A prominent gene activation role for C-terminal binding protein in mediating PcG/trxG proteins through Hox gene regulation

Cai-Li BI, Qian Cheng, Ling-Yue Yan, Hong-Yan Wu, Qiang Wang, Ping Wang, Lin Cheng, Rui Wang, Lin Yang, Jian Li, Feng Tie, Hao Xie and Ming Fang
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Original submission

First decision letter

MS ID#: DEVELOP/2021/200153

MS TITLE: A prominent gene activation role for C-terminal binding protein in mediating PcG/trxG proteins through Hox gene regulation

AUTHORS: Cai-Li BI, Qian CHENG, Ling-Yue YAN, Hong-Yan WU, Qiang WANG, Ping WANG, Lin CHENG, Rui WANG, Lin YANG, Jian LI, Feng TIE, Hao XIE, and Ming FANG

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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.

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Please attend to all of the reviewers' comments and 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. 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

CtBP has been known to be a transcriptional corepressor. However, the authors found that reduced CtBP functions suppresses PcG homeotic transformation and enhances that of trxG in Drosophila, suggesting the role for CtBP in gene activation. Using cultured Kc cells, they show that CtBP activates many PcG targets including Hox genes upon knockdown of PcG genes. Furthermore, they indicated that CtBP physically interacts with many proteins, such as UTX, CBP, fly homologue of BRD4 and RNA pol II, presumably assisting in their recruitment to the regulatory regions of Hox genes.

Comments for the author

Although their findings are potentially interesting, the manuscript is still premature. The proposed model is quite speculative and lacks sufficient supporting evidence. In addition, some data did not support their claims and lacked proper statistical analyses.
Followings are my comments that would be helpful to improve the manuscript.
1. Figure 1A and 1B, Figure 2A-C They have to specify the mutants exhibiting these phenotypes in the Figure legends.
2. Figure 1C and 1E Why the degree of effect was so different between apparently null mutant CtBP[De10] and deficiency CtBP[Df]?
3. Figure 4D-K, Figure 5B-G, and Figure 6A-B They have to provide p-values for proper comparison. My concern is that some of their claims would be based on data without statistical significance.
4. Page 9, lines 216-219 They described "…, through modulating PcG complexes rather than any specific PRC component." This is too speculative as they provided no evidence for modulation of PcG complexes. They may say "…., not through modulating any specific PRC component.
5. They described "Interestingly, we found depletion of CtBP also produced a clear tendency for decreased CBP binding and H3K27ac…" However, upon knockdown of CtBP alone, the tendency is not clear at bxd PRE, bx PRE etc in Figure 5F and at bx PRE, Antp PRE etc in Figure 5G.
6. Figures 5H-J and 6C-F Curiously all Co-IP experiments displayed positive interactions, although these proteins are not subunits of a known complex. They included a cross-linking step in the Co-IP experiments but this is subject to artificial interactions. I would like to see Co-IP data without crosslinking.
7. Figure 6C Data on the non-phosphorylated form are not shown. They have to show the data.
8. Page 12, lines 292-296 They described "By combining the interactions between RNA pol II Ser2-P …, these results support a model that CtBP is required for active transcription of Hox genes by forming a complex with the epigenetic co-activators…" This is overstatement. To correlate physical interactions with transcriptional activation, they should specify domains of CtBP which are responsible for interaction to CBP and UTX, and examine whether mutations in the interacting domains affect Hox gene activation.
9. Page 18, line 433-page 19, line 435.
They described "An initial protein-protein cross-linking step by … may be included." I don't like to see such an uncertain description in the Methods section. It will be difficult for someone to reproduce their Co-IP experiments.

Reviewer 2

Advance summary and potential significance to field

In the study by Bi et al., the authors have investigated the role of C-terminal binding protein (CtBP), a well-known as transcriptional corepressor, during fly development. By using genetical

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analyses, the authors find that additional loss of CtBP clearly alleviated the phenotype seen in PcG mutant, whereas it enhanced that observed in trxG mutant. Although it is still unclear whether this is a direct or indirect effect of CtBP-loss, the results obtained from genetic analyses suggest corepressor CtBP also works as coactivator during fly development. To further examine the molecular mechanisms by which CtBP enables the PcG-target genes to derepress upon PcG-loss, they perform RNA-seq, ChIP-qPCR and co-IP assays using fly cell lines with knock-down strategy. The authors show that derepression of 73 PcG-target genes including Hox genes that they focused on the study are mostly dependent on the existence of CtBP. They also show that CtBP is required for alterations of histone modification status from H3K27me3 to H3K27ac via recruiting UTX and CBP to both Polycomb Response Elements and promoter regions at derepressed Hox locus caused by PcG-loss, which nicely corresponds to the changes of Hox expression status seen in embryos and cells.
Overall, the work is very interesting, and the genetic approach is particularly well-designed. By genetical approach, the authors clearly showed that CtBP antagonizes and enhances PcG mutant and trxG mutant phenotype, respectively, in dose-dependent manner, while the molecular analysis using fly cell line should be improved. Thus, I would ask the authors to provide more direct evidence which will support their hypothesis especially in the molecular mechanism of CtBP. With these comments described below, I believe the authors would be able to address my concerns, and with edits which are suitable for publication.

Comments for the author

Major Concerns 1) In Figs 3A and 4B, the authors predicted the target genes of CtBP from the alterations in gene expression in RNAi-treated Kc167 cells, but the authors did not assess if these changes were direct or indirect effect of CtBP-knockdown. Identification of CtBP-direct target genes is essential to assess the function of CtBP during fly development. Related to this point, the authors identified 73 genes out of 381 genes as direct target of PcG proteins in Fig. 4A when compared with results in Schwarts et al. (2006) in which the different cell line was used, and then they focused on the 73 genes in the subsequent experiments. I concerned this strategy was strictly biased and it appears that the authors just wanted to focus only on their favorite candidates (Hox genes). Thus, I would ask the authors to identify the direct target genes of CtBP and PcG proteins in Kc167 cells by ChIP-seq analysis and then recategorize genes as “CtBP-associated PcG targets” and “CtBP-unassociated”. In addition to see GO term annotation of the CtBP-target genes, it would be interesting to annotate CtBP-enriched regions in the genome whether CtBP prefers the Polycomb response elements than promoter as the authors showed in Fig. 5B. I think it would be a great addition to the paper.
2) Regarding the results obtained from RNAi experiment, especially in the case of CtBP, the authors did not consider that the function of CtBP as activator for derepression of Hox genes upon PcG-loss was due to the off-target effects of shRNA. To exclude this possibility, I would ask the authors to validate whether the observations from mRNA expression, ChIP and physical interaction of proteins in ph-p+E(z)+CtBP RNAi-treated Kc167 cells with exogenous tagged-CtBP reproduce those in ph-p+E(z) RNAi-treated ones. If tagged-CtBP enrichments are observed at PRE in ph-p+E(z)+CtBP RNAi-treated Kc167 cells with exogenous tagged-CtBP and the physical interaction among exogenous tagged-CtBP, UTX and CBP are detected, these would strongly support the author’s hypothesis.
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Minor Concerns 4) Putting arrowheads showing which of parts is sex combs in Fig. 1A would be better for the readers who are not familiar with fly development.
5) In Fig. 2M-P, I understand Abd-B was not expressed in each mutant, however, black in the picture would make the readers confused. I would suggest the authors to add the result of DAPI staining.
6) I wonder what kinds of effects did knockdown of CtBP or both PcG and CtBP induce the treated cells? Cell proliferation and cell morphology are affected?
7) ChIP-qPCR data is quite difficult to read since all results are put together in the same panel. Furthermore statistical analysis for ChIP-qPCR data should be performed to assess the results

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correctly. I also wonder how many biological replicates were performed. Some ChIP-qPCR data shown in Fig5, Fig6 and Fig S1G did not show error bars, especially in CtBP RNAi-treated and ph-p+E(z) RNAi-treated Kc167 cells. This information should be also properly written in material and methods.
8) It would be nicer to give GEO access link with password for the reviewers, enabling them to analyze the author's NGS data and assess the data analysis.

First revision

Author response to reviewers' comments

POINT TO POINT RESPONSES TO THE COMMENTS OF REVIEWERS

Reviewer 1 Advance Summary and Potential Significance to Field:
CtBP has been known to be a transcriptional corepressor. However, the authors found that reduced CtBP functions suppresses PcG homeotic transformation and enhances that of trxG in Drosophila, suggesting the role for CtBP in gene activation. Using cultured Kc cells, they show that CtBP activates many PcG targets including Hox genes upon knockdown of PcG genes. Furthermore, they indicated that CtBP physically interacts with many proteins, such as UTX, CBP, fly homologue of BRD4 and RNA pol II, presumably assisting in their recruitment to the regulatory regions of Hox genes.

Reviewer 1 Comments for the Author:
Although their findings are potentially interesting, the manuscript is still premature. The proposed model is quite speculative and lacks sufficient supporting evidence. In addition, some data did not support their claims and lacked proper statistical analyses. Followings are my comments that would be helpful to improve the manuscript.

1. Figure 1A and 1B, Figure 2A-C
They have to specify the mutants exhibiting these phenotypes in the Figure legends.
We have done so in the figure legends, Figure1A-B (page 27, line 686-690) and Figure 2A-C (Page 28, Line 710-711).

2. Figure 1C and 1E
Why the degree of effect was so different between apparently null mutant CtBP[De10] and deficiency CtBP[Df]?
We have to admit that we do not understand the phonotypical bias shown in between *CtBP[De10]* and deficiency *CtBP[Df]*. However, we would not see *CtBP[De10]* as an apparent null, *CtBP[De10]* bears a point mutation resulting a coding change from Q299 to stop, could potentially have a truncated form existed. Therefore, we would describe *CtBP[De10]* as a strong allele, i.e. compared with *CtBP[P32]*. *CtBP[Df]*, on the other hand, in addition to the entire C*tBP* sequence deleted, it also lose a number of other genes including *CG7966*, *CG11668*, *CG11670*, *CG46280*, *CG31157*, *Hsc70-2*, *l(3)87Df*, *ry*, *snk*, *CG46281* and CG45122. This difference between the two alleles in molecular nature might explain phonotypical biases seen in these results. In most cases, *CtBP[Df]* shows more dramatic effects than *CtBP[De10]* which fits our estimation that *CtBP[Df]* is a stronger allele. We have also shown a dosage curve of sex comb transformations in revised Figure 1E

3. Figure 4D-K, Figure 5B-G, and Figure 6A-B
They have to provide p-values for proper comparison. My concern is that some of their claims would be based on data without statistical significance.

We have now shown p-values as the reviewer required, either in revised Figure 4, or as a supplemental table 4 for Figures 5-6.

4. Page 9, lines 216-219
They described "..., through modulating PcG complexes rather than any specific PRC component."

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This is too speculative as they provided no evidence for modulation of PcG complexes. They may say "…., not through modulating any specific PRC component.
We have done so accordingly (Page 9, Line 218).

5. They described "Interestingly, we found depletion of CtBP also produced a clear tendency for decreased CBP binding and H3K27ac…" However, upon knockdown of CtBP alone, the tendency is not clear at bxd PRE, bx PRE etc in Figure 5F and at bx PRE, Antp PRE etc in Figure 5G.
We agree with the reviewer that these decreases are too weak to pronounce. We therefore rephrase this part, largely drop the related description and conclusion

6. Figures 5H-J and 6C-F
Curiously all Co-IP experiments displayed positive interactions, although these proteins are not subunits of a known complex. They included a cross-linking step in the Co-IP experiments but this is subject to artificial interactions. I would like to see Co-IP data without crosslinking.
We have now shown a supplemental Figure S4 with co-IP without beforehand cross- linking treatment.

7. Figure 6C
Data on the non-phosphorylated form are not shown. They have to show the data.
We would argue that the non-phosphorylated version of Pol II is not so relevant here. Our purpose of the experiment is to obtain further support for a direct link of CtBP to gene activation, however, non-phosphorylated form of Pol II does not play a role in such process, as shown by the published studies. For example, one study suggest that two serines (serine 2 and serine 5) of the CTD in Pol II are phosphorylated, and sometimes serine 7 is phosphorylated as well. The transcription complexes near the promoter have CTDs in which serine 5 is phosphorylated, but this phosphorylation shifts to serine 2 as transcription progresses (Komarnitsky, P., et al., 2000, DOI: 10.1101/gad.824700.).

8. Page 12, lines 292-296
They described "By combining the interactions between RNA pol II Ser2-P …, these results support a model that CtBP is required for active transcription of Hox genes by forming a complex with the epigenetic co-activators…" This is overstatement. To correlate physical interactions with transcriptional activation, they should specify domains of CtBP which are responsible for interaction to CBP and UTX, and examine whether mutations in the interacting domains affect Hox gene activation.
We have now tone-down the inference as shown in (Page 12, Line 286-288).

9. Page 18, line 433-page 19, line 435.
They described "An initial protein-protein cross-linking step by … may be included." I don't like to see such an uncertain description in the Methods section. It will be difficult for someone to reproduce their Co-IP experiments.

We have now changed the statement as "An initial protein-protein cross-linking step by incubating cells with a 5 mM dimethyl 3,30-dithio-bis (propionimidate) dihydrochloride (DTBP, Sigma-Aldrich) solution for 30-60 min on ice was included in the Co-IP experiments between CtBP and UTX, CBP, as well as Fs(1)h" (Page 18-19, Line 429-432).

Reviewer 2 Advance Summary and Potential Significance to Field:
In the study by Bi et al., the authors have investigated the role of C-terminal binding protein (CtBP), a well- known as transcriptional corepressor, during fly development. By using genetical analyses, the authors find that additional loss of CtBP clearly alleviated the phenotype seen in PcG mutant, whereas it enhanced that observed in trxG mutant. Although it is still unclear whether this is a direct or indirect effect of CtBP-loss, the results obtained from genetic analyses suggest corepressor CtBP also works as coactivator during fly development. To further examine the molecular mechanisms by which CtBP enables the PcG-target genes to derepress upon PcG-loss, they perform RNA-seq, ChIP-qPCR and co-IP assays using fly cell lines with knock-down strategy. The authors show that derepression of 73 PcG-target genes including Hox genes that they focused on the study are mostly dependent on the existence of CtBP. They also show that CtBP is required for alterations of histone modification status from H3K27me3 to H3K27ac via recruiting UTX and CBP to both Polycomb Response Elements and promoter regions at

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derepressed Hox locus caused by PcG-loss, which nicely corresponds to the changes of Hox expression status seen in embryos and cells. Overall, the work is very interesting, and the genetic approach is particularly well-designed. By genetical approach, the authors clearly showed that CtBP antagonizes and enhances PcG mutant and trxG mutant phenotype, respectively, in dose-dependent manner, while the molecular analysis using fly cell line should be improved. Thus, I would ask the authors to provide more direct evidence which will support their hypothesis, especially in the molecular mechanism of CtBP. With these comments described below, I believe the authors would be able to address my concerns, and with edits which are suitable for publication.

Reviewer 2 Comments for the Author:

Major Concerns

1) In Figs 3A and 4B, the authors predicted the target genes of CtBP from the alterations in gene expression in RNAi-treated Kc167 cells, but the authors did not assess if these changes were direct or indirect effect of CtBP-knockdown. Identification of CtBP-direct target genes is essential to assess the function of CtBP during fly development. Related to this point, the authors identified 73 genes out of 381 genes as direct target of PcG proteins in Fig. 4A when compared with results in Schwarts et al. (2006) in which the different cell line was used, and then they focused on the 73 genes in the subsequent experiments. I concerned this strategy was strictly biased and it appears that the authors just wanted to focus only on their favorite candidates (Hox genes). Thus, I would ask the authors to identify the direct target genes of CtBP and PcG proteins in Kc167 cells by ChIP-seq analysis and then recategorize genes as "CtBP-associated PcG targets" and "CtBP-unassociated". In addition to see GO term annotation of the CtBP-target genes, it would be interesting to annotate CtBP-enriched regions in the genome whether CtBP prefers the Polycomb response elements than promoter as the authors showed in Fig. 5B. I think it would be a great addition to the paper.

We totally agree with the reviewer's comments. We were very much to expect a ChIP- seq results of CtBP, which would presumably be able to unambiguously characterize the genome distribution of CtBP and therefore its direct targets. Unfortunately, despite our efforts before and during this revision period, we could not get a good enough result, probably due to the antibody problem. This is the reason why we didn't meet the three- month deadline for the revision.

2) Regarding the results obtained from RNAi experiment, especially in the case of CtBP, the authors did not consider that the function of CtBP as activator for derepression of Hox genes upon PcG-loss was due to the off-target effects of shRNA. To exclude this possibility, I would ask the authors to validate whether the observations from mRNA expression, ChIP and physical interaction of proteins in ph-p+E(z)+CtBP RNAi- treated Kc167 cells with exogenous tagged-CtBP reproduce those in ph-p+E(z) RNAi-treated ones. If tagged- CtBP enrichments are observed at PRE in ph-p+E(z)+CtBP RNAi-treated Kc167 cells with exogenous tagged- CtBP and the physical interaction among exogenous tagged-CtBP, UTX and CBP are detected, these would strongly support the author's hypothesis.

It is a good comment and we agree that the dsRNA-mediated knockdown exists off-target effects. We have previously adequately address this scenario by synthesizing two dsRNAs for CtBP targeting the ORF and 5' UTR of the gene respectively. The knockdown efficiency was confirmed by the previous work of our lab (Fang M et al., 2006, DOI: 10.1038/sj.emboj.7601153). We chose the one with better knockdown efficiency targeting the open reading frame (ORF) of the CtBP gene in this study. Furthermore, the point that we did these RNAi experiments is to collaborate our date in genetic studies, at least we have shown at protein level that CtBP stays in the Hox loci upon derepression and is required for Hox gene expression, which might indicate the role of CtBP is direct. We appreciate your invaluable suggestions on experiments with exogenous tagged-CtBP, however, what we have observed is that CtBP stays in PRE/Promoter in Hox loci rather than been enriched. We sincerely hope these explanations would satisfy the reviewer's concern.

3) In Fig.5 and Fig.6, to reveal the function of CtBP, the authors focused on the change of epigenetic status only at Hox genes as CtBP-associated PcG targets and suggested CtBP is required for the switch from H3K27me3 to H3K27ac via recruitment of CBP and UTX to the target gene loci. Is this specific to Hox loci? It would be better to clarify whether this molecular event is common phenomenon to CtBP-associated PcG targets or not. Furthermore, at CtBP- unassociated

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target loci (8 genes), do the author think if the opposite molecular mechanism, that is, the switch from H3K27ac to H3K27me3 by CtBP is exist as previously described by Kim et al., Stem Cells 2006?

This is an excellent comment. While our work has been mainly focused on homeotic genes, it will be fascinate to know the answer that you ask. However, it is only possible if we could get a successful ChIP-seq data, in which we put a lot of efforts and were without a luck to get one. We figured what you were referring was Kim et al, Stem cell 2015, we have now add a few lines in discussion. (Page 16, Line 371-376)

Minor Concerns

4) Putting arrowheads showing which of parts is sex combs in Fig. 1A would be better for the readers who are not familiar with fly development.

We have added the arrowheads in revised Figure 1.

5) In Fig. 2M-P, I understand Abd-B was not expressed in each mutant, however, black in the picture would make the readers confused. I would suggest the authors to add the result of DAPI staining.

We have added the DAPI staining in revised Figure2.

6) I wonder what kinds of effects did knockdown of CtBP or both PcG and CtBP induce the treated cells? Cell proliferation and cell morphology are affected?

Thanks a lot for the reviewer's constructive comments. Kc cells generally stop proliferate for a few days after RNAi treatment (even for control dsRNA) and there is not much morphology to see due to largely been cultured as floating cells. No apparent difference was seen in cells with different RNAi treatments.

7) ChIP-qPCR data is quite difficult to read since all results are put together in the same panel. Furthermore, statistical analysis for ChIP-qPCR data should be performed to assess the results correctly. I also wonder how many biological replicates were performed. Some ChIP- qPCR data shown in Fig5, Fig6 and Fig S1G did not show error bars, especially in CtBP RNAi- treated and ph-p+E(z) RNAi-treated Kc167 cells. This information should be also properly written in material and methods.

Thanks for your comments and kind advice. We performed at least three biological replicates for each experiment. And the error bars of the ChIP-qPCR data shown in Fig 5, Fig 6 and Fig S1G were too small to display. We have now added the information in the material and methods.

The statements are shown as follows (Page 20, Line 476-477):

For statistical analysis, experiments were performed at least three times. Data was presented as the means ± standard error of means (S.E.M).

8) It would be nicer to give GEO access link with password for the reviewers, enabling them to analyze the author's NGS data and assess the data analysis.

Thanks a lot for your appropriate suggestions. The following secure token has been created to allow review of record GSE88807 while it remains in private status: yzybiyswxrorrwn.

Second decision letter

MS ID#: DEVELOP/2021/200153

MS TITLE: A prominent gene activation role for C-terminal binding protein in mediating PcG/trxG proteins through Hox gene regulation

AUTHORS: Cai-Li BI, Qian CHENG, Ling-Yue YAN, Hong-Yan WU, Qiang WANG, Ping WANG, Lin CHENG, Rui WANG, Lin YANG, Jian LI, Feng TIE, Hao XIE, and Ming FANG

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I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.
As you will see, the referees still have some significant criticism, particularly for statistic processes, and recommend a revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. 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 attend to all of the reviewers' comments and 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. 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

CtBP has been known to be a transcriptional corepressor. However, the authors found that reduced CtBP functions suppresses PcG homeotic transformation and enhances that of trxG in Drosophila, suggesting the role for CtBP in gene activation. Using cultured Kc cells, they show that CtBP activates many PcG targets including Hox genes upon knockdown of PcG genes. Furthermore, they indicated that CtBP physically interacts with many proteins, such as UTX, CBP, fly homologue of BRD4 and RNA pol II, presumably assisting in their recruitment to the regulatory regions of Hox genes.

Comments for the author

The authors revised their manuscript responding to my comments. However, there still remain problems in the manuscript.
Comment 3 They referred to differences in the chromatin state between promoters and PREs in the text (p10, line 234-235 and p12, line 275-278). However, p-values are estimated based on the means of the five loci including promoters and PREs in Table S4. They have to consider the statistical significance more seriously.
Comment 6 I appreciate their efforts for co-IP experiments without crosslinking. However, co-IP of CtBP with anti-CBP
(Figure S4C) is not convincing as the signal is barely detectable. They need to show a reciprocal result (i.e. co-IP of CBP with anti-CtBP) to verify the physical interaction between CtBP and CBP.
Comment 7 Their response is “We would argue that the non-phosphorylated version of Pol II is not so relevant here”.
Then I would suggest to remove “but not the non-phosphorylated form (data not shown)” from the text.
Comment 8 I was disappointed to know that they did not test functional correlation between the physical interactions and transcriptional activation. Instead, they toned-down the inference. Unfortunately, they lost a chance to improve the manuscript.
The corresponding description in the revised text consists of two phrases (By confirming… and suggesting that …) but lacks any subject and predicate verb (p12, line 283-293). English should be corrected.

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Reviewer 2

Advance summary and potential significance to field

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Comments for the author

The authors addressed my questions satisfactorily except for statistical analysis.
In the Supplemental Table 4, it was written that "P values are derived from the comparison of the means of the five loci between the two corresponding treatments by paired t-test."
It is difficult for the reviewer to agree this analysis. Why didn't the author compare the mean at each loci instead of the mean of the five loci? I would ask the authors to show the p-value derived from the comparison at "each" loci and revise the text according to the results of p-value if it is needed.

Second revision

Author response to reviewers' comments

POINT TO POINT RESPONSES TO THE COMMENTS OF REVIEWERS

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We have now done statistics for Figure.5-6 with one-way ANOVA, followed by Tukey's multiple comparison for each locus, and the statistics are shown in revised TableS4. We also revised the text based on our new analysis. (p10, line 248-251).

Comment 6
I appreciate their efforts for co-IP experiments without crosslinking. However, co-IP of CtBP with anti-CBP (Figure S4C) is not convincing as the signal is barely detectable. They need to show a reciprocal result (i.e. co-IP of CBP with anti-CtBP) to verify the physical interaction between CtBP and CBP.
We have done this in Figure S4D.

Comment 7
Their response is “We would argue that the non-phosphorylated version of Pol II is not so relevant here”. Then I would suggest to remove “but not the non-phosphorylated form (data not shown)” from the text.
Thank you for your suggestion and we have revised the manuscript accordingly (p12, line 299-300).

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Comment 8
I was disappointed to know that they did not test functional correlation between the physical interactions and transcriptional activation. Instead, they toned-down the inference. Unfortunately, they lost a chance to improve the manuscript. The corresponding description in the revised text consists of two phrases (By confirming… and suggesting that …) but lacks any subject and predicate verb (p12, line 283-293). English should be corrected.

Thank you for your suggestion and we have revised the manuscript according to this point (p12, line 302-306).

Reviewer 2 Comments for the Author:
The authors addressed my questions satisfactorily except for statistical analysis.
In the Supplemental Table 4, it was written that "P values are derived from the comparison of the means of the five loci between the two corresponding treatments by paired t-test." It is difficult for the reviewer to agree this analysis. Why didn't the author compare the mean at each loci instead of the mean of the five loci? I would ask the authors to show the p-value derived from the comparison at "each" loci and revise the text according to the results of p-value if it is needed.
We have now done statistics for Figure.5-6 with one-way ANOVA, followed by Tukey's multiple comparison for each locus, and the statistics are shown in revised TableS4. We also revised the text based on our new analysis. (p10, line 248-251).

Third decision letter

MS ID#: DEVELOP/2021/200153

MS TITLE: A prominent gene activation role for C-terminal binding protein in mediating PcG/trxG proteins through Hox gene regulation

AUTHORS: Cai-Li BI, Qian CHENG, Ling-Yue YAN, Hong-Yan WU, Qiang WANG, Ping WANG, Lin CHENG, Rui WANG, Lin YANG, Jian LI, Feng TIE, Hao XIE, and Ming FANG

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

The same as my previous review.

Comments for the author

The authors satisfactorily responded to my comments and revised the manuscript.

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<u>Reviewer 2</u>

Advance summary and potential significance to field

-

Comments for the author

The authors revised the statistical analysis for ChIP-qPCR data according to the reviewer's requirement however, I think the revision of text based on ChIP-qPCR analysis was not done sufficiently. I would ask the authors to revise the text as followings:

Line 244:
In Supplemental Table 4, the adjusted p-value for PRE (bx) and the p-value of ANOVA for PRE (bxd) are not significant, thus the author's expression in line 244 "dramative decrease in CtBP ChIP signals at <all of these sites>" was not reflected their results of statistical analysis correctly. This sentense should be revised as "CtBP RNAi alone cause dramatic decreases in CtBP ChIP signals at <some regulatory sites for Antp and Ubx>", for example.

Lines 253-254:
Since there were no significant changes in UTX binding levels at all sites when PcG RNAi compared with control RNAi if my understanding for Supplemental Table 4-4 is correct, the word "UTX" should be removed from the sentence "with higher levels of CBP and UTX bindings and H3K27ac modifications (lines 253-254)".
And Supplemtental Table 4-4 showed almost no significant change of UTX levels, thus it would be an option to move Fig 5E to Supplemental Figure, I think.

Lines 263-264:
Because the significant alterations in UTX bindings was only observed at PRE of bx when PcG RNAi compared with CtBP RNAi or PcG/CtBP RNAi with PcG RNAi, the sentence "One possible explanation could be that CtBP helped the recruitment of UTX, the major H3K27me3 demethylase, to the derepressed Hox sites. (lines 263-264)" would mislead the readers.
I would suggest the authors to revise it as "One possible explanation could be that CtBP, at least partially contributed the recruitment of UTX, the major H3K27me3 demethylase, to the specific regularoty site, such as PRE of bx."

Lines 268-269:
Likewise in the above comments, based on the results of statistical analysis, the sentense "loss of CtBP caused moderate reductions of Pc and H3K27me3 levels (lines 268-269)" would be better to revise as "loss of CtBP caused moderate reduction of Pc and subtle decrease of H3K27me3 level without significant difference, when the Hox genes ...".

Lines 289-291:
The sentence "Knock down of PcG caused remarkable increase of αCTD signals in the proximal promoter regions (Figure 6A) and αSer2-P signals in both PREs and promoters (Figure 6B)," would be better to revise as "Knock down of PcG caused remarkable increase of αCTD signals in the proximal promoter region of Ubx (Figure 6A) and αSer2-P signals in both PREs and promoters of Antp and bxd (Figure 6B),"., for example.

Lines 303-304:
"CtBP may be directly required and achieve" is "CtBP may be directly required to achieve"?

The statistical analysis for ChIP-qPCR by using ANOVA followed by Tukey's multiple comparison tests (Supplemental Table S4) should be written in Mateial Methods and cited in the Main text and Figure legends.

Suppemental Fig 4 is not cited in the Main text.

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Third revision

Author response to reviewers' comments

POINT TO POINT RESPONSES TO THE COMMENTS OF REVIEWERS

Reviewer 1 Advance Summary and Potential Significance to Field:
The same as my previous review.

Reviewer 1 Comments for the Author:
The authors satisfactorily responded to my comments and revised the manuscript.
Thank you very much for your careful review.

Reviewer 2 Advance Summary and Potential Significance to Field:

Reviewer 2 Comments for the Author:
The authors revised the statistical analysis for ChIP-qPCR data according to the reviewer's requirement, however, I think the revision of text based on ChIP-qPCR analysis was not done sufficiently. I would ask the authors to revise the text as followings:

Line 244:
In Supplemental Table 4, the adjusted p-value for PRE (bx) and the p-value of ANOVA for PRE (bxd) are not significant, thus the author's expression in line 244 "dramative decrease in CtBP ChIP signals at sites>" was not reflected their results of statistical analysis correctly. This sentense should be revised as "CtBP RNAi alone cause dramatic decreases in CtBP ChIP signals at Ubx>", for example.
We have revised the manuscript accordingly. (p 10, line 239)

Lines 253-254:
Since there were no significant changes in UTX binding levels at all sites when PcG RNAi compared with control RNAi if my understanding for Supplemental Table 4-4 is correct, the word "UTX" should be removed from the sentence "with higher levels of CBP and UTX bindings and H3K27ac modifications (lines 253-254)". And Supplemtental Table 4-4 showed almost no significant change of UTX levels, thus it would be an option to move Fig 5E to Supplemental Figure, I think.
Thank you very much for your careful reading. We find it is weird that the significance in Figure5E is more obvious than Figure5F, but the statistical results are not. So we rechecked all the statistics and found that the statistics for Figure5E (Supplemental Table 4-4) were wrong, probably due to a copying error, and we have revised Supplemental Table 4-4. We also checked the other statistics and there are no more errors. We are so sorry for the trouble caused by this error. In that case, we don't think this sentence needs to be revised.

Lines 263-264:
Because the significant alterations in UTX bindings was only observed at PRE of bx when PcG RNAi compared with CtBP RNAi or PcG/CtBP RNAi with PcG RNAi, the sentence "One possible explanation could be that CtBP helped the recruitment of UTX, the major H3K27me3 demethylase, to the derepressed Hox sites. (lines 263-264)" would mislead the readers. I would suggest the authors to revise it as "One possible explanation could be that CtBP, at least partially, contributed the recruitment of UTX, the major H3K27me3 demethylase, to the specific regularoty site, such as PRE of bx."
Same reason as above

Lines 268-269:
Likewise in the above comments, based on the results of statistical analysis, the sentense "loss of CtBP caused moderate reductions of Pc and H3K27me3 levels (lines 268-269)" would be better to revise as "loss of CtBP caused moderate reduction of Pc and subtle decrease of H3K27me3 level without significant difference, when the Hox genes ...".
We have revised the manuscript accordingly. (p 11, line 265)

Lines 289-291:
The sentence "Knock down of PcG caused remarkable increase of αCTD signals in the proximal promoter regions (Figure 6A) and αSer2-P signals in both PREs and promoters (Figure 6B)," would be

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better to revise as "Knock down of PcG caused remarkable increase of αCTD signals in the proximal promoter region of Ubx (Fig 6A) and αSer2-P signals in both PREs and promoters of Antp and bxd (Figure 6B),"., for example.
We have revised the manuscript accordingly. (p 12, line 287-288)

Lines 303-304:
"CtBP may be directly required and achieve" is "CtBP may be directly required to achieve"? The statistical analysis for ChIP-qPCR by using ANOVA followed by Tukey's multiple comparison tests (Supplemental Table S4) should be written in Mateial Methods and cited in the Main text and Figure legends.
We have revised the manuscript accordingly. (p 12, line 300)
We have added the method of statistical analysis in Mateial Methods. (p 22, line 500-501)
We have cited Supplemental Table S4 in the Main text and Figure legends.

Suppemental Fig 4 is not cited in the Main text.
We have cited Supplemental Figure S4 in the Main text.

Fourth decision letter

MS ID#: DEVELOP/2021/200153

MS TITLE: A prominent gene activation role for C-terminal binding protein in mediating PcG/trxG proteins through Hox gene regulation

AUTHORS: Cai-Li BI, Qian CHENG, Ling-Yue YAN, Hong-Yan WU, Qiang WANG, Ping WANG, Lin CHENG, Rui WANG, Lin YANG, Jian LI, Feng TIE, Hao XIE, and Ming FANG
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

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