nature portfolio

Peer Review File

Calcium modulates the tethering of BCOR-PRC1.1 enzymatic core to KDM2B via liquid-liquid phase separation

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Chen et al. have demonstrated the crystal structure of the BCOR/PCGF1/KDM2B complex and provided a model for the BCOR/PCGF1/KDM2B/SKP1 tetramer, supported by mutagenesis experiments. They also suggest a hypothesis regarding the potential relevance of BCOR/PCGF1 liquid-liquid phase separation (LLPS) to PRC1 function in vivo. While the structural work appears more solid, the LLPS findings warrant further investigation.

Major Points:

1. The LLPS studies were conducted using truncated domains. It remains to be determined whether full-length proteins can also undergo phase separation. Additionally, the observed condensates derive from overexpressed truncated proteins in the cases of in vitro and in cells, raising questions about their biological relevance.
2. The impact of the BCOR WFY/A mutation on PRC1's enzymatic activity or chromatin binding affinity has not been assayed in vitro. Additionally, the derepression of Hox genes shown in Figure 6H could be due to indirect effects. ChIP of PRC1 subunits and H2AK119ub might clarify this.
3. Replicates were not performed for ITC and WB experiments. For Figure 6H, it is unclear whether the error bars represent biological or technical replicates.
4. In Figure 5A, are they the correct images? and this also applies to the top two panels of Figure 6F.

Minor Points:

1. “The determined Kd values for BCORN1607/PCGF1RAWUL dimer, BCORN1612/PCGF1RAWUL dimer and BCORN1624 /PCGF1RAWUL dimer to KDM2B/SKP1 are 0.29 μM, 0.77 μM and 4.5 μM, respectively (Fig.1D).”, based on the figure, the 0.77 should be 0.97.
2. The top panel of Figure 5B is not clear.
3. The plot in Figure 6G appears to be truncated.

Reviewer #2 (Remarks to the Author):

The study titled "Calcium modulates the tethering of BCOR-PRC1.1 enzymatic core to KDM2B via liquid-liquid phase separation" provides a comprehensive analysis of how calcium influences the recruitment of the non-canonical BCOR-PRC1.1 complex to non-methylated CpG islands through KDM2B. Employing structural methods, the research successfully models the BCOR/PCGF1/KDM2B/SKP1 hetero-tetramer and underscores the importance of BCOR's Poly-D/E regions in its interaction with KDM2B. The authors claim that calcium, by neutralizing the negative charges within these regions, reduces the interaction between BCOR/PCGF1 and KDM2B, facilitating the co-condensation of the BCOR-PRC1.1 enzymatic core with KDM2B via a liquid-liquid phase separation (LLPS) mechanism. This study advances our understanding of how calcium modulates BCOR-PRC1.1 enzymatic core recruitment on KDM2B target loci, shedding light on the regulatory mechanisms affecting BCOR-PRC1.1 and its involvement in transcriptional regulation during development and cancer progression.

Comments:

- The Kd values presented lack error bars. It is crucial for the authors to specify in the figure legend whether the ITC experiments were conducted multiple times. If so, details on whether these measurements were performed in duplicate or triplicate should be provided, along with the corresponding error bars. This level of detail is essential, as the paper's key conclusions are drawn from these ITC measurements.

- The calcium concentrations utilized in Figures 4A and B appear exceedingly high, raising concerns about their physiological relevance. The elevated calcium levels used in Figure 4C further complicate the physiological relevance of calcium's role in disrupting the protein-protein interactions. It may be worthwhile to consider magnesium (Mg^{2+}) as a potential divalent cation disrupting these interactions, especially given its higher physiological concentration compared to calcium. I recommend conducting parallel experiments with Mg^{2+} to validate the findings.

- The statement that calcium is the divalent cation responsible for modulating the BCOR-PRC1.1 complex is not entirely convincing, especially since magnesium (Mg^{2+}) could produce similar effects, albeit at higher concentrations. This point needs further exploration to conclusively establish the specific role of calcium in this context. I suggest the authors to write about Mg^{2+} in the discussion section.

Major Points

(1) The LLPS studies were conducted using truncated domains. It remains to be determined whether full-length proteins can also undergo phase separation. Additionally, the observed condensates derive from overexpressed truncated proteins in the cases of in vitro and in cells, raising questions about their biological relevance.

A：We thank the reviewer for the comments. Several literatures had revealed that overexpressed BCOR could form condensates in live cells (Huynh KD., et al. Genes Dev. 2000, 14(14):1810-1823; Buchberger E., et al. PLoS One. 2013, 8(10):e76845). Our unpublished data also demonstrated that condensates of endogenous BCOR can be detected by immunofluorescence (unpublished Figure1).

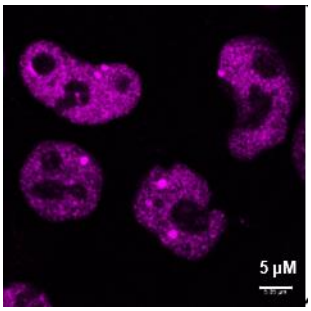


Figure1 Immunofluorescence for endogenous BCOR condensates in HeLa cells (Unpublished data)

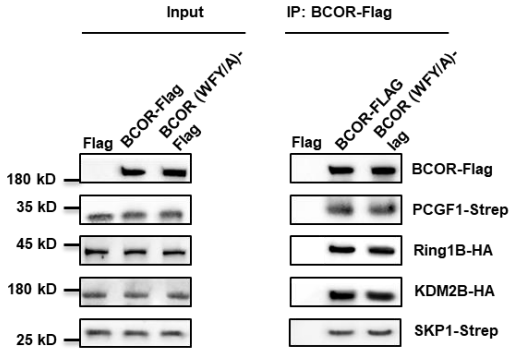
(2) The impact of the BCOR WFY/A mutation on PRC1's enzymatic activity or chromatin binding affinity has not been assayed in vitro. Additionally, the derepression of Hox genes shown in Figure 6H could be due to indirect effects. ChIP of PRC1 subunits and H2AK119ub might clarify this.

A：We thank the reviewer for the comments. Our ITC data have confirmed that BCOR$^{\text{ANK-linker-PUFD}}$/PCGF1$^{\text{RAWUL}}$ binding to KDM2B$^{\text{F-box-LRRs}}$/SKP1 cannot be altered by mutating aromatic residues (WFY) on linker of BCOR to alanine (Fig 6G in the manuscript). Moreover, in the revised manuscript, our Co-IP experiment further confirmed that the aromatic residues (WFY) on linker of BCOR are not necessary for the affinity of enzymatic core of BCOR-PRC1.1 binding to KDM2B (Supplementary Fig. 7, see below).

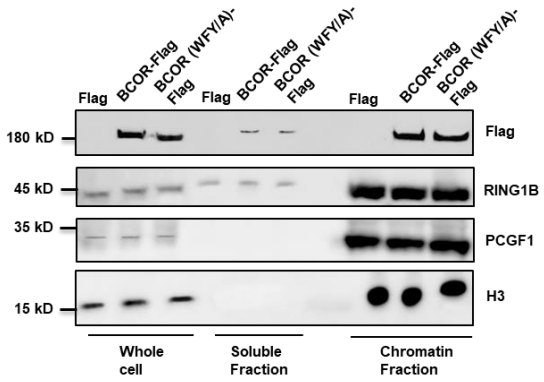
To further investigate BCOR WFY/A mutation on chromatin binding affinity of core subunits of BCOR-PRC1.1, we performed subcellular fractionation isolation analysis for subunits of BCOR-PRC1.1 in the presence of mutated BCOR. As shown in Supplementary Fig. 8, the chromatin binding of Ring1B and PCGF1 cannot be impaired by mutating aromatic residues (WFY) on linker of BCOR.

With all these data, we can conclude that BCOR WFY/A mutation induced de-repression of Hox genes is caused by effecting LLPS of BCOR/PCGF1, but not disrupting the assembly or chromatin affinity of

BCOR-PRC1.1.



Supplementary Figure 7. The importance of aromatic residues (WFY) on linker for BCOR binding to KDM2B/SKP1 is assessed using Co-IP assay. Expressing plasmids for BCOR (wild or mutant), PCGF1, KDM2B or SKP1, were co-transfected into HEK293T cells. Co-IP was performed with anti-Flag magnetic beads, after 48h transfection. The western-blotting data is representative of two independent experiments.



Supplementary Figure 8. The subcellular localizations of BCOR, RING1B and PCGF1 are determined by a biochemical fractionation assay in 293T cells upon transient expression of the Flag-tagged BCOR (wild type or mutant). Whole-cell extracts, the soluble and chromatin-binding fractions are analyzed by western-blotting. The western-blotting data is representative of two independent experiments.

(3) Replicates were not performed for ITC and WB experiments. For Figure 6H, it is unclear whether the error bars represent biological or technical replicates.

A：We thank the reviewer for the comments. In the revision all ITC measurements were performed in triplicate. The WB experiments were in triplicate (Fig 1E-F, and Fig 4A-D) or duplicate (Supplementary Fig. 7 and 8). For Figure 6H, the error bars represent biological replicates.

(4) In Figure 5A, are they the correct images? and this also applies to the top two panels of Figure 6F.

A：We thank the reviewer for the comments. We have double-checked the figures, the Figure5A is not the same as Figure 6F.

Minor Points:

(1) “The determined Kd values for $BCOR^{N1607}$/$PCGF1^{RAWUL}$ dimer, $BCOR^{N1612}$/$PCGF1^{RAWUL}$ dimer and $BCOR^{N1624}$/$PCGF1^{RAWUL}$ dimer to KDM2B/SKP1 are 0.29 μM, 0.77 μM and 4.5 μM, respectively (Fig.1D).”, based on the figure, the 0.77 should be 0.97.

A：We apologize for the mistake. We have corrected it in the revised manuscript.

(2) The top panel of Figure 5B is not clear.

A：We thank the reviewer for the comments. The clarity for that figure has been improved in the revised manuscript.

(3) The plot in Figure 6G appears to be truncated.

A：We thank the reviewer for the comments. This figure panel has been improved in the revised manuscript.

Reviewer 2

(1) The Kd values presented lack error bars. It is crucial for the authors to specify in the figure legend whether the ITC experiments were conducted multiple times. If so, details on whether these measurements were performed in duplicate or triplicate should be provided, along with the corresponding error bars. This level of detail is essential, as the paper’ s key conclusions are drawn from these ITC measurements.

A：We thank the reviewer for the comments. In the revised manuscript, all ITC measurements were performed in triplicate. The K_d values are represented as mean ± SD for triplicate experiments.

(2) The calcium concentrations utilized in Figures 4A and B appear exceedingly high, raising concerns about their physiological relevance. The elevated calcium levels used in Figure 4C further complicate the physiological relevance of calcium's role in disrupting the protein-protein interactions. It may be worthwhile to consider

magnesium (Mg2+) as a potential divalent cation disrupting these interactions, especially given its higher physiological concentration compared to calcium. I recommend conducting parallel experiments with Mg2+ to validate the findings.

A：We thank the reviewer for the comments. In the revised manuscript, we also performed co-IP to experiments to evaluate the impact of magnesium on BCOR/PCGF1 binding to KDM2B/SKP1. The binding affinity of KDM2B and BCOR/PCGF1 was significantly reduced in the presence of Mg^{2+} in IP buffer with calcium concentration higher than 300 μM (Fig 4A-B). Indeed, with higher physiological concentration, Mg^{2+} is also a potential divalent cation disrupting this interaction, although is inhibitory potency is weaker than Ca^{2+} (Fig 4)。

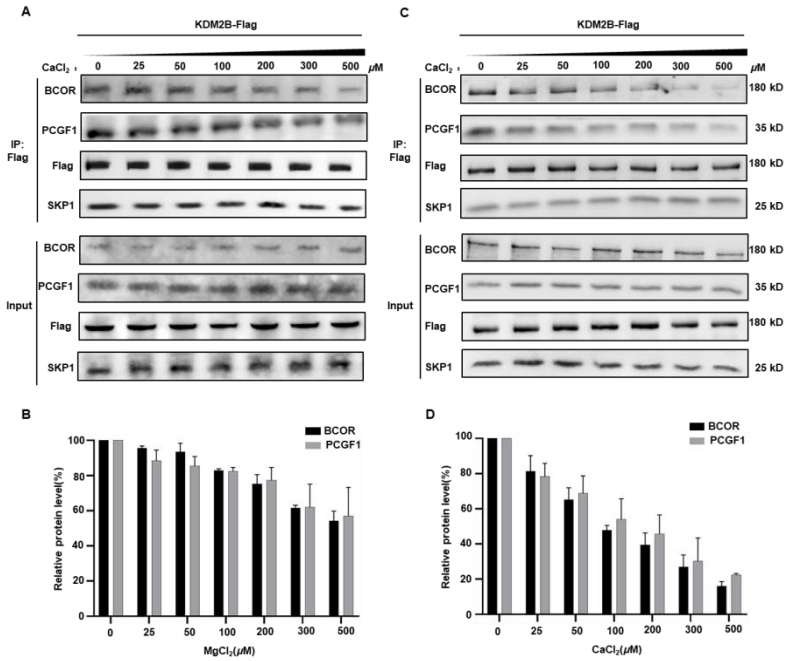


Figure 4. Effect of magnesium ion or calcium ion on BCOR binding to KDM2B. (A) Analyzing the effect of magnesium ion on over-expressed KDM2B-Flag binding to endogenous BCOR, PCGF1 and SKP1 using Co-IP assay. The western-blotting data is representative of three independent experiments. (B) Relative level of BCOR or PCGF1 protein co-purified with KDM2B-Flag as shown in (A). Data are represented as mean ± SD for triplicate experiments. (C) Analyzing the effect of calcium on over-expressed KDM2B-Flag binding to endogenous BCOR, PCGF1 and SKP1 using Co-IP assay. The western-blotting data is representative of three independent experiments. (D) Relative level of BCOR or PCGF1 protein co-purified with KDM2B-Flag as shown in (C). Data are represented as mean ± SD for triplicate experiments. Expressing plasmid for KDM2B-Flag was transfected into HEK293T cells. Co-IP was performed with anti-Flag magnetic beads, after 48h transfection. $MgCl_2$ or $CaCl_2$ at indicated concentration is supplemented to IP buffer.

(3) The statement that calcium is the divalent cation responsible for modulating the BCOR-PRC1.1 complex is not entirely convincing, especially since magnesium (Mg2+) could produce similar effects, albeit at higher concentrations. This point needs further exploration to conclusively establish the specific role of calcium in this context. I suggest the authors to write about Mg2+ in the discussion section.

A：We thank the reviewer for the suggestion. In the revised manuscript, we discussed the role of Mg^{2+}. "Our ITC experiments and Co-IP assays clearly showed that BCOR/PCGF1 interaction with KDM2B could also be weakened by Mg^{2+}. In additional, the LLPS of $BCOR^{ANK\text{-}linker\text{-}PUFD}$/$PCGF1^{RAWUL}$ also can be induced by high concentration of Mg^{2+}. Unlike calcium, free magnesium is abundant in cellular, with concentration in the range of 203.68 to 673.50 μM (in platelets). This suggests that Ca^{2+} may cooperate with Mg^{2+} to reduce the affinity of enzymatic core of BCOR-PRC1.1with KDM2B, and to trigger the LLPS of $BCOR^{ANK\text{-}linker\text{-}PUFD}$/$PCGF1^{RAWUL}$".

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Thanks to the authors for their efforts, most of my comments have been resolved. However, regarding Fig 5A, could you please confirm if we are supposed to see the black field there?

For the experiments with replicates, if a representative is shown, please ensure to add all other replicates to the supplementary figures or supplementary datasets.

Reviewer #2 (Remarks to the Author):

The authors addressed my questions and concerns. I recommend it for publication.

Reviewer 1:

Q (1): Thanks to the authors for their efforts, most of my comments have been resolved. However, regarding Fig 5A, could you please confirm if we are supposed to see the black field there?

A: We thank the reviewer's comments. We double checked the Fig5A, we realized that the Fig 5A was presented as black field in the tracked-change version of the manuscript due to the format conversion error (from MS word to PDF), while the Fig5A was correctly presented in the clean version manuscript. We apologized for the mistake. In this current submission, we double check and make sure that there is no error in the conversion, though the content of the manuscript has not changed.

Q (2): For the experiments with replicates, if a representative is shown, please ensure to add all other replicates to the supplementary figures or supplementary datasets.

A: We thank the reviewer's comments. Replicated ITC data were provided in supplementary data1. Replicated western-blot data were provided in supplementary data2.