Phospho-KNL-1 recognition by a TPR domain targets the BUB-1-BUB-3 complex to C. elegans kinetochores

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RE: JCB Manuscript #202402036

Dr. Arshad Desai
University of California, San Diego
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Dear Dr. Desai:

Thank you for submitting your manuscript entitled "Phospho-KNL-1 recognition by a TPR domain targets the BUB-1-BUB-3 complex to C. elegans kinetochores". Your study has been assessed by three expert reviewers whose comments are appended below. Based on their feedback we would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below) along with a response to their minor comments.

As you will see, the reviewers are all very positive about the significance and quality of your study. Please respond to all of their comments in your final version with text revisions as appropriate. While we would welcome new data and can provide additional time if you would like to conduct any suggested experiments, this is not necessary.

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Sincerely,

Aaron Straight
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Reviewer #1 (Comments to the Authors (Required)):

This interesting manuscript by Houston and colleagues focuses on the role of the tetratricopeptide repeat (TPR) domain in the localization of BUB-1, a kinase involved in the spindle assembly checkpoint, in biorientation, and in the protection of sister chromatid cohesion.

The authors find that the BUB-1 TPR recognizes a phosphorylated motif in KNL-1, a kinetochore scaffold protein, and is essential for kinetochore localization of the BUB-1-BUB-3 complex. This finding is interesting and novel, because the current model of recruitment of BUB1 to kinetochores in various model systems emphasizes a crucial role of its tightly bound phospho-aminoacid adaptor BUB3, which is known to bind different phosphorylated motifs known as MELT. The KNL-1 motifs additionally characterized here, the TF motifs, are substrates of PLK-1 (like the MELT motifs), and the authors demonstrate that a 2-point high-affinity interaction engaging at the same time the BUB-1 TPR domain and BUB-3 on KNL-1 is required for super-stoichiometric recruitment of BUB-1 to kinetochores.

Understanding the kinetochore recruitment mechanism of BUB-1 and of other kinetochore regulators is very important for their functional dissection. By revealing a significantly more nuanced view of the mechanism of kinetochore recruitment of BUB-1 in C. elegans, an established model system for cell division, this study explains why loss of BUB-1 is more severe than loss of BUB-3. The experiments are technically excellent and nicely controlled (e.g. panels 1C-D). The conclusions are persuasive, the illustrations very clear, and the text accessible and logical. Overall, I consider this manuscript a very strong candidate for the JCB and recommend its publication essentially in its present form.

Minor points:

Figure 3B, the direction of the 90º rotation is opposite to the one indicated

Reviewer #2 (Comments to the Authors (Required)):

In this study, Houston et al. have identified the N-terminal TPR domain is the primary kinetochore targeting motif in C.elegans BUB1. This is in contrast to the prevailing dogma that BUB3 directs BUB1 to kinetochores through binding phosphorylated MELT motifs. The authors used AlfaFold to identify a motif on KNL1 that binds the TPR and show that this depends on PLK1 phosphorylation and is necessary for proper chromosome segregation. This finding could have broader implications because the TPR motif in BUB1 is highly conserved.

As expected from the Desai lab, this is a very solid piece of work. The data are admirably clear and convincing and all the appropriate controls are included. I recommend publication as the paper stands.

Reviewer #3 (Comments to the Authors (Required)):

This manuscript reports a very interesting study of how the Bub1 TPR domain interacts directly with phosphorylated TF motifs in the kinetochore protein KNL1, in nematode worms. These motifs are interspersed with, but distinct from, the well known MELT motifs in KNL1 that are bound by Bub3.
Whilst these TF motifs may not be very widely conserved, beyond nematodes, they do provide an interesting mechanism for TPR domain function and phosphoreader function. As a consequence, this mechanism will be of interest far beyond the kinetochore/mitosis/checkpoint signalling communities.

The data are excellent and extremely clearly presented and the paper is very well written. I have only minor comments to be addressed:

1) Fig 2E. It is not clear why the C.elegans Bub1 TPR should interact with a novel motif in the same way as human Bub1 interacts with the KI motif. The mutations made have a clear impact and phenotype, but might that not be true of many other miutations made in the TPR fold?

2) Fig 2G. Where do these mutant Bub1 proteins localize?

3) Fig 3B: why alphafold 'multimer' - isn't this simply a 1:1 complex?

4) I find the phrase super-stoichiometric somewhat confusing here (page 9-10). Could it be that (on average) only one molecule of Bub1 binds directly via the KNL1 TF motifs and the other 4 via Bub3-MELTs on each molecule of KNL1?
If so, why are there 6 TF motifs? Is one more important than the others?

The authors model in 5D is quite different to this, with a single Bub1 molecule interacting with both TF (directly) and MELT (indirectly via Bub3) motifs. Can they provide direct biophysical evidence for this higher affinity interaction?

Related to their proposed mechanism: do we know how many of the TF and MELT motifs get phosphorylated by Plk1 in a single molecule of KNL1? From mass-spec analysis of intact KNL1 molecules.

5) Discussion: the authors propose that T motifs in human KNL1 may carry out a similar function to the nematode TF motifs: can they provide any evidence for this? Has anybody mutated these motifs and reported a phenotype?

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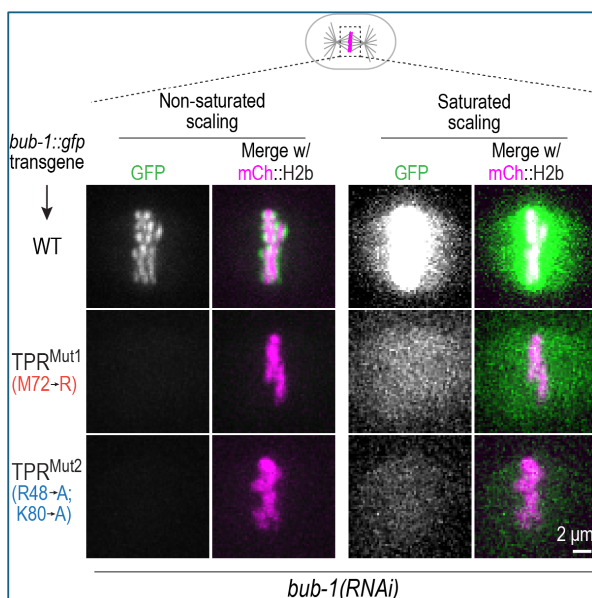
We designed the BUB-1 TPR mutants prior to the introduction of Alphafold and based them on comparing a structural model of the *C. elegans* TPR to the structure of the human Bub1 TPR-KI1 complex. Given the results with the ΔTPR and TPR-only fragments, we endeavored to design mutants on the convex surface of the TPR (M72R and R48A;K80A) that potentially disrupted an interaction analogous to the Bub1-KI1 interaction in human cells. We additionally generated and screened E63A, R67A and K99A mutants on the concave TPR surface. Only the 2 mutants on the convex surface disrupted BUB-1 localization. As Alphafold modeling, conducted after we obtained results with the TPR mutants, revealed an interface similar to the Bub1-KI1 motif interface on the convex surface of the TPR, we did not analyze the concave surface mutants in sufficient depth to include in the manuscript. Instead, we focused on analyzing the interface from the KNL-1 side, by conducting parallel in vivo and biochemical analysis of the TF motifs.



2) Fig 2G. Where do these mutant Bub1 proteins localize?

The BUB-1 mutants do not exhibit chromosomal localization but are weakly enriched in the nuclear/spindle region (*see images to the left*).

3) Fig 3B: why alphafold 'multimer' - isn't this simply a 1:1 complex?

The term was based on the name for the implementation of Alphafold used to generate models of protein complexes, instead of single polypeptide chains (https://doi.org/10.1101/2021.10.04.463034). To avoid confusion, we have removed “multimer” from the label.

4) I find the phrase super-stoichiometric somewhat confusing here (page 9-10). Could it be that (on average) only one molecule of Bub1 binds directly via the KNL1 TF motifs and the other 4 via Bub3-MELTs on each molecule of KNL1?

Our analysis of the TPR mutants in the presence of endogenous BUB-1 indicated that these mutants, which do not alter the BUB-3 binding interface, nonetheless fail to localize (**Fig. S1E**). This observation excludes the possibility raised by the reviewer and indicates that TPR engagement of each individual BUB-1 molecule with a TF motif on KNL-1 is required for localization. This conclusion is consistent with BUB-3 not localizing significantly when the BUB-1 TPR was unable to engage with the KNL-1 TF motifs (**Fig. 4A**).

If so, why are there 6 TF motifs? Is one more important than the others?

In light of the requirement of TPR domain engagement with a TF motif for individual BUB-1 molecule localization (*see response to first set of comments in point 4 above*), our data quantifying *in situ* GFP-tagged proteins suggest that ~5 of the TF motifs in each KNL-1 molecule must engage BUB-1–BUB-3 complexes. In terms of importance, we have previously shown that partial truncations of the KNL-1 N-terminus that remove different sets of TF motifs exhibited partial BUB-1 localization (PMID: 24567362). These data, interpreted in light of what we elucidate in the current manuscript, indicate that there may be a greater contribution of TF motifs 4,5 & 6 to BUB-1 localization. However, we would need to repeat these analysis with the significantly better probes (such as *in situ*-tagged GFP::BUB-1) and believe this is best done in future work that combines such an effort with mutation of individual TF motifs.

The authors model in 5D is quite different to this, with a single Bub1 molecule interacting with both TF (directly) and MELT (indirectly via Bub3) motifs. Can they provide direct biophysical evidence for this higher affinity interaction?

The model is based on three pieces of data: TPR interaction with a TF motif is required for each BUB-1 molecule to localize (*see response to first set of comments in point 4 above*), mutating BUB-3's ability to recognize pMELT motifs prevents the rapid increase in localization, and preventing PLK-1 docking on BUB-1 prevents the rapid increase in localization. These observations are best reconciled by the model that is depicted in which a TF motif is phosphorylated by a PLK-1 pool distinct from BUB-1-bound PLK-1 that, via the TPR interface, brings BUB-1-associated PLK-1 activity to drive phosphorylation of MELT and possibly other TF motifs. Engagement of BUB-3 with the pMELT motif leads to a higher affinity bound state that enables rapid increase as each TF motif + MELT motif recruits a BUB-1-BUB-3 complex.

We do not currently have the tools (a reconstituted *C. elegans* BUB-1–BUB-3 complex) to test the two-point interaction biochemically but note that this has been shown for human Bub1-Bub3 engagement with KI and pMELT motifs (see *Fig. 2E* of PMID: 24361068).

Related to their proposed mechanism: do we know how many of the TF and MELT motifs get phosphorylated by Plk1 in a single molecule of KNL1? From mass-spec analysis of intact KNL1 molecules.

We have conducted proteolysis-based mass spec and detected phosphorylation of 4 TF motifs (see *Fig. S3E*). Unfortunately, a major challenge with mass spec analysis of KNL-1 is the absence / failure to detect a large part of the phosphorylated protein. We suspect this is because it is heavily phosphorylated by PLK-1 (see the shift in electrophoretic mobility in *Fig. 3F*

& G). We additionally show mutation of the threonine in TF motifs to nonphosphorylatable alanines enhanced electrophoretic mobility following PLK-1 phosphorylation (*Fig. 3G*), which is evidence for phosphorylation of these residues.

5) Discussion: the authors propose that T motifs in human KNL1 may carry out a similar function to the nematode TF motifs: can they provide any evidence for this? Has anybody mutated these motifs and reported a phenotype?

In the context of the 3-MELT repeat fragment of human Knl1 analyzed by Vluegel et al. (PMID: 25661489), no mutation of the hydrophobic residue of the TΩ was analyzed. They did mutate the T and an S that follows the hydrophobic residue to alanines and showed that, when monitoring kinetochore localization, these alanine mutations enhanced mutation of the “SHT” motif immediately C-terminal to the MELTs that is recognized by human Bub3. As the SHT mutant by itself significantly compromised checkpoint signaling, no functional analysis of the TΩ motifs was conducted and, to our knowledge, has not been pursued in subsequent work.