Cabut/dTIEG associates with the transcription factor Yorkie for growth control

Marina Ruiz-Romero, Enrique Blanco, Nuria Paricio, Florenci Serras and Montserrat Corominas

Corresponding author: Montserrat Corominas, Universitat de Barcelona

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<tr>
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Transaction Report:

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

Editor: Esther Schnapp

1st Editorial Decision 17 July 2014

Thank you for your patience while your manuscript was peer-reviewed at EMBO reports. We have now received the full set of referee comments as well as cross-comments that are copied below.

As you will see, while the referees acknowledge that the findings are interesting, they point out that the functional relevance of the interaction between Cbt/dTIEG and Yorkie and the data presented in figure 2F need to be strengthened, and that Yki expression needs to be analyzed more carefully or the data removed from the manuscript. The referees further pinpoint a number of missing controls.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. I agree that it would significantly strengthen the manuscript if you can show that Cbt/dTIEG plays a more general role in Hippo pathway-mediated growth control, for example in the adult and/or in other tissues/organs. Also, please remember that splicing of gel bands needs to be clearly indicated by a black line and white space between the bands. In this case, please also send us the source data for the new figure 2F along with the revised manuscript.

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. Please contact us if a 3 months time frame is not sufficient for the revision. Also, the revised manuscript may not exceed 30,000 characters (including spaces, references and figure legends) and 5 main plus 5 supplementary figures. The current manuscript text slightly exceeds our limits, and the text therefore needs to be somewhat shortened. Commonly used materials and methods can be moved to the supplementary information, but please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. mean +/- SEM, SD) and the statistical tests used to calculate p-values in the respective figure legends. This information is currently incomplete and must be provided in the figure legends. Please also add scale bars to all microscope images and define their length in the figure legends.

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

REFEREE REPORTS:

Referee #1:

This manuscript describes a role for the Cbt/dTEIG transcription factor in the Hippo signalling pathway.

The authors show that Cbt binds to Yki target gene promoters and is required for certain targets to be normally expressed and for cell proliferation in the wing. Overexpression of Cbt is sufficient to increase expression of certain Yki target genes. Thus, Cbt appears to be a partner factor for Yki on several of its target gene promoters.

These are novel and significant findings that will be of interest to the rapidly growing Hippo pathway field. The experiments are well performed and the data look solid. I recommend publication in EMBO Reports.

The authors may wish to consider the following minor comments, none of which are essential to publication:

1. No adult phenotypes are shown for cbt mutant clones (e.g.: ey.flp over a Minute), Cbt RNAi, or Cbt overexpression. This may be due to the pleiotropy of the phenotype, but would still be a useful supplementary figure.

2. It looks like overexpression of Cbt increases nuclear density in the wing disc. This could be because Cbt promotes cell proliferation/cell cycle progression. This would be interesting to test in clonal overexpression or by EdU or pH3 staining or by FACS.

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Referee #2:

The manuscript by Corominas and colleagues identifies the Drosophila Cabut/dTIEG transcription factor as a novel interaction partner of the Hippo pathway member Yorkie. The authors show that
Cabut/dTIEG and Yorkie colocalize on many gene promoters and that Cabut/dTIEG complexes with Yorkie in co-immunoprecipitation assays. They also show that overexpression of Cabut/dTIEG leads to upregulation of Yorkie target genes in wing discs and that Cabut/dTIEG mutant clones have reduced levels of Yorkie target genes. Finally, they show that Cabut/dTIEG is required for Yorkie driven overgrowth and vice versa.

The presented data are of adequate quality and the paper is well written and easy to follow. Conclusions largely follow from the data and I have only comment: Figure 2F shows co-immunoprecipitations, but I would like to see some negative controls with proteins that do not interact with the bait. Also, why are there two bands in the top panel but only one in the bottom panel? It would be good to indicate the size of these bands. In addition, Cabut/dTIEG proteins are not visible in the input lane.

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In this manuscript, Ruiz-Romero and colleagues identify target genes of the transcriptional regulatory protein Cbt/dTIEG by ChIPseq. Overexpression of Cbt/dTIEG leads to upregulation of selected target genes, suggesting that Cbt/dTIEG acts as an activator of transcription. Sequence motif enrichment analysis of the genomic regions bound by Cbt/dTIEG reveals enrichment of the GAGA motif. The GAGA motif is also bound by the transcriptional regulatory factors Yorkie and GAGA factor (GAF)/Trl. The authors find that many Cbt/dTIEG target genes are shared with Yorkie and GAF and provide evidence suggesting that Cbt/dTIEG physically interacts with Yorkie and GAF, at common target genes. As a central player in the growth-regulatory Hippo pathway, Yorkie regulates the expression of genes involved in tissue growth. Ruiz-Romero and colleagues present gain- and loss-of-function experiments that show that Cbt/dTIEG regulates a subset of canonical Yorkie target genes. Finally, Ruiz-Romero report the results of functional experiments suggesting that Cbt/dTIEG and Yorkie exhibit interdependency in the regulation of tissue growth.

The manuscript is well-written. The subject is relevant as Yorkie is a key regulator of organ size and cell fate and is the homolog of two human oncogenes, YAP and TAZ, whose mechanism of action is incompletely understood. The current study would benefit from a number of additions and modifications, as detailed below.

Major points:
1. Functional interaction between Cbt/dTIEG and Yorkie. The significance of the data described in figures 1-3 depends on the functional interactions between Cbt/dTIEG and Yorkie shown in figure 4. These analyses of clonal growth are interesting but confined to one larval tissue, the wing imaginal disc. There are two key questions:
   a. Do the same conclusions apply to growth of the adult organ? For example, does Cbt/dTIEG knockdown suppress the growth enhancing effects of Expanded knockdown and/or Yki overexpression in the wing? What is the effect of combined Cbt/dTIEG + Yki overexpression/activation? Do they cooperatively enhance wing growth?
   b. The second question is whether the observed effects are indicative of a more general function for Cbt/dTIEG in the regulation of Hippo-pathway mediated tissue growth, or whether they are specific to the wing. Experiments in the eye (adult/imaginal disc) analogous to the experiments suggested/performed in the wing could answer this question.
   Moreover, some essential controls are missing in these experiments:
      a. Fig 4A - D: To exclude RNAi off-target effects, it is essential to repeat the experiment with at least one additional Cbt/dTIEG RNAi line. Moreover, the exE1 only control should include a control non-targeting RNAi line.
      b. Fig 4E-H: Please include a control where UAS-YkiS168A is expressed in a Cbt/dTIEG wildtype background.
      c. Fig 4I-L: Please include a control where UAS-dbtdTIEG is expressed in a Yki wildtype background.

   A detailed description of the procedure followed to quantify the clonal area is lacking and should be added.
2. On the basis of the data presented in figure 1, the authors argue that Cbt/dTIEG can act as a transcriptional activator. However, these data are either correlative in nature, or dependent on Cbt/dTIEG overexpression, potentially resulting in overexpression artefacts. Moreover, in a previous paper from one of the authors of this manuscript, it is concluded that Cbt/dTIEG acts as a repressor (Belacortu et al, PLoS ONE 2012). To address this issue satisfactorily, an RNAseq experiment comparing control and Cbt/dTIEG loss-of-function wing discs would be ideal. The Cbt/dTIEG-dependent gene expression program could then be compared to its set of target genes. It is appreciated that this is not a trivial experiment and comes with technical challenges (e.g. the poor survival of dTIEG mutant tissue). Therefore, although it would likely add value to the manuscript, it is not viewed as necessary.

3. In figure 2F, the results of co-IP experiments are presented. These are the weakest data in the manuscript. The authors either immunoprecipitate overexpressed tagged GAF or tagged Yki, and perform western blotting analysis of endogenous Cbt/dTIEG. There are several concerns:
   a. The marked transition in background signal from the 'IP: GAF' lane to the 'mock' lane suggests that these lanes were not adjacent in the original blot but were spliced in post-hoc to appear so. This data, if indeed it has been generated from separate experiments and non-adjacent lanes on a gel, should be separated. Gel lanes should not be artificially juxtaposed.
   b. It is unclear what the 'mock' control IP is. It should be an IP using the same antibody (FLAG or HA, respectively) on lysates from empty vector-transfected cells.
   c. The authors should display Western blots for inputs and IPs for all three proteins.
   d. The authors could perform the reverse IP (of Cbt/dTIEG) and probe for GAF and Yki.
   e. It is unclear to us why there are two Cbt/dTIEG bands in the GAF IP and only one in the Yki IP. This should be clarified.
   f. The molecular Mass for each Western blot should be indicated.
   g. Finally, considering that Yki binds to GAF, and that Cbt/dTIEG binds to both Yki and GAF, the question arises whether the three proteins can interact with each other at the same time.

4. In Figure 4 O,P the authors show Yki localization from planar sections. This data is weak at present and needs to be addressed more carefully or removed. Yki localization should be imaged and quantified using X-Z sections as well, as morphological disruption of the tissue can very easily shift the position of nuclei and give misleading results. Assessing a larger area of tissue with another driver (e.g. en or hh) would also be useful.

Minor points:
In Figures 1, 2 and 4 it is more informative to show individual data points than a bar on the charts.
The n values should be specified for each experiment that uses replicates.
Page 3: The references cited in the first sentence of the Results and Discussion section do not describe the antibody used. Reference 42, correctly cited in the Materials and Methods section, does. Please change.
Fig 1 C. What is the unit on the y-axis? Number of reads? This needs to be included.
Fig 1 D. What is the negative control in this figure? It should be ChIP using neutral IgG. In addition, a FACS plot showing the selection of GFP+ cells should be included (if necessary in the supplementary figures).
Fig 2 D. Are these the promoters of common target genes, as in fig 2E? Please clarify in the legend.
Fig 4 Q. The Y axis legend would be clearer if altered to N/C ratio.
In general, I think Figs 3 and 4 would be easier to interpret if more of the wing discs were shown.

Cross-comments from referee 2:

Ref3 point1: these are good suggestions and not difficult to do. For the controls in point 1: for me they do not need to repeat the clones with another RNAi but show another RNAi has similar effects in a simple GAL4 driven experiment looking at Yki targets. I do not think the control with a non-targeting RNAi line would be much informative, I am ok if they do not do that. The controls b and c are good suggestions and would complete the experiment in a good way. These are also not difficult to do.

Ref3 point 3: I agree with all suggestions and my concerns were very similar.
Response to the reviewers

Referee #1:

This manuscript describes a role for the Cbt/dTEIG transcription factor in the Hippo signalling pathway.

The authors show that Cbt binds to Yki target gene promoters and is required for certain targets to be normally expressed and for cell proliferation in the wing. Overexpression of Cbt is sufficient to increase expression of certain Yki target genes. Thus, Cbt appears to be a partner factor for Yki on several of its target gene promoters.

These are novel and significant findings that will be of interest to the rapidly growing Hippo pathway field. The experiments are well performed and the data look solid. I recommend publication in EMBO Reports.

The authors may wish to consider the following minor comments, none of which are essential to publication:

1. No adult phenotypes are shown for cbt mutant clones (e.g.: ey.flp over a Minute), Cbt RNAi, or Cbt overexpression. This may be due to the pleiotropy of the phenotype, but would still be a useful supplementary figure.

Following the reviewer’s suggestion, we have overexpressed cbt for 24h under spaltE/Pv promoter (wing blade specific promoter). As expected, wings overexpressing cbt are larger than controls. These results are also in agreement with previous observations obtained by Rodriguez [1] using and EP line inserted in cbt. In the same direction, wings expressing cbt RNAi show smaller area. All these results are in new figure E4.

2. It looks like overexpression of Cbt increases nuclear density in the wing disc. This could be because Cbt promotes cell proliferation/cell cycle progression. This would be interesting to test in clonal overexpression or by EdU or pH3 staining or by FACS.

To answer this question we have induced clones either expressing cbt RNAi or overexpressing cbt in the wing and eye- antennal imaginal discs (new Figure E4). In both tissues, depletion of Cbt levels by cbt RNAi results in clones with reduced area compared to wt clones. In the same direction, homozygous clones mutant for cbt show reduced capacity of proliferation Moreover, clones overexpressing cbt display larger areas than wt clones. In agreement with our results, Song et al., [2] already suggested that overexpression of cbt promotes cell cycle after pupariation and regulates cell cycle genes together with dE2F1. In fact, enrichment of genes associated to cell cycle is observed among Cbt targets. Altogether our data points to Cbt as a modulator of proliferation/cell cycle progression during development.

3. The increased nuclear Yki upon Cbt overexpression the wing disc could be real, or could be an artefact caused by increased cell density (which might affect morphology to bring the apical cortex into the plane of the section). I would encourage the authors to be careful in their interpretation of this experiment, perhaps verifying it in Z-sections before publishing. Alternatively,
this experimental data could be removed from the manuscript, as it is not necessary to the main conclusions of the paper.

Following the reviewer’s comments and considering also reviewer’s 3 remarks, we have repeated this experiment acquiring images of the whole discs and performing 3D reconstructions to measure nuclear Yki levels in the entire nuclei. As they suggested, the previously observed results were likely due to increased cell density because no differences have been observed in the majority of the wing discs analyzed. In consequence, and following their indications, we have removed this result from the manuscript. Nevertheless, we include a graph at the end of this document to illustrate this result.

Referee #2:

The manuscript by Corominas and colleagues identifies the Drosophila Cabut/dTIEG transcription factor as a novel interaction partner of the Hippo pathway member Yorkie. The authors show that Cabut/dTIEG and Yorkie colocalize on many gene promoters and that Cabut/dTIEG complexes with Yorkie in co-immunoprecipitation assays. They also show that overexpression of Cabut/dTIEG leads to upregulation of Yorkie target genes in wing discs and that Cabut/dTIEG mutant clones have reduced levels of Yorkie target genes. Finally, they show that Cabut/dTIEG is required for Yorkie driven overgrowth and vice versa.

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To further strengthen results in Figure 2 including the suggested controls, we have performed several new co-immunoprecipitation experiments. More specifically, we have used negative controls using anti-Act5C and anti-αTub, proteins that should not interact with Cbt, Yki or GAF, to verify specificity of western blotting. Regarding the second band observed using Cbt antibody in the GAF-Flag immunprecipitation sample, we have repeated the same experiments changing immunoblotting conditions. Our results indicate that the second band was not specific since it disappears in more stringent conditions. Thus, new images, also containing the size of the bands, are now in new Figures 2 and E3. Moreover, we include the original data source, showing whole membranes at the end of this document. Altogether, these results allow us to conclude Cbt interaction with Yki and GAF.

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In this manuscript, Ruiz-Romero and colleagues identify target genes of the transcriptional regulatory protein Cbt/dTIEG by ChIPseq. Overexpression of Cbt/dTIEG leads to upregulation of selected target genes, suggesting that Cbt/dTIEG acts as an activator of transcription. Sequence motif enrichment analysis of the genomic regions bound by Cbt/dTIEG reveals enrichment of the GAGA motif. The GAGA motif is also bound by the transcriptional regulatory factors Yorkie and GAGA factor (GAF)/Trl. The authors find that
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   b. The second question is whether the observed effects are indicative of a more general function for Cbt/dTIEG in the regulation of Hippo-pathway mediated tissue growth, or whether they are specific to the wing. Experiments in the eye (adult/imaginal disc) analogous to the experiments suggested/performed in the wing could answer this question.

   a. To answer all these questions we have performed similar experiments both in wing and eye-antenna imaginal discs as well as in adult organs such as wing and eye. We have first expressed an activated form of yki together with cbt RNAi in the wing, using a spaltE/Pv promoter (bing blade specific promoter) for 24h (Figure 4P-T). Quantification of wing areas points out that reduction of cbt levels in yki overexpressing wings restores wing area to control size. In the same direction, clones expressing cbt RNAi and yki in the adult eye show similar clone size than control ones (Figure 5 P-T). These results demonstrate that depletion of cbt levels impairs the overgrowth phenotypes caused by yki overexpression in adult tissues.

   Moreover, to analyze the effect of cbt and yki overexpression we have induced clones in wing and eye-antennal imaginal disc overexpressing both genes. Massive growth is observed in both tissues where giant clones can be recovered (Figure E5U-V).

   b. As stated above (answer to reviewer 1) and following the reviewer’s suggestion, we have performed analogous experiments in the eye-antenna imaginal disc (Figure 5). The results obtained are similar to those observed in the wing imaginal disc, indicating a general function for Cbt in the regulation of Hippo mediated tissue growth.

   Moreover, some essential controls are missing in these experiments:
   a. Fig 4A - D: To exclude RNAi off-target effects, it is essential to repeat the...
experiment with at least one additional Cbt/dTIEG RNAi line. Moreover, the exE1 only control should include a control non-targeting RNAi line.

New experiments using another cbt RNAi line have been performed (new Figure E5). As expected Exe1 overgrowth is also reverted when expressing an independent cbt RNAi line. Furthermore, we have observed that depletion of cbt by RNAi reduces Yki target genes expression, as in the case of fj (Figure E3). We are convinced that this additional result has improved and consolidated the conclusion that Cbt is involved in Yki-dependent control of transcription.

b. Fig 4E-H: Please include a control where UAS-YkiS168A is expressed in a Cbt/dTIEG wildtype background.

c. Fig 4I-L: Please include a control where UAS-dbtdTIEG is expressed in a Yki wildtype background.

Controls overexpressing yki and cbt have been added (Figures 4 and 5).

A detailed description of the procedure followed to quantify the clonal area is lacking and should be added.

A detailed description of clonal analysis can be now found in expanded methods. We apologize for this lack of information.

2. On the basis of the data presented in figure 1, the authors argue that Cbt/dTIEG can act as a transcriptional activator. However, these data are either correlative in nature, or dependent on Cbt/dTIEG overexpression, potentially resulting in overexpression artefacts. Moreover, in a previous paper from one of the authors of this manuscript, it is concluded that Cbt/dTIEG acts as a repressor (Belacortu et al, PLoS ONE 2012). To address this issue satisfactorily, an RNAseq experiment comparing control and Cbt/dTIEG loss-of-function wing discs would be ideal. The Cbt/dTIEG dependent gene expression program could then be compared to its set of target genes. It is appreciated that this is not a trivial experiment and comes with technical challenges (e.g. the poor survival of dTIEG mutant tissue). Therefore, although it would likely add value to the manuscript, it is not viewed as necessary.

We agree that the assay proposed by this reviewer will be insightful to assess the role of Cbt as an activator or an inhibitor, however this experiment is technically very difficult as cbt homozygous mutants dye during embryogenesis. Thus, other approaches, such as another tissue, or even whole embryos will have to be used for this experiment, which would likely require to perform new Chip-Seqs as targets could be different in different contexts. On the other hand, although it is true that according to published data Cbt seems to act as a repressor in certain contexts, as it happens with its mammalian orthologs, here we present evidences that, at least in the context of the wing disc, Cbt seems to be acting as an activator of several genes involved in growth and patterning (Figures 1, 3 and E3). Further experiments will clarify the role of Cbt in transcriptional regulation. We thank the reviewer’s comment indicating that this is not viewed as necessary for the present manuscript.

3. In figure 2F, the results of co-IP experiments are presented. These are the weakest data in the manuscript. The authors either immunoprecipitate overexpressed tagged GAF or tagged Yki, and perform western blotting analysis of endogenous Cbt/dTIEG. There are several concerns:
a. The marked transition in background signal from the 'IP: GAF' lane to the 'mock' lane suggests that these lanes were not adjacent in the original blot but were spliced in post-hoc to appear so. This data, if indeed it has been generated from separate experiments and non-adjacent lanes on a gel, should be separated. Gel lanes should not be artificially juxtaposed.

We apologize for this figure because, although lanes were from the same gel in the original picture, they were not adjacent. We have now repeated the same experiment and results are properly shown in new figure 2.

b. It is unclear what the 'mock' control IP is. It should be an IP using the same antibody (FLAG or HA, respectively) on lysates from empty vector-transfected cells.

c. The authors should display Western blots for inputs and IPs for all three proteins.

d. The authors could perform the reverse IP (of Cbt/dTIEG) and probe for GAF and Yki.

e. It is unclear to us why there are two Cbt/dTIEG bands in the GAF IP and only one in the Yki IP. This should be clarified.

f. The molecular Mass for each Western blot should be indicated.

g. Finally, considering that Yki binds to GAF, and that Cbt/dTIEG binds to both Yki and GAF, the question arises whether the three proteins can interact with each other at the same time.

As mentioned above and taking into consideration all comments, we have performed several new co-immunoprecipitation experiments. More specifically, we have used negative controls using anti- Act5C and anti-αTub, proteins that should not interact with Cbt, Yki or GAF, to verify specificity of western blotting and performed reverse IPs. Regarding the second band observed using Cbt antibody in GAF-Flag immunprecipitation sample, we have repeated the same experiments changing immunoblotting conditions. Our results indicate that the second band was not specific since it disappears in more stringent conditions. Thus, new images, also containing the molecular mass, are now in new Figures 2 and E3. Moreover, we have included the original data source, showing whole membranes, at the end of this document.

Altogether, these results let us to conclude that Cbt interacts with Yki and GAF (IP with Cbt allows detection of both Yki and GAF in the same lane of the Western blot). The question of whether Cbt-Yki-GAF act together at the same genomic region or associate in different protein complexes is an interesting point but technically difficult to approach in a short term as this will require a thorough protein domain analysis, mutant generation or functional studies. Thus, here we have focused on the ability of Cbt to associate with Yki for growth control.

4. In Figure 4 O,P the authors show Yki localization from planar sections. This data is weak at present and needs to be addressed more carefully or removed. Yki localization should be imaged and quantified using X-Z sections as well, as morphological disruption of the tissue can very easily shift the position of nuclei and give misleading results. Assessing a larger area of tissue with another driver (e.g. en or hh) would also be useful.

We agree with the reviewer that this data is rather weak and because, as pointed also by reviewer 1, does not seem necessary for the main conclusions of our work, we have removed this data from the manuscript.

Minor points:
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a bar on the charts.
The n values should be specified for each experiment that uses replicates.
Page 3: The references cited in the first sentence of the Results and Discussion section do not describe the antibody used. Reference 42, correctly cited in the Materials and Methods section, does. Please change.
Fig 1 C. What is the unit on the y-axis? Number of reads? This needs to be included.

We apologize for these errors that have currently been fixed and the missing information included.

Fig 1 D. What is the negative control in this figure? It should be ChIP using neutral IgG. In addition, a FACS plot showing the selection of GFP+ cells should be included (if necessary in the supplementary figures).

Negative control is a sample with no antibody. Normalization is performed comparing to input sample as it was previously done with the ChIP-Seq (Methods section). Fig E1 contains now the FACS information.

Fig 2 D. Are these the promoters of common target genes, as in fig 2E? Please clarify in the legend.

This question has been clarified in the figure legend.

Fig 4 Q. The Y axis legend would be clearer if altered to N/C ratio.
In general, I think Figs 3 and 4 would be easier to interpret if more of the wing discs were shown.

As stated above we have removed previous figure 4Q. In Figures 3 and 4 we show images of the wing pouch because it corresponds to the region analyzed.

Cross-comments from referee 2:

Ref3 point1: these are good suggestions and not difficult to do. For the controls in point 1: for me they do not need to repeat the clones with another RNAi but show another RNAi has similar effects in a simple GAL4 driven experiment looking at Yki targets. I do not think the control with a nontargeting RNAi line would be much informative, I am ok if they do not do that. The controls b and c are good suggestions and would complete the experiment in a good way. These are also not difficult to do.

Ref3 point 3: I agree with all suggestions and my concerns were very similar.

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the comments from referee 3 who was asked to assess it.

As you will see, this referee is still not happy with the quality of the IPs. Importantly, the gel bands should not be "overcropped", they should not be cropped at all, if possible. You can also upload source data that show entire gels with the manuscript, which will be linked to the respective figure panels. I suggest that you address these remaining concerns and improve the quality of the data and their presentation before we can proceed with the official acceptance of the manuscript.

Please remember to define all scale bars, as the definition is missing in some of the figure legends. In Figure 2G n=2 but you show error bars. For n<3 no error bars should be shown. Please show the actual data points instead, along with the mean.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions or comments.

REFEREE REPORT:

Referee #3:

The IPs are still not of the highest quality. They don't look terribly convincing and the input lanes show a lot of background. The bands are also overcropped. They should show at least 6 band widths above and below the band in question. The rest of the manuscript is satisfactory for publication.

2nd Revision - authors' response 26 November 2014

Thanks a lot for your kind help. We have just submitted the final version of all files. I believe we have now addressed all raised issues.

Correspondence - author 04 December 2014

Following our conversation here you have:
- source data for figure 2F
- manuscript including the accession number for the ChIP-Seq data in the methods section
I hope you'll find now the manuscript suitable for publication.
Thank you very much for your help.

Correspondence - editor 05 December 2014

Thank you for sending the new files, which look good.
I have heard back from the referee now, please see her/his comments:

"I re-looked over the data. The controls are actually OK, it is just very hard to follow exactly what they have done. For each IP they state whether or not one protein is added (+ or -) but don't say whether the other
protein was present in both. It would help to add this in the legend
and/or text.
Regarding inputs: Typically inputs for both proteins in the IP are shown.
In all of the experiments in Fig 2F, only one input protein is shown."

Can you please add the first information to the figure legend. Would it be possible that you show the inputs for both proteins for Fig 2F?

Correspondence - author 05 December 2014

In order to clarify our experiments regarding co-immunoprecipitations, we have modified the figure legend of figure 2F, in agreement with the information present in the Expanded View. We think is now clear the way experiments were performed (individual transfections with plasmids containing either Cbt-V5, GAF-flag or Yki-HA and detected in the western blot by antibodies against Cbt, GAF or Yki). By performing these experiments, we understand that inputs for both proteins in the IP could not be shown.
Our apologies if we didn’t explain this properly in previous versions.
Therefore, we are sending back the manuscript with figure legend 2F modified.

3rd Editorial Decision 08 December 2014

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.
Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.