm E, Stierhof YD, Nigg EA (2013) Human Cep192 and Cep152 cooperate in Plk4 recruitment and centriole duplication. *Journal of cell science* 126: 3223-3233

2nd Editorial Decision 23 November 2017

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see both referees are now positive about the study and request mostly minor changes to clarify text and figures and one control experiment (referee 1, comment 7) to address a remaining concern.

I look forward to seeing a final version of your manuscript as soon as possible.

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REFEREE REPORTS

Referee #1:

Overall the authors have done a good job addressing most of my issues. I note the following minor points to consider before acceptance of the manuscript for publication.
Comment 1 and 2:
The authors show Fbxl13 regulated cell migration depends on its catalytic activity as suggested. Moreover, the authors show that CEP192 siRNA treatment could partially rescue the delay of cell migration induced by FBXL13 siRNA. This supports the model that FBXL13 regulates cell migration though the control of CEP192 levels. This is not in line with previous roles for CEP192 as a positive regulator of cell migration and although I respect the authors arguments here it is still (in my opinion) strange. Nonetheless they have done the propoer controls so the data is what it is, so as long as they thead carefully on this I'm OK with it. Having said this, the model brought forth by the authors in Fig7G looks a bit odd in terms of the MT radiating from a blank region. Is that Golgi? This could be claridied.
Comment 6:

The authors now show the quantification of CEP192 level in CHX as suggested, and demonstrate that the expression of Fbxl13 increased CEP192 degradation rate. However, I find the number a little bit difficult to follow, since the authors set "0h CHX in empty vector" as 100%. Could they use both 0h of CHX in empty and Fbxl13 vector as 100%, and comparing the corresponding condition to their own controls?
Comment 7:
The author's data indicate that Fbxl13 does not regulate endogenous CEP192 degradation at centrosome. They argue that it may be due to Fbxl13 specific regulation through CEP192 isoform 3 degradation, and that the reason they could not see CEP192 level changed at centrosome is due to "background" picked up by non-specific antibodies. To address that, author could show the CEP192 intensity at centrosome after treating with siRNA specifically targeting isofom3.
Minor concerns:
Fig 4:
The authors need to change it to a more representative blot, if they argue the isoform 2 downregulation was not repeatable.

Referee #2:

EMBO Reports
Manuscript #: EMBOR-2017-44799V2

This manuscript authored by Fung et al. titled "Fbxl13 directs ubiquitin-mediated proteolysis of Cep192 to regulate centrosome homeostasis and cell migration" has now been resubmitted as a revised manuscript. The authors have done extensive work to address my, as well as the other two reviewers, concerns. After reading the manuscript and rebuttal to reviewers comments, I believe that with the incorporation of the new data and added discussion/methods, the manuscript is now suitable for publication in EMBO Reports.

Referee #3:

The authors have thoroughly addressed my concerns and appear to have satisfactorily addressed the concerns of the other reviewers.

2nd Revision - authors' response 8 December 2017

Reviewer #1:

This reviewer states "*Overall the authors have done a good job addressing most of my issues*" but indicates the following remaining points:

"Comment 1 and 2:
The authors show Fbxl13 regulated cell migration depends on its catalytic activity as suggested. Moreover, the authors show that CEP192 siRNA treatment could partially rescue the delay of cell migration induced by FBXL13 siRNA. This supports the model that FBXL13 regulates cell migration though the control of CEP192 levels. This is not in line with previous roles for CEP192 as a positive regulator of cell migration and although I respect the authors arguments here it is still (in my opinion) strange. Nonetheless they have done the propoer controls so the data is what it is, so as long as they thead carefully on this I'm OK with it. Having said this, the model brought forth by the authors in Fig7G looks a bit odd in terms of the MT radiating from a blank region. Is that Golgi? This could be claridied."

With regard to the model depicted in Fig. 7G, we do not know whether the microtubules are coming from Golgi or other sites. Therefore, at this stage, we would prefer to depict the microtubules only as extracentrosomal (coming from a blank space).

"Comment 6:
The authors now show the quantification of CEP192 level in CHX as suggested, and demonstrate

that the expression of Fbxl13 increased CEP192 degradation rate. However, I find the number a little bit difficult to follow, since the authors set "0h CHX in empty vector" as 100%. Could they use both 0h of CHX in empty and Fbxl13 vector as 100%, and comparing the corresponding condition to their own controls?"

We would prefer to leave the quantification as it is, as this numbering also reflects differences of CEP192 expression at time point 0h in both the empty vector and FBXL13 overexpression samples.

"Comment 7:
The author's data indicate that Fbxl13 does not regulate endogenous CEP192 degradation at centrosome. They argue that it may be due to Fbxl13 specific regulation through CEP192 isoform 3 degradation, and that the reason they could not see CEP192 level changed at centrosome is due to "background" picked up by non-specific antibodies. To address that, author could show the CEP192 intensity at centrosome after treating with siRNA specifically targeting isofom3."

Regarding this comment, we are afraid that the reviewer has not fully understood our reply. The experiment he proposes will not clarify the issue for the following reasons: siRNA of FBXL13 induces upregulation of isoform 3 but not isoform 1, the most abundant isoform. The isoforms of CEP192 are separated on the SDS page due to the differences in their molecular weight. Because of the differences in their molecular weight, we can detect upregulation of CEP192 isoform 3 after siRNA of FBXL13 by Western blotting (Figure 4E). However, by Immunofluorescence, the isoforms all reside at the centrosome. So, if isoform 3 is more abundant, this will not be detected by immunofluorescence as it is masked by the presence of isoform 1. Notably, the antibodies for CEP192 do not pick up background, as otherwise stated by the reviewer, but instead detect different, more abundant CEP192 isoforms.
Against this background, the reviewer asks to perform siRNA of CEP192 isoform 3 and see if the signal at the centrosome changes. We can't see how this experiment will contribute or reinforce the main finding of the manuscript. It is an experiment designed to obtain a negative result.

"Fig 4:
The authors need to change it to a more representative blot, if they argue the isoform 2 downregulation was not repeatable"

In our reply to this point, we did not mean to indicate that this effect was not repeatable, but instead that isoform 2 of CEP192 was not consistently detectable (e.g. Fig. 4G, where only CEP192 isoforms 1 and 3 are detectable).
Given the function of FBXL13 as a ubiquitin E3 ligase, the effect, if any, is certainly not directly mediated by FBXL13. It is conceivable that there is a negative feedback loop caused by high levels of CEP192 isoform 3 which downregulates the expression of other isoforms.

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: D'Angiolella Vincenzo/ Florian Basserman
Journal Submitted to: Embo Reports
Manuscript Number: EMBOR-2017-44799V2

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 $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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.

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
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http://ClinicalTrials.gov
http://www.consort-statement.org
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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

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

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http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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?	Experiments were repeated 3 times. In the case of centrosome duplication and migration assays experiments were repeated three times and size was based on previous publications using the same approaches.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Animal experiments were not performed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animal experiments were not performed.
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.	Animal experiments were not performed.
For animal studies, include a statement about randomization even if no randomization was used.	Animal experiments were not performed.
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.	Animal experiments were not performed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Animal experiments were not performed.
5. For every figure, are statistical tests justified as appropriate?	Yes. We used more than one statistical analysis to ensure reproducibility of observations. Quantitative analysis of band intensity was performed using Image J. Data are reported as mean ± SD. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc.). Differences between groups were compared using unpaired Student's t-test. Statistical analysis of relative ratios was performed by using one-sample t-tests with hypothetical means of 1.0. Statistically insignificant results are indicated as ns for P>0.05. Statistically significant results are indicated as * for P≤0.05, ** for P≤0.01, *** for P≤0.001, and **** for P≤0.0001.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. We used T-test, non-parametric Mann-Whitney test. Indicated in the figure legends
Is there an estimate of variation within each group of data?	Samples were always compared using a negative control, which provide an estimate of variance.
Is the variance similar between the groups that are being statistically compared?	Samples were always compared using a negative control, which provide an estimate of variance.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following monoclonal antibodies were used: anti-Myc (9B11, mouse, Cell Signaling Technology), anti-HA (05-904, mouse, Millipore and 16B12, Biolegend), anti-GAPDH (MA5-15738, rabbit, Sigma-Aldrich), anti-Cyclin B (AHF0052, Life Technologies), anti-CUL1 (32-2400, Thermo Fisher Scientific), anti-PLK1 (33-1700, Thermo Fisher Scientific), anti-β-actin (A1978, Sigma-Aldrich) and anti-Centrin-3 (SC100933, Santa Cruz). Polyclonal antibodies used were: anti-Flag (F7425, rabbit, Sigma-Aldrich), anti-Centrin-2 (SC27793, Santa Cruz), anti-FBXL13 (OAAB12542, Aviva), anti-FBXL16 (PA5-21094, Thermo Fisher Scientfic), phospho-Histone H3 (06-570, Millipore), anti-SKP1 (sc-7163, Santa Cruz), anti-α/β-Tubulin (2148, Cell Signaling), anti-CEP152 (unpurified, kind gift from Prof. Erich A. Nigg at the Biozentrum of University of Basel, Switzerland) and anti-CEP192 (kind gift from Prof. Laurence Pelletier at the University of Toronto, Canada). Polyclonal rabbit antibody against FBXL13 used for endogenous IP was raised and purified by Innovagen (aa111-121 and 695-707). Page 15
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were a kind gift of Michele Pagano. They were purchased from ATCC. Cell lines are routinely tested for mycoplasma contamination.

* 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.	Animal experiments were not performed.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal experiments were not performed.
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.	Animal experiments were not performed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Does not apply
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.	Does not apply
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Does not apply
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Does not apply
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Does not apply
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.	Does not apply
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.	Does not apply

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 MS data has been deposited and is available through the PRIDE public resource (px-submission #195154). Page 22
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).	All MS data has been deposited and is available through the PRIDE public resource (px-submission #195154). Page 22
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).	Does not apply
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.	Does not apply

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