The AAA+ ATPase TRIP13 remodels HORMA domains through N-terminal engagement and unfolding

Qiaozhen Ye, Dong Hyun Kim, Ihsan Dereli, Scott C. Rosenberg, Goetz Hagemann, Franz Herzog, Attila Tóth, Don W. Cleveland, and Kevin D. Corbett

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Review timeline:

Submission date: 07 May 2017
Editorial Decision: 22 May 2017
Revision received: 30 May 2017
Accepted: 06 June 2017

Editor: Hartmut Vodermaier

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.)

1st Editorial Decision 22 May 2017

Thank you for submitting your manuscript on HORMA domain remodeling by TRIP13 for our editorial consideration. It has now been seen by two expert referees, and I am happy to inform you that both of them consider this work well-executed and important. They do raise a number of specific issues, which I hope should be straightforward to address during a revision of this manuscript. Pending adequate revisions, we shall be pleased to accept the manuscript for publication in The EMBO Journal.

REFEEEREE REPORTS

Referee #1:

This is a lovely manuscript that sheds light on the mechanism of the interaction of the AAA ATPase Trip13 and its co-factor p31comet with the HORMA domain containing protein Mad2. By combining a variety of structural, biochemical, and biophysical methods, the authors identify the N-terminal region of Mad2 as a crucial target of the ATPase required to convert closed Mad2 to open Mad2, a reaction that may be required for regeneration of the pool of open Mad2 required for sustained checkpoint signaling. Besides testing the effects of N-terminal deletion mutants of Mad2 on the checkpoint response in vivo, with results that confirm the importance of this region for the Mad2 conversion, the authors go on to confirm the generality of their conclusions on a second HORMA domain protein, HORMAD1. They then extend this analysis to other HORMA domain proteins and predict that Rev7 and Atg13 may not be targets of Trip13 as they lack a network of hydrogen bonds that the authors implicate in the stabilization of closed Mad2.

Besides the few points discussed below, I feel that the manuscript meets all the standards required for publication in EMBO Journal. The work is excellent, thorough, and well illustrated. The
methodological pipeline and detailed mechanistic insight reported in the manuscript have the potential to become a reference for researchers on a rather broad front, ranging from the SAC to AAA ATPases.

Specific points:

- Introduction: I would like to ask the authors to consider giving credit to work that they partly cite - and not too precisely - and partly forgot to cite. Part of that work was even published in the EMBO Journal. In particular, neither the term 'safety belt', nor the terminology 'open' and 'closed' Mad2, nor the realization that Mad2 dimerization involves open and closed conformers were ever proposed in the three Luo et al. papers discussed early in the Introduction together with a review by the authors. Rather, these terms and concepts were respectively introduced by Sironi et al. 2002 and by De Antoni et al. 2005. The authors cite the first of these papers (not the second) but later and in a different context, and to me (A. Musacchio) it seems that they could find their home at this point, at risk of making the argument come across as being exclusively pro domo mea. Also, the interaction of p31comet with C-Mad2 was the main subject of Mapelli et al. EMBO J 2006, which the authors do not elect to cite. Finally, the characterization of the deltaN15 mutant as a binder of Cdc20/closure motifs is described already in Mapelli et al. Cell 2007 and is not a new finding of this paper.

- Page 7: 'Data' is plural

- Page 11: As originally shown by Fang, Yu and Kirschner in their 1998 G&D paper, wild type Mad2 in the absence of closure motifs forms oligomers that were later shown to consist of open and (empty) closed Mad2. This dimerization likely influences the equilibrium of the open and closed forms. The Yu laboratory also proposed the existence of a C-Mad2 dimer, whose functional and structural significance has however remained somewhat elusive. I do not have a strong opinion on the question of which between the open and closed Mad2 conformer is more stable, but I think that this is an element of consideration before concluding that empty closed Mad2 is the more stable conformer. Is it clear that the high salt peak from the ion exchange column does not contain this oligomer originally described by the Kirschner lab? Or can the authors conclude that it is pure empty C-Mad2?

Figure 3: What are the faster migrating bands detected by the anti Mad2 antibody and observed with the 17C and 106C mutants?

Referee #2:

Activated by improper kinetochore-microtubule attachment, the spindle assembly checkpoint halts the metaphase-to-anaphase transition, providing time for error correction to maintain chromosomal stability. The activation of the checkpoint results in the protein MAD2 changing from 'open' to 'closed' conformation, which is then incorporated into protein complexes that inhibit mitosis progression. Once the kinetochore-microtubule attachment error is corrected, the AAA-ATPase TRIP13 works with its binding partner p31comet to convert C-MAD2 back to O-conformation and meanwhile disassemble the inhibitory complexes. How this conversion is achieved mechanistically is unclear and is addressed in this manuscript.

The authors previously have solved the crystal structure of C. elegans TRIP13 (Pch2) and performed biochemical assays confirming the role of TRIP13 and p31comet in MAD2 C-O conversion. In this manuscript they first obtained a crystal structure of human TRIP13 hexamer, and then mapped potential binding sites of MAD2 and p31comet on TRIP13 through cross-linking and modeling. Their results suggested that the disordered MAD2 N-terminus is engaged by the TRIP13 "pore loops". Further experiments indicated MAD2 N-terminus is required for MAD2 conformational change and consequently its checkpoint activity. They also suggested that engaging with a small fragment at the N-terminus might be sufficient for inducing unfolding/refolding of large fragment of MAD2 protein to change its conformation. They extended the result to HORMAD1, another member of HORMA domain containing proteins and an important meiosis regulator, and suggested that the N-terminus of HORMAD1 might also be important for its extraction from meiotic chromosomes by TRIP13.
Overall the experiments are well designed, the results are clear and well-presented. They have carried on the tradition of nice integration of structural and biochemical analyses for the TRIP13 working mechanism, and added some functional assays in cells through microscopic imaging. As the MAD2 conformational change (both O-C and C-O) is the key to a productive and faithful mitosis, the insights garnered from the work on how the conformation remodeling occurs are valuable. Any extension to other HORMA domain proteins is a plus. I do have two major points that need to be clarified (#1) and a few minor points for the authors’ attention.

1. Structure of TRIP13: does every subunit in the TRIP13 hexamer obtained in the presence of ATP bind to ATP? Does the helical configuration provide any explanation that each TRIP13 hexamer binds only one p31:MAD2 complex? What does it mean when the authors say “Crystallization trials with TRIP13:p31:MAD2 complex yielded only crystals of TRIP13E253Q alone”? It seemed that p31 and MAD2 are present in the crystals but not producing any signals (Materials and Methods). Does that mean the two proteins are probably not binding at the same subunit of TRIP13? It would be puzzling if the two proteins’ presence did not even change the NTD conformation (meaning no flipping "upward" as they suggested later? But the HDX-MS indicated even p31 alone could provide some protection to a "pore loop" region on TRIP13). They previously used a dimerization defective MAD2 (R133A) but did not state in this manuscript whether this is still the case. What is the purpose to do the modeling of a "closed" TRIP13 hexamer (Fig 1F)? What is the basis that this model instead of the real crystal structure used in interpreting the crosslinking data (p6, Fig 2F)?

2. Cysteine crosslinking result (Fig 3): The reasoning behind the conclusion that "MAD2 Cys106 crosslinking to the pore loop of TRIP13" means MAD2 unfolding to 106 residues is not clear to me. The authors do state that MAD2 conformational change may need only partial unfolding, and the differences in O-MAD2 and C-MAD2 structures mainly concerns the N- and C-terminus not the core where C106 is situated.

3. P8, the beginning sentence of the section “TRIP13 requires the...”: “TRIP13... maintains the soluble pool of O-MAD2 for MCC assembly...” (Similar statement in the beginning sentence of the next section). This could be confusing. I understand they echoed Ma and Poon’s results on TRIP13 knock-out suggesting a rigid C-MAD2 conformation might not be good in maintaining the spindle assembly checkpoint, but it’s not O-MAD2 that gets incorporated in the MCC. Also note that Hongtao Yu’s lab recently solved the ΔN10-MAD2 structure (Hara et al PNAS, 2015).

4. Discussion: second paragraph, Figure EV12 should be EV11. I am surprised they discussed so little about the MAD2 results (thus my questions in #1), but spent two paragraphs on meiotic HORMAD proteins. Also note in rice p31comet has been identified as a component of the synaptonemal complex (Ji et al, PNAS, 2016).

5. Citations: some sources were not properly credited. For example, they did not mention at all the first report on TRIP13-p31comet interaction (Tipton et al, BMC Cell Biol, 2012) and the first report proposing that TRIP13 and p31comet work together to utilize ATP hydrolysis to power MAD2 C-O conformation change (Wang et al., JBC, 2014).

Please find attached a revised version of our manuscript titled “The AAA+ ATPase TRIP13 remodels HORMA domains through N-terminal engagement and unfolding,” which was previously reviewed for publication in The EMBO Journal with manuscript # EMBOJ-2017-97291.

We were very pleased to see that both reviewers found the work interesting and important. Both reviewers pointed out references that we had regrettably failed to cite, and for that we thank them. We have responded in detail to each of their other points in the attached response document. Overall, the changes and additions prompted by both reviewers have significantly improved the revised manuscript.

(Point by point response shown on the following pages.)
Response to Reviewers:

The AAA+ ATPase TRIP13 remolds HORMA domains through N-terminal engagement and unfolding

Qiaozhen Ye, Dong Hyun Kim, Ihsan Dereli, Scott C. Rosenberg, Goetz Hagemann, Franz Herzog, Attila Tóth, Don W. Cleveland, Kevin D. Corbett

We were very pleased to read the positive assessments of both reviewers, and thank them for their helpful comments. In particular, we apologize that in several cases, ideas and concepts were not credited to their proper sources, or key studies were inadvertently not cited. We have altered the manuscript accordingly, and below address each comment and criticism individually. All text and citations that have been altered or added are highlighted in red text in the revised manuscript.

Reviewer #1:

Introduction: I would like to ask the authors to consider giving credit to work that they partly cite - and not too precisely - and partly forgot to cite. Part of that work was even published in the EMBO Journal. In particular, neither the term 'safety belt', nor the terminology 'open' and 'closed' Mad2, nor the realization that Mad2 dimerization involves open and closed conformers were ever proposed in the three Luo et al. papers discussed early in the Introduction together with a review by the authors. Rather, these terms and concepts were respectively introduced by Sironi et al. 2002 and by De Antoni et al. 2005. The authors cite the first of these papers (not the second) but later and in a different context, and to me (A. Musacchio) it seems that they could find their home at this point, at risk of making the argument come across as being exclusively pro domo mea. Also, the interaction of p31comet with C-Mad2 was the main subject of Mapelli et al. EMBO J 2006, which the authors do not elect to cite. Finally, the characterization of the deltaN15 mutant as a binder of Cdc20/closure motifs is described already in Mapelli et al. Cell 2007 and is not a new finding of this paper.

We apologize for our incomplete citations of important MAD2-related concepts and findings in the introduction. We have added a citation of Sironi et al. *EMBO J.* 2002 to the first paragraph of the introduction, at the first mention of open and closed MAD2 conformations, along with a seminal review on MAD2 conformations and their functions (Mapelli et al. *Current Biology* 2007). We also cite two of the Luo et al. papers at that point (*NSB* 2000 and *Mol. Cell* 2002), as these are the first reported structure of open and closed MAD2 (despite not being referred to by those names in these papers), along with our recent review (Rosenberg & Corbett *JCB* 2015).

Upon re-reading, it became clear to us that our originally-submitted manuscript did not provide enough detail into SAC activation and MCC assembly. We have expanded the second paragraph of the discussion accordingly, and included a number of new primary references including the De Antoni et al. (*Current Biology* 2005) paper mentioned by the reviewer.

Regarding the p31comet-MAD2 interaction, we have also expanded the section introducing TRIP13 and p31comet, and added the Mapelli et al. *EMBO J.* 2006 reference plus two others dealing with TRIP13 (Tipton et al. *BMC Cell Biology* 2012; Wang et al. *JBC* 2014) that we had inadvertently failed to cite.
Finally, we apologize for failing to cite both Mapelli et al. *EMBO J.* 2006 and Mapelli et al. *Cell* 2007 for their characterization of the structure and CDC20-binding activity of the ΔN15-MAD2 construct. These references have been added to the revised manuscript on page 8.

Page 7: 'Data' is plural.

We have altered the sentence in question to read “Our HDX-MS data show that p31comet residues 156-165…”

Page 11: As originally shown by Fang, Yu and Kirschner in their 1998 G&D paper, wild type Mad2 in the absence of closure motifs forms oligomers that were later shown to consist of open and (empty) closed Mad2. This dimerization likely influences the equilibrium of the open and closed forms. The Yu laboratory also proposed the existence of a C-Mad2 dimer, whose functional and structural significance has however remained somewhat elusive. I do not have a strong opinion on the question of which between the open and closed Mad2 conformer is more stable, but I think that this is an element of consideration before concluding that empty closed Mad2 is the more stable conformer. Is it clear that the high salt peak from the ion exchange column does not contain this oligomer originally described by the Kirschner lab? Or can the authors conclude that it is pure empty C-Mad2?

We agree that in the case of wild-type MAD2, O-MAD2:C-MAD2 dimerization likely plays a role in determining the equilibrium between open and closed MAD2 in solution, as this complex is potentially more stable than either monomeric species. We would point out that our *in vitro* assays used exclusively the MAD2 R133A mutant, which cannot stably dimerize (Sironi et al. *EMBO J.* 2001 and many others). In our purifications of MAD2 R133A, both the early-eluting from ion-exchange (O-MAD2) and late-eluting (C-MAD2) populations are exclusively monomeric when further examined by size-exclusion chromatography. Thus, we are confident that the late-eluting species does in fact represent pure, empty, monomeric C-MAD2 R133A. Moreover, both the Yu lab and our earlier tests with MAD2 R133A (Luo et al. *NMB* 2004; Ye et al., *eLife* 2015) showed that, when incubated at room temperature or 37°C, MAD2 R133A is eventually converted to ~90% C-MAD2, regardless of whether it started as O-MAD2 or C-MAD2. Regardless of the pathway of conversion and the potential role of O-MAD2:C-MAD2 dimerization in this pathway, we feel that this is strong evidence that (at least for MAD2 R133A) the C-MAD2 conformer is more stable in solution, even in the absence of a closure motif/MIM. Nonetheless, in recognition of the complexity of this issue, we have removed several references to C-MAD2 as the “more thermodynamically-stable” state of MAD2 from the manuscript.

Figure 3: What are the faster migrating bands detected by the anti Mad2 antibody and observed with the 17C and 106C mutants?

As the full-gel images in Appendix Figure S3 show, several bands are detected by the anti-MAD2 antibody between monomeric MAD2 at ~25 kDa, and the MAD2-TRIP13 crosslinked species migrating at ~120 kDa. It is likely that one of these (probably the species migrating at ~40 kDa) represents a MAD2-MAD2 dimer. Other species likely represent crosslinked MAD2-p31comet complexes. We were unable to generate an active cysteine-free p31comet construct, forcing us to use wild-type *H. sapiens* p31comet. This protein contains 8 cysteine residues, several of which are predicted to be solvent-exposed (from the structure of human p31comet; PDB ID 2QYF). In
addition to 1:1 complexes, it is also possible that complexes containing multiple copies of either p31\textsuperscript{comet} or MAD2 are formed at low levels, due to the number of cysteines per p31\textsuperscript{comet} protomer. We have added a note to this effect in the legend to Appendix Figure S3.

Reviewer #2:

1. Structure of TRIP13: does every subunit in the TRIP13 hexamer obtained in the presence of ATP bind to ATP?

Our crystals of TRIP13\textsuperscript{E253Q}, both in the presence and absence of ATP, are in space group P6\textsubscript{1} with a single TRIP13 protomer per asymmetric unit. We see clear density for ATP in this single subunit when the crystals are grown in the presence of ATP. As to the broader question of ATP occupancy in TRIP13 hexamers in solution, we cannot confidently speculate. Our prior structure of C. elegans PCH-2 (Ye et al. eLife 2015) suggested that the active TRIP13 hexamer may have ATP bound to two subunits, ADP to another two subunits, and no nucleotide bound to the final two subunits, which adopt the more-strained “open” conformation. This state would be very difficult to capture in a crystal structure. In hexamers of TRIP13\textsuperscript{E253Q}, ATP, we suspect that four subunits are ATP-bound, with two subunits in the “open” conformation (see model, Figure 1F). This, however, is probably only one of many states adopted by this dynamic machine during catalysis.

Does the helical configuration provide any explanation that each TRIP13 hexamer binds only one p31:MAD2 complex? What does it mean when the authors say "Crystallization trials with TRIP13:p31:MAD2 complex yielded only crystals of TRIP13\textsuperscript{E253Q} alone"? It seemed that p31 and MAD2 are present in the crystals but not producing any signals (Materials and Methods). Does that mean the two proteins are probably not binding at the same subunit of TRIP13? It would be puzzling if the two proteins' presence did not even change the NTD conformation (meaning no flipping "upward" as they suggested later? But the HDX-MS indicated even p31 alone could provide some protection to a "pore loop" region on TRIP13).

We apologize for the confusion on these related points. First, we speculate that TRIP13 can transiently associate with several copies of p31\textsuperscript{comet} through its six N-terminal domains. Only one copy of p31\textsuperscript{comet}:MAD2, however, could be stably accommodated in a “flipped-up” conformation in which MAD2 is engaged with the hexamer pore (Figure 2F).

Second, we speculate that the helical configuration of TRIP13 seen in our crystals arises because the “closed” conformation of TRIP13 is a lower-energy state than the “open” conformation. In solution at relatively low concentration, the competing energetics of: (1) satisfying subunit-subunit interfaces, versus (2) protein conformation, tends to stabilize hexamers. At the extremely high protein concentrations of a crystal lattice, a helical filament forms that both satisfies the native subunit interfaces and allows all subunits to adopt the lowest-energy conformation.

When we set up crystallization trials of the TRIP13:p31\textsuperscript{comet}:MAD2 complex, we obtained only crystals of TRIP13 alone. There is no indication of p31\textsuperscript{comet}:MAD2 in the resulting electron density, and indeed the crystals do not require p31\textsuperscript{comet}:MAD2 to form (though in our initial trials, p31\textsuperscript{comet}:MAD2 appeared to function essentially as a “chaperone”, helping maintain TRIP13 solubility during crystallization). We speculate that formation of the helical TRIP13 filament, and the lateral packing of filaments into a crystal, combine to dissociate p31\textsuperscript{comet}:MAD2 from TRIP13. Based on our rough 3D model of the complex (Figure 2F), this makes sense: if a
large part of the TRIP13-substrate interface involves the top surface of the hexamer, then filament formation essentially destroys this surface, likely destabilizing substrate binding. These points are noted more fully in the revised manuscript on page 18-19 (Materials and Methods).

They previously used a dimerization defective MAD2 (R133A) but did not state in this manuscript whether this is still the case.

We apologize for this omission. Initial purification of the TRIP13:p31comet:MAD2 complex, crystallization trials, and XLMS/HDX-MS experiments were all done using wild-type human MAD2. This MAD2 was always used in the presence of p31comet, a closure motif-containing peptide, or both, thereby minimizing the tendency of MAD2 to dimerize in these experiments. For biochemical assays, the R133A dimerization-defective mutant of both human and mouse MAD2 was used. This is more clearly noted on page 8 of the revised manuscript, and more fully explained in the Materials and Methods section.

What is the purpose to do the modeling of a "closed" TRIP13 hexamer (Fig 1F)? What is the basis that this model instead of the real crystal structure used in interpreting the crosslinking data (p6, Fig 2F)?

We modelled a closed TRIP13 hexamer model essentially to show that the helical filament does, in fact, show the biologically-relevant subunit-subunit interfaces, and that the difference between closed hexamer and helical filament lies in the conformation of subunits (Figure EV3). The cross-linking data was in fact interpreted in the context of three successive subunits of the helical filament form (Figure 2D and 2F), which is essentially the same as a “half-hexamer” in Figure 1F. This point is noted in the legend to Figure 2D.

2. Cysteine crosslinking result (Fig 3): The reasoning behind the conclusion that "MAD2 Cys106 crosslinking to the pore loop of TRIP13" means MAD2 unfolding to 106 residues is not clear to me. The authors do state that MAD2 conformational change may need only partial unfolding, and the differences in O-MAD2 and C-MAD2 structures mainly concerns the N- and C-terminus not the core where C106 is situated.

While alternative explanations are possible, the fact that we observed cross-linking between the TRIP13 pore loops and MAD2 residues 17, 25, 40, 79, and 106 only in the presence of ATP, and not ATP-γS, strongly suggests that these cross-links form as a result of processive unfolding by TRIP13. We agree with the reviewer that unfolding of the HORMA domain core is probably not required for MAD2 conformational conversion, but we cannot rule out complete MAD2 unfolding as a possible (if minor) mechanism. We discuss these points in the first paragraph of the Discussion, on page 12.

3. P8, the beginning sentence of the section "TRIP13 requires the...": "TRIP13... maintains the soluble pool of O-MAD2 for MCC assembly..." (Similar statement in the beginning sentence of the next section). This could be confusing. I understand they echoed Ma and Poon's results on TRIP13 knock-out suggesting a rigid C-MAD2 conformation might not be good in maintaining the spindle
assembly checkpoint, but it’s not O-MAD2 that gets incorporated in the MCC. Also note that Hongtao Yu's lab recently solved the ΔN10-MAD2 structure (Hara et al PNAS, 2015).

We agree that the wording in both sections was somewhat confusing, and have altered the wording in both cases (pages 8 and 9). Regarding the point that O-MAD2 is not incorporated into the MCC, this is essentially a matter of semantics. The reviewer is correct that, in the MCC, MAD2 is in the C-MAD2 conformation. However, the bulk of evidence indicates that O-MAD2 is the only form that can be recruited to kinetochores and incorporated into the MCC (along with conversion to the closed conformation). Pre-existing “empty” C-MAD2, while competent to bind closure-motif peptides in vitro, is most likely unable to bind full-length CDC20.

We are aware of two structures of ΔN10-MAD2: an NMR structure of a ΔN10-MAD2 monomer in the O-MAD2 conformation (Luo et al. NSB 2000), and the more recent crystal structure of an asymmetric ΔN10 O-MAD2:C-MAD2 dimer (Hara et al. PNAS 2015). We would point out that in both cases, the first 10 residues of MAD2 were removed but an N-terminal tag (MRGSHHHHHHGS) was present, meaning that this construct actually has a longer N-terminus than wild-type MAD2. We purified a similar ΔN10-MAD2 construct with a Ulp1-cleavable tag, and found that the tag could not be cleaved even with an extended incubation with Ulp1 (noted in Materials and Methods); this implies that the recognition sequence in the tag is involved in secondary structure, most likely forming a β1-like strand. Supporting this idea, some residues from the tag are ordered in both structures mentioned above (GS in the O-MAD2 monomer structure; as much as HHGS in the O-MAD2 protomers of the dimer structure). We don’t include discussion of these points in the manuscript, as they likely have little bearing on the behavior of the ΔN10-MAD2 construct we generated in DLD1 cells, which has no tag. Based on MAD2 structures, however, we expect that tagless ΔN10-MAD2 can likely adopt both open and closed conformations.

4. Discussion: second paragraph, Figure EV12 should be EV11. I am surprised they discussed so little about the MAD2 results (thus my questions in #1), but spent two paragraphs on meiotic HORMAD proteins. Also note in rice p31comet has been identified as a component of the synaptonemal complex (Ji et al, PNAS, 2016). We thank the reviewer for catching the figure reference error, and have corrected it (indeed, all EV figures have been renumbered according to EMBO Journal requirements). Regarding the discussion’s focus on meiotic HORMADs, we feel that the focus on these proteins in this section is entirely appropriate, given the number of outstanding questions regarding TRIP13’s mechanism of regulating these proteins. We also thank the reviewer for noting the recent finding that p31comet localizes to the synaptonemal complex in rice meiosis; we have added a reference to, and discussion of this finding to the manuscript (page 12-13).

5. Citations: some sources were not properly credited. For example, they did not mention at all the first report on TRIP13-p31comet interaction (Tipton et al, BMC Cell Biol, 2012) and the first report proposing that TRIP13 and p31comet work together to utilize ATP hydrolysis to power MAD2 C-O conformation change (Wang et al., JBC, 2014). We apologize for inadvertently excluding these important references, which were the key works first showing TRIP13’s role in the SAC, and in MCC disassembly in particular. As noted in our
response to reviewer #1, we have expanded the introduction of p31\textsuperscript{comet} and TRIP13 and added the noted references (page 3-4 of the revised manuscript).
Thank you for submitting your final revised manuscript for our consideration. I have now gone through your responses and revised manuscript, and I am pleased to inform you that in light of the satisfactory revisions, we have now accepted the paper for publication in The EMBO Journal!

Thank you again for this contribution to The EMBO Journal and congratulations on a successful publication! Please consider us again in the future for your most exciting work.
b) Statistics and general methods

5. For every figure, are statistical tests justified as appropriate?

6. Is the variance similar between the groups that are being statistically compared?

7. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

8. Are there adjustments for multiple comparisons?

9. Are error bars shown for technical replicates?

10. Are error bars not shown for technical replicates.

C) Reagents

11. Are all figures, table data, and information about sources of reagents provided?

12. Are the names of all reagents and the reference to the vendors’ web sites or antibodypedia or the vendors’ web sites provided?

D) Animal Models

13. Are all animals, both males and females, housed together in the same environment?

14. Are all monkeys, both males and females, housed together in the same environment?

E) Humans Subjects

15. Are all human studies, including both males and females, housed together in the same environment?

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### Data Accessibility

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**Examples:**

- Crystallographic data for small molecules (see link list at top right).
- Functional genomics data (see link list at top right).
- Macromolecular structures (see link list at top right).
- Primary diffraction data has been deposited to the Protein Data Bank (https://www.rcsb.org) under accession codes 409 and 410, as noted in the “Data Availability” section.
- The reported crystal structures can be accessed at the wwPDB (http://www.rcsb.org) under accession numbers 409 and 410.
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