Crumbs, Galla and Xpd are required for Kinesin-5 regulation in mitosis and organ growth in *Drosophila*

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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to:

https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area.

(Corresponding author only has access)

As you will see from their reports, both reviewers raise a number of substantial criticisms that prevent me from accepting your paper for publication.

I am very sorry to give you such disappointing news, but we are currently under great pressure for space and it takes a very enthusiastic recommendation by the referees for a manuscript to be accepted.

I do hope you find the comments of the reviewers helpful in allowing you to revise the manuscript. I suggest two routes forward: either you work on addressing the referees' comments with the aim of re-submitting to JCS in which case I would regard the paper as a new submission; or you might consider transferring the paper to Biology Open.

Reviewer 1

Advance summary and potential significance to field

Evidence presented in this manuscript suggests that Drosophila galla-2, xpd, and crumbs (crb) act through Klp61F and that these genes are required for the stability of Klp61F, the Drosophila tetrameric Kinesin-5 motor protein. Stabilization of this kinesin-5 appears to be their key function in regulating spindle formation and function because the spindle defects seen upon knockdown (or overexpression of a probable dominant-negative form) can be restored by overexpressing Klp61F. These findings provide an interesting interpretation of previously reported phenotypes and they point to a pathway through which these genes and proteins normally contribute to spindle formation in mitosis.

Comments for the author

Overexpression of a Crumbs fragment containing only the cytoplasmic and the transmembrane domain (Crbintra; without the extracellular domain) causes an apparently dominant-negative effect that can be enhanced by RNAi knockdown (kd) of klp61F. In contrast, overexpression of klp61F rescues the phenotype.
Thus, the two genes show an interesting genetic interaction.

In Drosophila embryos, antibody staining for Crb and Klp61F suggests colocalization to microtubule structures, particularly the mitotic spindle.
\ Unfortunately, the resolution of the images provided is low (Figure 2) and there is no mentioning whether and which precautions had been taken to ascertain that there is no bleed-through from one channel (e.g. tubulin) to another.

Immunoprecipitations and GST binding assays show that Crbintra indeed interacts with Klp61F. The authors claim that this is not the case for their control, the kinesin-2a, Klp64D.
\Unfortunately, there is no loading control showing that there was indeed the same amount of soluble Klp64D in this assay. Without this data, this conclusion cannot be made.

The paper describes mitotic defects in embryos with maternally overexpressed Crbintra, and it refers to previous publications that showed the same phenotype when klp61F function was reduced. As in the initial experiment, the Crbintra overexpression phenotype can be rescued by overexpressing Klp61F, revealing again this genetic interaction.

Galla-2 kd causes also spindle defects and nuclear loss in embryos, and the spindle defects are also rescued very well by overexpressing Klp61F (the nuclear loss phenotype partially, too). Galla-2 also interacts with Klp61F \ (although there is something wrong with the 4th column in Figure 4B, where there should be a Klp61F band if the labeling is correct).
Galla-2 thus seems to be part of the same mechanism. Consistent with this, Galla-2 staining lights up the mitotic spindle in metaphase (Figure 4C), just like the tubulin staining of the same sample.
\The strong resemblance of the two signals requires that the authors provide careful controls that the Galla-2 signal is not a bleed-through signal from the tubulin channel. Comparable to the genetic interaction reported for Galla-2, spindle defects produced by xpd kd can be rescued by overexpression of Klp61F (but “defective embryos” not), and Xpd can be pulled down by Klp61F.
The authors go on to show that heterozygous mutants in two genes that contribute to proteasomal degradation can rescue the spindle defects caused by galla-2, xpd and klp61F kd (or by overexpression of Crbintra), suggesting that these genes are required for the stability of Klp61F.
This result is very reminiscent of a previous report that found that the stability of another transport molecule, myosin V, was dependent on crb (J. Cell Biol. 195(5): 827—838).
\ In the present (Hwang et al.) experiments, the effect on the spindle phenotype is impressive, but the Western blots, designed to test Klp61F levels, lack a loading control and can therefore not be interpreted.
Similarly, the statement that alpha-tubulin levels also changed by the treatments remains to be proven by comparison to a loading control.
\IPs, GST pull-down experiments and Western blots need (more) loading controls to allow the

authors to come to various presented conclusions.

\ The Materials and Methods section is too brief for my taste. It lacks details and other important information.

\ Figure legends should also include more details to allow the reader to interpret the Figures better.

Potential extensions of the study:
It would also be interesting to know whether knocking down any of these factors affects the (spindle) localization of the other proteins.

Minor points:
Figure 2E): is the first lane input? (as in F).

The paper refers to embryonic stages (stage 10, stages 12-13) when it should refer to nuclear cycles 10, 12-13.

Two “intra” became inta” (Figure legend Figure 1)
Figure 3: last sentence: adding “overexpression” would make the sentence clear. “Nuclear loss phenotypes by Crbintra overexpression are partially suppressed by Klp61F overexpression.”
The GST pull down with GST-Xpd (Figure 5B) is less important for this paper, but there is the worry that the MBP-Klp61F band shown is caused by run-over from the input loading (note the asymmetric intensity of this band). The authors should double-check whether this interaction is real.

Clearly state when overexpressing a protein maternally and when performing RNAi kd maternally. Similarly, (in the figure legend of Figure 6) “rpt504210b/+ embryos” is presumably not correct (genotype of the mother).

Discussion: “This suggests that Galla-2 is required for the function of Xpd and Klp61F in embryo(s).” For the function of Klp61F, yes, but why for the function of Xpd?

Reviewer 2

Advance summary and potential significance to field

In this manuscript authors extended their previous finding that apical polarity protein Crb binds Galla/MIP18 and XPD to form a “CGX” complex controlling the proper chromosome segregation during nuclear division.
Authors now identified kinesin-5/Klp61F as the major effector downstream of CGX complex based on the genetic interactions between Klp61F and components of CGX complex. Furthermore, authors showed that components of CGX complex physically bind Klp61F and such interactions prevent Klp61F from degradation.

Overall authors have done a solid, albeit relatively simple and straight forward work, to demonstrate the physical and functional interactions between Klp61F and Crb/Galla/XPD complex. The data presented in the manuscript are consistent with their previous finding, and provide a new insight into the mechanisms regarding how polarity protein Crb could also function in controlling spindle stability and chromosome segregation. I support its publication on JCS, with some minor revisions.

Major comments:
1. Given the transmembrane nature of Crb, it is kind of surprising or counter-intuitive that Crb would localize to spindles during nuclear division and functions to control chromosome segregation. Could there be certain features of Crb that make it a good candidate for this function? Are there any precedents that other transmembrane proteins were found on spindle and chromosomes during mitosis? I would appreciate authors to discuss and elaborate on this matter in the revised manuscript.

2. Many proteins are well characterized for interacting and regulating Crb, among them Stardust, Par-6, Patj etc, just to name a few. Have authors looked at potential nuclear division defects in syncytial embryos mutant of sdt or par-6? It will be beneficial to have such data in the revised manuscript, although further phonotypical analysis are not necessary as such studies would be major projects on their own.
3. Although discussed by authors, it remains a bit puzzling that crb-RNAi and Crb-intra overexpression yielded similar nuclear division phenotypes. In particular, galla-RNAi can rescue the Crb-intra overexpression phenotype in eyes and this does not appear to be immediately consistent with the model that Crb, Galla and XPD form a complex to stabilize Klp61F, and that crb-RNAi and Crb-intra somehow both act to reduce Crb activity/function. It is again beneficial that revised manuscript can show the nuclear division phenotypes and Klp61F expression in {Crb-intra + galla-RNAi} embryos.
4. The discussion section overall is fine, but can be more articulated and better structured in revised manuscript.

Comments for the author

Minor errors/typos that need to corrected:
1. Figure 3 title: “Spindle defects and nuclear loss caused by Crb-intra overexpression by Klp61F overexpression” should be “.... are suppressed by Klp61F overexpression”

2. Duplicated references:
YEOM, E., HONG, S. T. & CHOI, K. W. 2014. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777.
YEOM, E., HONG, S. T. & CHOI, K. W. 2015a. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777-2789.
YEOM, E., HONG, S. T. & CHOI, K. W. 2015b. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777-89.

Reviewer 3

Advance summary and potential significance to field

Xeroderma pigmentosum group D (XPD) is a protein of great clinical relevance; patients with mutations in XPD show increased photo sensitivity and increased cancer incidence. XPD functions in DNA repair and transcription but it also resides in alternate complexes that appear to have a role in mitosis. Studies in Drosophila identified an complex of XPD, Galla (MIP18 homolog) and Crumbs, and this GCX complex is the focus of the manuscript. Hwang et al. report that GCX complex members regulate the protein level of mitotic kinesin KLP61F in Drosophila, which could explain why depletion of Crumbs, Galla-2 or Xpd results in mitotic chromosome segregation defects. In principle, this work is suitable for the Journal of Cell Science.

Comments for the author

But the data shown fall short of supporting many of the conclusions, so I cannot recommend publication in its current form.

One major weakness throughout the manuscript is the lack of supporting data that the tools work as intended. What is the evidence that RNAi lines knock out the intended target? What is the evidence that antibodies recognize the intended protein? Controls such as vector only or a second line (for RNAi) and mutant/depleted samples (for antibody specificity) need to be included. Methanol fixation is great for preserving the spindle but also tends to precipitate out proteins and give false signals, so controls are particularly important.

Another major weakness is the lack of statistical analysis. In the graphs in figures 3-6, how many embryos were analyzed, now many independent replicates were done, and are the differences statistically significant?

Even if supported by rigorous analysis, the magnitude of many effects observed is small. For example, spindle defects increase from ~10% in controls to 20% with Crb-Intra in Fig. 3D. The rescue by KLP61F overexpression brings it back down to 10%. Even if statistically significant, does 10% of spindles being defective tell us anything about how Crb and KLP61F function? This low level also does not agree with the near complete depletion of KLP61F shown in Fig. 6. How can embryos (for example, mat>Crb-Intra) have no detectable KLP61F (Fig. 6A) but show only 20% of spindles with defects (Fig. 3D)? How are they even reaching cortical syncytial cycles with 80% of spindles normal when there is little or no KLP61F?

Interaction studies were done with Crb-Intra or overexpressed proteins. Do endogenous proteins interact?
The authors have antibodies and could try immune-precipitation from embryo extracts.

Fig. 6 shows that KLP-61F protein disappears upon depletion of GCX proteins but recovers when proteasome is depleted. This leads the authors to conclude that GCX proteins 'are required for KLP61F stability'. But there are alternate explanations. For example, the GCX complex could regulate the synthesis of KLP61F. In the absence of synthesis, degradation depletes the protein. Reducing the degradation then restores the protein. In fact, reduced KLP61F protein in KLP61F RNAi was rescued by an rpt5 mutation; RNAi is working in this case to affect synthesis.

The Methods section lack important information, for example, description or source of the antibody against Crb or how embryo extracts were prepared for Western blotting, and how S2 cells were fixed for antibody staining.

Resubmission

Author response to reviewers' comments

Point-by-point response to Reviewers' comments

We would like to thank the Reviewers for constructive criticisms and suggestions. We addressed Reviewers' questions with additional experiments and clarification. Below, we provide our point-by-point response in the order of issues raised.

Reviewer 1 Comments for the Author:

1. In Drosophila embryos, antibody staining for Crb and Klp61F suggests colocalization to microtubule structures, particularly the mitotic spindle. Unfortunately, the resolution of the images provided is low (Figure 2) and there is no mentioning whether and which precautions had been taken to ascertain that there is no bleed-through from one channel (e.g. tubulin) to another.

- Thanks for this comment. Indeed, we found significant bleeding from Tubulin-FITC channel. To eliminate such bleeding effect, we repeated immunostaining in the absence of anti-tubulin antibody and found similar overlapping localization between Crb and Klp61F, although Crb staining was much weaker and diffused. Hence, we replaced Fig.1 with new Fig. 1 without tubulin staining and move the tubulin staining image to Supplementary information (Fig. S1) for comparison.

2. Immunoprecipitations and GST binding assays show that Crbintra indeed interacts with Klp61F. The authors claim that this is not the case for their control, the kinesin-2a, Klp64D. Unfortunately, there is no loading control showing that there was indeed the same amount of soluble Klp64D in this assay. Without this data, this conclusion cannot be made.

- We repeated experiments in Fig. 2E-F and Fig. 5A-B with similar amounts of input control for Klp61F and Klp64D. New data show that Crbintra and Xpd interact with Klp61F but not with Klp64D. Accordingly, Fig. 2E-F and Fig. 5A-B were replaced with new data containing input controls.

3. Galla-2 KD causes also spindle defects and nuclear loss in embryos, and the spindle defects are also rescued very well by overexpressing Klp61F (the nuclear loss phenotype partially, too). Galla-2 also interacts with Klp61F (although there is something wrong with the 4th column in Figure 4B, where there should be a Klp61F band if the labeling is correct).
- Thanks for finding this error in Fig. 4B. "+" should not be there for MBP-Klp64D in the 4th column. We fixed the error in the revised manuscript. We replaced Fig. 4B with a new blot showing inputs for both MBP-Klp61F and MBP-Klp64D.

4. Galla-2 thus seems to be part of the same mechanism. Consistent with this, Galla-2 staining lights up the mitotic spindle in metaphase (Figure 4C), just like the tubulin staining of the same sample. The strong resemblance of the two signals requires that the authors provide careful controls that the Galla-2 signal is not a bleed-through signal from the tubulin channel.
- We repeated immunostaining in the absence of anti-tubulin antibody to avoid bleeding from tubulin staining. We replaced Fig. 4C image with new one stained in the absence of tubulin staining.

5. The authors go on to show that heterozygous mutants in two genes that contribute to proteasomal degradation can rescue the spindle defects caused by galla-2, xpd and klp61F kd (or by overexpression of Crbintra), suggesting that these genes are required for the stability of Klp61F. This result is very reminiscent of a previous report that found that the stability of another transport molecule, myosin V, was dependent on crb (J. Cell Biol. 195(5): 827—838). In the present (Hwang et al.) experiments, the effect on the spindle phenotype is impressive, but the Western blots, designed to test Klp61F levels, lack a loading control and can therefore not be interpreted. Similarly, the statement that alpha-tubulin levels also changed by the treatments remains to be proven by comparison to a loading control. IPs, GST pull-down experiments and Western blots need (more) loading controls to allow the authors to come to various presented conclusions.
- Yes, proteasome-dependent changes in the Klp61F level by crb RNAi seems to be similar to the case of myosin V, although cellular contexts reported in the JCB paper are different (nuclear division in embryo vs differentiating retina). We cited this paper with a brief discussion on the similarity in the Discussion section.
In our Western blot experiments for testing Klp61F level, we found consistent reduction in Klp61F levels by knockdown of Crb, Galla-2 and Xpd, but the extent of reduction was highly variable from experiment to experiment, even though we tried carefully to load same amount of quantified protein extracts in each lane. We think that there are two major reasons for such variations: (i) Syncytial embryos collected for 2h are unsynchronized, and their nuclear division stages are variable in different RNAi conditions, and more importantly (ii) knockdown of Crb, Galla-2 and Xpd have severe nuclear loss phenotypes as we showed in Fig.S6C, which can cause severe variations. We addressed these problems in two ways. First, we repeated the western blot experiments a number of times (n=20), and presented the raw quantitative data of gel scan from 20 blots in a Table and a bar graph. Klp61F levels were normalized to the level of tubulin for each genotype. Similarly, effects of proteasome mutation on the Klp61F level were also measured from 20 western blots and shown as a Table and a graph. The new data are shown in Fig. S5 and Table S2, S3. Secondly, we figured that the best way to show the effects of crb/galla-2/Xpd RNAi is to examine Klp61F levels in spindles from individual embryo rather than checking the Klp61F protein levels in heterogeneous populations of embryos. Hence, we provide an addition data that show reduced Klp61F levels in mitotic spindles, as presented in new Fig. 6.
We also added Klp64D control in IPs and GST pull-down experiments, as mentioned above in response to the comment #2.

6. The Materials and Methods section is too brief for my taste. It lacks details and other important information. Figure legends should also include more details to allow the reader to interpret the Figures better.
- We added additional information for genetic crosses, immunostaining, and immunoprecipitation and statistical analysis.

7. Potential extensions of the study:
It would also be interesting to know whether knocking down any of these factors affects the (spindle) localization of the other proteins.

- We tested whether knocking down of any of CGX/Klp61F factors affects the spindle localization of the other proteins. As expected from their physical interaction, our data show that RNAi of any CGX gene affects the localization of others. These results suggest that spindle localization of Crb, Galla2, Xpd and Klp61F is dependent on each other. These data are presented as follows:
(i) Crbintra overexpression, galla-2 RNAi, or Xpd RNAi: reduces Klp61F spindle staining (Fig. 6)
(ii) Klp61F, galla-2 and Xpd RNAi reduces Crb staining (Fig. S1 and S8)
(iii) Crbintra overexpression, Xpd RNAi, or Klp61F RNAi reduces Galla-2 staining (Fig. S4).

Minor points:
1. Figure 2E): is the first lane input? (as in F).
- Yes, it is. We labeled the input lanes. We also added the input for Klp64D.

2. The paper refers to embryonic stages (stage 10, stages 12-13) when it should refer to nuclear cycles 10, 12-13.
- We changed ‘stages’ to ‘nuclear division cycles’.

3. Two “intra” became inta” (Figure legend Figure 1)
- We fixed the error.

4. Figure 3: last sentence: adding “overexpression” would make the sentence clear.
- We added “overexpression” to make the sentence more clear.

5. The GST pull down with GST-Xpd (Figure 5B) is less important for this paper, but there is the worry that the MBP-Klp61F band shown is caused by run-over from the input loading (note the asymmetric intensity of this band). The authors should double-check whether this interaction is real.
- We repeated this GST-pulldown experiment and confirmed that MBP-Klp61F band is not caused by run-over from the adjacent lane. We replaced it with an improved blot.

6. Clearly state when overexpressing a protein maternally and when performing RNAi kd maternally. Similarly, (in the figure legend of Figure 6) “rpt504210b/+ embryos” is presumably not correct (genotype of the mother).
- We specified “maternal” knockdown or overexpression whenever necessary. We also changed Rpt504210b/+ embryos” to “embryos produced from Rpt504210b/+ females.

Discussion: “This suggests that Galla-2 is required for the function of Xpd and Klp61F in embryo(s).” For the function of Klp61F, yes, but why for the function of Xpd?
- We showed that Xpd RNAi eye phenotype is suppressed by overexpression of Klp61F but not Galla2. This suggests that Galla2 may act upstream to Xpd. To test this possibility, we need to check whether galla-2 RNAi eye phenotype can be suppressed by Xpd overexpression. However, galla-2 RNAi does not have any phenotype in the eye. Hence, we tested this possibility using nuclear division phenotype in embryo. Indeed, galla-2 RNAi phenotype is significantly suppressed by maternal Xpd overexpression. We added this new data in Fig. 8B.

Reviewer 2 Advance Summary and Potential Significance to Field:

Overall authors have done a solid, albeit relatively simple and straight forward work, to demonstrate the physical and functional interactions between Klp61F and Crb/Galla/XPD complex. The data presented in the manuscript are consistent with their previous finding, and provide a new insight into the mechanisms regarding how polarity protein Crb could also function in controlling spindle stability and chromosome segregation. I support its publication on JCS, with some minor revisions.

Major comments:
1. Given the transmembrane nature of Crb, it is kind of surprising or counter-intuitive that Crb would localize to spindles during nuclear division and functions to control chromosome segregation. Could there be certain features of Crb that make it a good candidate for this function? Are there any precedents that other transmembrane proteins were found on spindle and chromosomes during

mitosis? I would appreciate authors to discuss and elaborate on this matter in the revised manuscript.
- The transmembrane protein Crb is unlikely to be directly associated with spindle microtubules. Instead, we speculate that Crb might be associated with intracellular vesicles involved in endosomal trafficking for two reasons: (i) apical localization of Crb as well as DE-cad in epithelia of Drosophila embryo is regulated by endosomal trafficking (Roeth et al., 2009). (ii) Endosomes have been implicated in early mitotic process (Das et al., 2014; Hehnly and Doxsey, 2014). We included this possibility in Discussion.
Roeth, J.F, Sawyer, J.K., Wilner, D.A., & Peifer, M. Rab11 helps maintain apical crumbs and adherens junctions in the Drosophila embryonic ectoderm. PLoS One. 2009 Oct 28;4(10):e7634. doi: 10.1371/journal.pone.0007634 (2009).
Hehnly, H. & Doxsey, S. Rab11 endosomes contribute to mitotic spindle organization and orientation. Dev Cell 28, 497-507, doi:10.1016/j.devcel.2014.01.014 (2014).
Das, S., Hehnly, H. & Doxsey, S. A new role for Rab GTPases during early mitotic stages. Small GTPases 5, doi:10.4161/sgtp.29565 (2014)

2. Many proteins are well characterized for interacting and regulating Crb, among them Stardust, Par-6, Patj etc, just to name a few. Have authors looked at potential nuclear division defects in syncytial embryos mutant of sdt or par-6? It will be beneficial to have such data in the revised manuscript, although further phonotypical analysis are not necessary as such studies would be major projects on their own.
- We have examined the effects of sdt RNAi and par-6 RNAi. Our data show that knockdown of Sdt or Par-6 resulted in relatively normal spindles. Although some embryos showed patches of nuclear loss, the frequency of such embryos was not significantly different from that of mat>GFP control. We show these data in new Fig. S7.

3. Although discussed by authors, it remains a bit puzzling that crb-RNAi and Crb-intra overexpression yielded similar nuclear division phenotypes. In particular, galla-RNAi can rescue the Crb-intra overexpression phenotype in eyes and this does not appear to be immediately consistent with the model that Crb, Galla and XPD form a complex to stabilize Klp61F, and that crb- RNAi and Crb-intra somehow both act to reduce Crb activity/function. It is again beneficial that revised manuscript can show the nuclear division phenotypes and Klp61F expression in {Crb-intra + galla-RNAi} embryos.
- As Reviewer 2 correctly pointed out, it is unexpected that Crbintra overexpression eye phenotype is suppressed by galla RNAi rather than Galla overexpression. This apparent inconsistency seems to be due to different effects of Crbintra overexpression in the eye. Although both Crbintra and crb RNAi promote tissue growth in Hippo signaling, they lead to distinct phenotypes in retinal morphogenesis during late stage of eye development (Izaddoost et al.,2003; Pellikka et al., 2003). For example, while loss of Crb does not alter the apical basal polarity in the retina (it is mainly required for rhabdomere morphogenesis), overexpression of Crbintra causes severe disruption of retinal cell polarity and cell death. That's why Crbintra adult eyes are severely rough and small rather than enlarged, as shown in Fig. 1. In contrast, external morphology of crb RNAi adult eyes is relatively normal. The observed suppression of Crbintra eye phenotype by galla RNAi suggests that Crbintra may interact with Galla to disrupt cell polarity and retinal morphogenesis in eye. Thus, dominant effects of Crbintra overexpression can be bypassed for an unknown mechanism(s) by reducing the Galla level. We briefly discussed this issue in Discussion. This is an interesting topic to be studied in the future.
Regarding the second issue, we have attempted to examine the nuclear division phenotype of Crbintra; galla RNAi, as suggested by Reviewer 2. For this experiment, we need to put three transgenes (mat-Gal4, UAS-Crbintra and UAS-galla RNAi) together in females. Because all these transgenes are located on the second chromosome, it was necessary to generate recombinant chromosomes to construct a proper genotype that carries all three transgenes. Unfortunately, we were unable to establish the recombinant lines due to their female sterility. Hence, we could not check nuclear division phenotype of Crbintra; galla RNAi.
However, we have shown an alternative experiment to test the relationship between Crb and Galla, using crb RNAi. Since crb RNAi and Crbintra overexpression show similar nuclear division phenotype, use of the loss of function condition would be an alternative or perhaps better approach than using Crbintra overexpression. In this test, crb RNAi phenotype was suppressed by Galla overexpression (Yeom et al., 2015). This result is consistent with the suppression of Crbintra phenotype by Klp61F overexpression (Fig. 3 in this study).

4. The discussion section overall is fine, but can be more articulated and better structured in revised manuscript.

- We revised the Discussion section to improve its flow and structure.

Reviewer 2 Comments for the Author:

Minor errors/typos that need to corrected:
1. Figure 3 title: “Spindle defects and nuclear loss caused by Crb-intra overexpression by Klp61F overexpression” should be “.... are suppressed by Klp61F overexpression”
- We corrected the Figure 3 title to “Spindle defects and nuclear loss caused by Crbintra overexpression are suppressed by Klp61F overexpression”.

2. Duplicated references:
YEOM, E., HONG, S. T. & CHOI, K. W. 2014. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777.
YEOM, E., HONG, S. T. & CHOI, K. W. 2015a. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777-2789.
YEOM, E., HONG, S. T. & CHOI, K. W. 2015b. Crumbs interacts with Xpd for nuclear division control in Drosophila. Oncogene, 34, 2777-89.
- We fixed this error.

1. One major weakness throughout the manuscript is the lack of supporting data that the tools work as intended. What is the evidence that RNAi lines knock out the intended target? What is the evidence that antibodies recognize the intended protein? Controls such as vector only or a second line (for RNAi) and mutant/depleted samples (for antibody specificity) need to be included. Methanol fixation is great for preserving the spindle but also tends to precipitate out proteins and give false signals, so controls are particularly important.
- We added following supplementary figures to show the effects of RNAi lines and antibody specificity.
(i) Klp61F RNAi reduces Klp61F level in spindles (Fig. S1F). For crb, we have reported crb RNAi and anti-Crb antibody test in our previous paper (Yeom, 2015).
(ii) galla-2 RNAi reduces the level of anti-Galla-2 staining in spindles (Fig. S3C).
(iii) We do not have anti-Xpd antibody. However, we show that Xpd RNAi by ey-Gal4 results in eye reduction, and the small eye phenotype is rescued by overexpressing Xpd (Fig. 8G).

2. Another major weakness is the lack of statistical analysis. In the graphs in figures 3-6, how many embryos were analyzed, now many independent replicates were done, and are the differences statistically significant?
- We added t-test results for statistical significance for quantitative data in the legends for Figures 3 to 5 and 7-8. For more detail statistics for each of phenotype classes, we provide all p-values in Table S1

3. Even if supported by rigorous analysis, the magnitude of many effects observed is small. For example, spindle defects increase from ~10% in controls to 20% with Crb-Intra in Fig. 3D. The rescue by KLP61F overexpression brings it back down to 10%. Even if statistically significant, does 10% of spindles being defective tell us anything about how Crb and KLP61F function? This low level also does not agree with the near complete depletion of KLP61F shown in Fig. 6. How can embryos (for example, mat>Crb-Intra) have no detectable KLP61F (Fig. 6A) but show only 20% of spindles with defects (Fig. 3D)? How are they even reaching cortical syncytial cycles with 80% of spindles normal when there is little or no KLP61F?
- As we indicated in the text, we scored embryo phenotypes in two categories: (i) spindle defects in cortical nuclei that were maintained near the surface of embryo, and (ii) nuclear loss in large patches. In such areas of nuclear loss, spindle defects cannot be scored because nuclei were lost from their normal position. Although Crbintra overexpression causes about 20% defective spindles, 85% embryos show gross defects with patches of nuclear loss (Fig. 3H) and the majority of these embryos fail to hatch to first instar larvae. Therefore, low frequency of spindle phenotypes scored in cortical dividing nuclei are underestimates of the strong Crbintra effects. The same is true for phenotypes of galla-2, Xpd and Klp61F RNAi.

Regarding the second question on Fig. 6, we revised our data based on a number of repeat experiments, as mentioned earlier in response to the Reviewer 1's comment #5. Quantitative analysis of 20 western blots shows approximately 55% reduction in Klp61F in all tested genotypes (crb, galla-2, Xpd, and Klp61F RNAi). This data is shown in new Fig. S5A.

4. Interaction studies were done with Crb-Intra or overexpressed proteins. Do endogenous proteins interact? The authors have antibodies and could try immune-precipitation from embryo extracts.
- Unfortunately, we do not have enough anti-Crb for IP. Anti-Crb antibody used in this study was made in Hugo Bellen's lab, but his lab ran out of this antibody. We have very little left just enough for a few tissue immunostaining. Recently, we produced anti-Crb antibody against the extracellular domain of Crb. However, IP with this antibody has not been successful. Anti-Xpd antibody has been unavailable to us even though we have tried to obtain it.
Although we could not perform endogenous co-IP, we have shown co-IP between Crbintra and Flag-Klp61F, using Flag-Klp64D as a negative control to support the specificity of interactions.
In addition, using anti-Klp61F and anti-Galla-2, we carried out endogenous interaction between Galla-2 and Klp61F. In the revised manuscript, we show endogenous co-IP between Galla-2 and Klp61F in embryo (new Fig. S3A).

Fig. 6 shows that KLP-61F protein disappears upon depletion of GCX proteins but recovers when proteasome is depleted. This leads the authors to conclude that GCX proteins 'are required for KLP61F stability'. But there are alternate explanations. For example, the GCX complex could regulate the synthesis of KLP61F. In the absence of synthesis, degradation depletes the protein. Reducing the degradation then restores the protein. In fact, reduced KLP61F protein in KLP61F RNAi was rescued by an rpt5 mutation; RNAi is working in this case to affect synthesis.

- In Discussion, we added the possibility that CGX complex proteins may regulate Klp61F expression. "An alternative possibility is that CGX proteins might be involved in the regulation of Klp61F synthesis. In this case, proteasome-dependent degradation will facilitate the depletion of Klp61F since Klp61F synthesis is impaired. However, Klp61F RNAi phenotype is also suppressed by an Rpt5 mutation when Klp61F synthesis is affected by RNAi. Hence, regulation of Klp61F stability may be more critical for syncytial nuclear division when its synthesis is impaired."

The Methods section lack important information, for example, description or source of the antibody against Crb or how embryo extracts were prepared for Western blotting, and how S2 cells were fixed for antibody staining.
- We added description of antibody source, preparation of embryo extracts, and immunostaining procedures. We also provided more information on statistical analysis.

First decision letter

MS ID#: JOCES/2020/246801

MS TITLE: Crumbs, Galla and Xpd are required for kinesin-5 regulation in mitosis and organ growth in *Drosophila*

AUTHORS: Ji-hyun Hwang, Linh Thuong Vuong, and Kwang-Wook Choi
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

(Same as the original review)
In this manuscript authors extended their previous finding that apical polarity protein Crb binds Galla/MIP18 and XPD to form a “CGX” complex controlling the proper chromosome segregation during nuclear division. Authors now identified kinesin-5/Klp61F as the major effector downstream of CGX complex based on the genetic interactions between Klp61F and components of CGX complex. Furthermore, authors showed that components of CGX complex physically bind Klp61F and such interactions prevent Klp61F from degradation.

Comments for the author

I found that overall the authors have satisfyingly addressed my concerns with addition experiments and revisions in the manuscripts. I support its publication as the results are an interesting step forward in understanding how Crb-CGX complex may regulate the mitosis during cell divisions. Nonetheless, the discussion is still a bit too convoluted to my taste. I’d strongly suggest authors to consider dividing the long discussion in the final manuscript into several sections each with its own summarizing subtitles focusing on different aspects of how Crb/Galla-2/XPD interact with Klp61F to regulate mitosis.

Reviewer 2

Advance summary and potential significance to field

Evidence presented in this manuscript suggests that Drosophila galla-2, xpd, and crumbs (crb) act through Klp61F and that these genes are required for the stability of Klp61F, the Drosophila tetrameric Kinesin-5 motor protein. Stabilization of this kinesin-5 appears to be their key function in regulating spindle formation and function because the spindle defects seen upon knockdown (or overexpression of a probable dominant-negative form) can be restored by overexpressing Klp61F. These findings provide an interesting interpretation of previously reported phenotypes and they point to a pathway through which these genes and proteins normally contribute to spindle formation in mitosis.

Comments for the author

The authors did a good job fixing the issues pointed out in the previous version. In my mind, the paper is now sound and needs only minor changes or additions.

Figure 1 shows an impressive genetic interaction between CrumbsIntra and Klp61F. These results were obtained with the GMR-Gal4 driver that is mainly active in the eye disc. However, Li et al. (Genet Mol Res. 2012 Aug 6;11(3):1997-2002. doi: 10.4238/2012.August.6.4.) showed that this driver still has considerable activity in other tissues, for instance in the brain. If the authors have additional evidence that this all happens in the eye disc (e.g. defects visible already in discs? Are eye defects fully present in freshly eclosed flies or is it a more degenerative phenotype?), it would be worth mentioning this here. Alternatively, it could be pointed out that this might reflect defects in the eye disc or possibly the brain. As this figure only serves as an introduction to studying this interaction, these suggested modifications are not crucial to the paper, but nice to have.

Fig 2 & 3. Physical interaction, co-localization at the spindle and genetic data showing the Crbintra interaction with Klp61F are convincing, even if the physical interaction is seen in an artificial situation.
The remaining figures show that Crb somehow acts on Galla-2, which acts through Xpd to stabilize Klp61F, a Kinesin-5 that acts in spindle dynamics. Physical and genetic interactions are explored and appear correctly controlled. This leads to the conclusion that “knockdown of any of the Crb/Galla/Xpd proteins results in (the) reduction of Klp61F levels in mitotic spindles.
Figure 8 would deserve a more interesting title. And in the text I got confused by «In contrast, Galla-2 overexpression did not show any noticeable defects in adult eyes (Fig. 8E), implying that Galla-2 is essential for nuclear division in (the) embryo but may be dispensable for (the) development of (the) adult eye.» The first part of the sentence seems to be in the wrong context here and the second part cannot be concluded from it, because overexpression does not test for requirement (downregulation does). Alternatively, this might be a misspelling and “overexpression” should be RNAi.
3rd paragraph of discussion: TFHII > TFIIH

Some parts in the discussion are too redundant with the result section and in my mind not worth repeating. I would suggest streamlining the discussion along these lines.

The last paragraph of the discussion deviates too much from the main project and becomes very speculative.
In my mind, it does not improve the paper.

First revision

Author response to reviewers' comments

Point-by-point response to Reviewers’ comments.
Our point-by-point response is provided below.

Reviewer 1 Comments for the author

I found that overall the authors have satisfyingly addressed my concerns with addition experiments and revisions in the manuscripts. I support its publication as the results are an interesting step forward in understanding how Crb-CGX complex may regulate the mitosis during cell divisions. Nonetheless, the discussion is still a bit too convoluted to my taste. I’d strongly suggest authors to consider dividing the long discussion in the final manuscript into several sections each with its own summarizing subtitles, focusing on different aspects of how Crb/Galla-2/XPD interact with Klp61F to regulate mitosis.
- As suggested, we divided Discussion into three sections with subtitles. We also shortened or removed redundant statements to make the Discussion more concise.

Reviewer 2 Comments for the author

Figure 1 shows an impressive genetic interaction between CrumbsIntra and Klp61F. These results were obtained with the GMR-Gal4 driver that is mainly active in the eye disc. However, Li et al. (Genet Mol Res. 2012 Aug 6;11(3):1997-2002. doi: 10.4238/2012.August.6.4.) showed that this driver still has considerable activity in other tissues, for instance in the brain. If the authors have additional evidence that this all happens in the eye disc (e.g. defects visible already in discs? Are eye defects fully present in freshly eclosed flies or is it a more degenerative phenotype?), it would be worth mentioning this here. Alternatively, it could be pointed out that this might reflect defects in the eye disc or possibly the brain. As this figure only serves as an introduction to studying this interaction, these suggested modifications are not crucial to the paper, but nice to have.
- Yes, Li et al reported that GMR can drive GAL4 expression in wing disc and weakly in larval brain. However, eye imaginal disc and brain develop independently, although development of the first optic lobe (lamina) is dependent on the retinal innervation. Hence, defects in eye disc can affect the lamina part of brain, but it is unlikely that brain defects affect eye development. All eye phenotypes shown in Fig. 1 are fully present in newly eclosed flies. We and others have reported that GMR-Crbintra overexpression impairs the integrity of developing retina during mid-pupal stages (Izaddoost et al., 2002, Pellikka et al., 2002, Grzeschik and Knust, 2005) and Yorkie target gene expression in larval eye disc (Grzeschik et al., 2010). Hence, as suggested by the Reviewer, we modified the introductory sentence as the following: “Overexpression of Crb (Crbintra) in eye disc driven by GMR-Gal4 causes severe roughening of adult eyes by affecting the integrity of differentiating retinal epithelium (Izaddoost et al., 2002, Pellikka et al., 2002, Grzeschik and Knust, 2005).”
Fig 2 & 3. Physical interaction, co-localization at the spindle and genetic data showing the Crbintra interaction with Klp61F are convincing, even if the physical interaction is seen in an artificial situation. The remaining figures show that Crb somehow acts on Galla-2, which acts through Xpd to stabilize Klp61F, a Kinesin-5 that acts in spindle dynamics. Physical and genetic interactions are explored and appear correctly controlled. This leads to the conclusion that “knockdown of any of the Crb/Galla/Xpd proteins results in (the) reduction of Klp61F levels in mitotic spindles».
- Thank you for positive evaluation of our data.

Figure 8 would deserve a more interesting title.
- We changed the title to a more specific one: “Genetic interaction between galla-2, Xpd and Klp61F in embryo and eye” to “Xpd RNAi phenotypes are suppressed by overexpression of Klp61F but not by Galla-2”.

And in the text I got confused by «In contrast, Galla-2 overexpression did not show any noticeable defects in adult eyes (Fig. 8E), implying that Galla-2 is essential for nuclear division in (the) embryo but may be dispensable for (the) development of (the) adult eye.» The first part of the sentence seems to be in the wrong context here and the second part cannot be concluded from it, because overexpression does not test for requirement (downregulation does). Alternatively, this might be a misspelling and “overexpression” should be RNAi.
- Thanks for this comment. The sentence “Galla-2 overexpression did not show any noticeable defects in adult eyes (Fig. 8E)” should be “Galla-2 overexpression or galla-2 RNAi did not show any noticeable defects in adult eyes (Fig. 8E)”. We added a panel for galla-2 RNAi eye (Fig. 8F), although both Galla-2 overexpression and galla-2 RNAi show normal eyes.

3rd paragraph of discussion: TFHII > TFIIH
- We fixed this error.

Some parts in the discussion are too redundant with the result section and in my mind not worth repeating. I would suggest streamlining the discussion along these lines. The last paragraph of the discussion deviates too much from the main project and becomes very speculative. In my mind, it does not improve the paper.
- We deleted the last paragraph and revised the discussion by shortening or removing redundant parts.

Second decision letter

MS ID#: JOCES/2020/246801

MS TITLE: Crumbs, Galla and Xpd are required for kinesin-5 regulation in mitosis and organ growth in *Drosophila*

AUTHORS: Ji-hyun Hwang, Linh Thuong Vuong, and Kwang-Wook Choi
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.