nature portfolio

Peer Review File

Structures of KEOPS bound to tRNA reveal functional roles of the kinase Bud32

Corresponding Author: Professor Frank Sicheri

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Chuquimarca, Beenstock et al. perform an extensive characterization of the assembly of the KEOPS complex, building on the excellent earlier work of the Sicheri lab in this area. In a sense, this study brings together their other findings and rationalizes a number of mysterious earlier observations via thorough interpretation of the cryoEM structures of the complex in two conformations and extensive biochemical validation presented in the current manuscript. The insights are such that the authors were able to perform some engineering of enzyme complex and substrate to be able to restore catalysis. This was an enjoyable paper to read and I have only minor suggestions for improvements.

Major-ish points
1. The authors have generated perhaps too much compelling data and therefore have relied heavily on the supplement for including additional data. This breaks up the reading experience. Perhaps it's a necessary evil, but if possible the authors should try to move as much to the main text as possible. One avenue could be to amalgamate Figures 3 and 4 to create room for another figure. Minimally, the other useful panels to include in the main text would be the cartoons to guide the reader understanding of the assembly of the complex and the conformation changes observed.

2. The SEC-MALLS shows the SEC part but not the MALLS. There are summary data (i.e. MW estimates) but not the MALLS itself.

3. The methods are incomplete in places. The methods should be detailed, not refer solely to previous literature. There is no description of the antibiotics used in expression, whether the proteins were co-expressed in E. coli or whether the complexes were assembled through mixing. Details of the complex assembly, the concentration of components (ratios if mixed), conditions, and whether 2uM of each component were added to enzymatic assays or a 2uM concentration of purified complex, etc. If individually purified, what were the final concentrations and how were they measured?

Minor
4. I believe the authors will be required to include maps for each structure as supplemental figures under current Nature Comm guidelines, and there's only one currently in Figure S4. It would be nice to see what the maps look like regardless.

5. Abstract. I understand the word limit but there needs to be definition of KEOPS, ANN and GAMOS in the abstract for accessibility to a broader readership.

6. Line 89- please elucidate why Bud32 is a pseudokinase rather than a kinase

7. Line 121- further detail of the mutant is warranted in the main text, rather than the methods.

8. Line 130- 4 fold by what assay?

9. Line 224- PKA is the usual comparator for a protein kinase structure. Is there a reason for choosing phosphorylase kinase?

Some typos – rationale (line 108), grooves (line 155), early on the authors have used backslash rather than forward slash for

listing names of KEOPS proteins across species – is this intended?

Reviewer #2

(Remarks to the Author)
In this manuscript, the authors describe the first structure of the entire KEOPS complex including tRNA which is essential for modifying the anticodon loop of tRNAs across archaea and eukaryotes. The authors report three cryo-EM structures: the apo KEOPS structure, KEOPS with a tRNA bound in its canonical conformation, and KEOPS bound to a distorted tRNA. Furthermore, they characterize the interactions with KEOPS and with tRNA through detailed mutagenesis studies including tRNA binding, modification activity and ATPase activity of the KEOPS subunit Bud32. The main conclusion of the authors is that Bud32 plays a role in recognizing tRNA and in KEOPS regulation, and they imply a role of Bud32 in reorganizing the tRNA structure to facilitate modification by Kae1. Given the importance and complexity of KEOPS in modifying tRNAs and in causing a severe, inherited diseases, it is of high interest to gain insight into KEOPS structure and function. Therefore, this manuscript has the potential for high impact.

While the structures shared in this manuscript are generally interesting and the large number of biochemical experiments are conducted in an appropriate manner, the manuscript is not suitable for publication in Nature Communication since the main conclusions are not supported by the experiments. The main issue is that the KEOPS-tRNA structures have low resolution (3.5 – 3.9 Angstrom with the tRNA having only a resolution of about 5.5 Angstrom) limiting interpretation. Moreover, the biochemical experiments do not support the main conclusions derived from the structure. Importantly, there is no direct evidence that Bud32 alters the tRNA conformation as claimed in the abstract. In particular, the summarizing statement on page 11, line 318 is not supported by data: “these results suggest that a key role of the tail region of Bud32 is to promote conformational changes in tRNA structure by inducing the flip of the G26 base, which in turn causes an extension of the anticodon domain and disordering of the anticodon loop. These effects collectively facilitate t6A modification by KEOPS.”

1. Based on the electron densities, it is convincing that the tRNA exists in at least two different conformations. However, the structure with the distorted tRNA conformation has a resolution of only 3.9A allowing us to hypothesize the rough backbone conformation of the tRNA and possibly the flipped-out conformation of G26, but not more. It is important to note that the tRNA itself has a resolution of only 5.5 Angstrom (Fig. S2F) further limiting the interpretation of this structure.

2. It must be emphasized that the anticodon loop is disordered in the structure of the distorted tRNA as density is missing. Therefore, no conclusions can be drawn about the anticodon loop; in particular, it is not justified to suggest that this conformation allows the anticodon loop to reach into the active site of Kae1 when it is obviously not in the active site (Fig 3).

3. There is no good evidence for the proposed interaction of G26 of the tRNA with R530 in Bud32. In figure 4D, we see that the proposed electron density for R530 is in contact wit the anticodon loop of the tRNA. The flipped-out density for G26 does not contact the protein density at all. The proposed distance between R530 and G26 is not stated. While I agree that G26 seems to be flipped out of the tRNA, I cannot see any evidence for the involvement of Bud32 in this conformational change.

4. There is no biochemical evidence that G26 is important for KEOPS functon(Fig. 5). Mutating G26 does not change tRNA binding to KEOPS, it does not affect the ATPase activity of Bud32, and it enhances (!) the modification activity of KEOPS. The authors claim that tRNA G26U “rescues” the R530D mutation in Bud32 with respect to tRNA modification (Figs. 5G), but this effect is simply explained by the higher activity of G26U: comparing Fig. 5F and G shows that regardless of the Bud32 mutation, the presence of G26U in tRNA leads to 0.01-0.02 in normalized levels of t6A in tRNA.

5. The authors further characterize a previously reported importance of nucleotides in the tRNA D arm for tRNA modification by KEOPS (Fig. 6). In particular, their data reveal that mutation of G24 severely impairs tRNA binding to KEOPS. This is interesting, but difficult to explain based on the reported structure as the D arm is not in contact with the KEOPS proteins. Other mutations in the D arm also disrupt Bud32 ATPase activity and tRNA modification. These effects are most easily explained by an altered tRNA conformation based on the mutations which may included alternative base-pairing. The authors must include mutations of G24 to A to maintain its size and stacking ability, they must predict the base-pairing of the mutated tRNA sequence, and they must check the structure of the mutated tRNA alone before drawing any conclusions regarding the recognition by KEOPS. Given that the importance of C10 and U11 was previously reported, this manuscript does not add significant additional insight into tRNA recognition by KEOPS.

6. In the current version of the manuscript, the authors imply that the sequence of the D arm is a recognition element for KEOPS (Fig. 7). This claim is not supported by structural or biochemical data. While it is interesting that they can convert tRNAAla into a KEOPS substrate by mutating the anticodon (!) triplet, this is no proof for the importance of the D arm sequence for KEOPS.

7. In their model (Fig. 7C), the authors imply that Bud32 activity is induced at the same time as a tRNA conformational change (step 3), but as explained above they do not provide any evidence for this mechanistic link. As a side note, G26 is shown in the wrong position in the schematic tRNA structure in Fig. 7C.

Additional comments:
• Why did the authors not include a substrate analog of TC-AMP to bind to Kae1 and an ATP analog to bind to Bud32 in their structures for cryo-EM?
• The authors state that a new contact is formed between C32 and G31 in the anticodon loop and loop 3 of Kae1. However,

this contact is not evident in Fig 5D, and the comparative structure of the undistorted tRNA is not shown.
• In the methods sections, the authors must provide equations which were used to determine dissociation constants and IC50 values.
• The description of the fluorescence polarization assays states that it was “done in triplicates”. Were these triplicates done on different days with different preparations? The reported IC50 data are highly variable raising concerns. For example, Fig. 5A and B report IC50 for the wild-type system (Bud32 or tRNA which should be the same) as 1.53 +/- 0.5 microM and 3.0 +/- 0.81 microM. These data suggest that the authors are overestimating the precision of their data which must be addressed.
• In Fig 2C and D: why are the variants R163E and Q160D labelled with an asterisk?
• Why is figure 5H labelled to include tRNAMet whereas the figure legend refers to tRNALys?
• In Figure 7B, why is the peak for t6A shifted for Ala36-UUA38 compared to Lys WT?
• In the supplementary material, the author should show the position of Bud32 E152R which was used to render the KEOPS complex inactive and explain the effect of this mutation.
• For Fig. S6B, the authors should clarify in the figure legends what is shown in the lanes labelled as “G” and “A”.

Reviewer #3

(Remarks to the Author)
In this work the authors report three novel cryo-EM structures of KEOPS complex (two with distinct tRNA conformations and one apo). Based on these structures and considerable biochemical follow up on the mutants based on the structures, the authors propose a catalytic cycle of this enzyme complex. The resulting cycle has a number of key features: (1) Bud32 ATPase regulation through Kae1 tRNA binding via Arg237 positioning (2) Order to disorder transition in the anticodon loop on the tRNA associated with a G26 base flip. Although the apo structure is very similar to the previous structures in the field (the ones that authors used to build their models, combination of PDBs 3enh and 3eno) the structure in the presence of tRNA together with resolution of multiple conformations and associated biochemistry, all resulting in a mechanistic model, certainly seems to be an advance for the field worth publishing. I think the work should be published with minor text/figure revisions addressing the following concerns.

The main concern is the fact that cryo-EM map resolutions are quite varied within and across the structures (based on examination of provided maps). Many of the key arguments about the specific interactions mentioned in the manuscript are not unambiguously supported by the experimental density. Furthermore, there seems to be slight “streaking” likely due to some degree of preferred orientations in the sample and likely resulting in the differential resolutions in different reconstruction directions. Examining the maps, there are side chain densities missing and extra densities present next to some residues. Often it seems that specific residue interaction arguments are made based on a roughly ~5A map resolution for those regions. Due to the rigorous biochemical experimental follow up, I do not think this invalidates the paper’s findings but the fact that many atomic interactions are based on map regions with resolution considerably worse than reported should be explicitly addressed and disclosed to the readers. For example, in the 3’ CCA interaction, side chain of a key, bulky interacting residue, Phe21 is not resolved at all in the distorted structure and barely resolved in the ordered tRNA structure. Side chain densities for Arg163 and and Arg237 are also quite ambiguous. Arg530 placement is also ambiguous with even the backbone not being very well defined. Additionally, the exact structure of the distorted tRNA is also ambiguous. Although there is a clear density for where flipped G26 base would be, the phosphate-sugar backbone for bases 11-18, 22-25, 41-45 is completely out of the cryo-EM density making overall positioning of G26 questionable, even if one low pass filters the map.

Here are concrete things that I think can make the manuscript better in this regard:
(1) When discussing specific interactions preface the discussion with a comment on the quality of the map in that region.
(2) Report 3D FSC plots for all the reconstructions to indicate resolution anisotropy
(3) Report per chain average Q-scores for all models and when discussing specific residues include that residue Q-score in the main text to provide a quantitative assessment of the map resolution in that region.

Beyond the above, below are some more general comments addressing which I think can make the manuscript better:
(1) Although authors get dimer/monomer equilibrium they use a mutant to focus on the monomer structure. Could it be that the physiologically relevant structure is the dimer? Could authors provide/cite some evidence that it is not and structure of the monomer is enough?
(2) The idea that tRNA binding on Kae1 activates Bud32 via repositioning of the Arg237 is cool. However, the protein structures seem to be nearly identical between the apo and tRNA bound complexes and although the authors place Arg237 side chain in a very different conformation in apo vs tRNA bound, I am not sure that is warranted based on the experimental density. But beyond that, can the authors provide some sort of mechanism to how this allosteric activation of Bud32 happens considering effectively identical Bud-Kae structures?
(3) Can the authors comment on how Asn156Ala, Gln160Asp, and Arg163Glu mutants would enhance the tRNA binding given their model, as that’s what they see experimentally but it doesn’t quite make sense structurally?
(4) Reporting specific distance from A37 to the TC-AMP in the distorted tRNA structure is beyond what the experimental evidence shows. That whole region is completely disordered (ie, completely missing density) in the distorted tRNA structure and coupled to the fact that the phosphate-sugar backbone for bases 11-18, 22-25, 41-45 is completely out of the cryo-EM density makes overall placement of that half of tRNA questionable. This is on top of the fact that TC-AMP is not present in any of these structures and its placement is inferred from the previous structures. Therefore reporting the exact distance between the base and TC-AMP in the tRNA distorted structure is certainly an overinterpretation of the data which will likely be missed by readers without structural biology background and will instead be interpreted as hard truth. Much more explicit language about what actually can be clearly resolved in the structures reported here and what is inferred should be used.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done an excellent job accommodating my suggestions.

Reviewer #2

(Remarks to the Author)
In their revised manuscript, the authors have addressed the three reviewers' comments that all centered around the limited resolution of the reported structures of the KEOPS complex bound to tRNA and the conclusions that can be drawn from the structure and the biochemical data. To this end, the authors have somewhat improved the resolution of the reported structure by analyzing more particles, and they have added some additional biochemical data, e.g. on tRNA and protein variants and their activity. But most importantly, they have significantly altered the writing of the manuscript changing many previous conclusions into much more carefully phrased speculations and hypotheses. In general, this approach has greatly increased the scientific quality of the manuscript!

At the same time, this approach also demonstrates that several mechanistic questions about t6A formation by KEOPS in tRNA remain. This is mostly the case because a static structure and multiple-turnover enzyme assays and tRNA binding assays cannot dissect the apparent dynamic conformational changes during KEOPS' mechanism and the timing of the different activities. Nevertheless, this manuscript clearly is a critical step forward towards understanding the complex mechanism of KEOPS and its disease relevance, and therefore it should be published in Nature Communications with a few remaining minor changes.

Minor comments:

Page 10 & Supplementary Movie 1:
The authors state: "We observed evidence of contact between the tRNA D-arm and Bud32 and Kae1 that was not captured in one of the two structures reported (Supplementary movie 1), however the details of these interactions await further investigation."
I assume that the movie models the conformational changes necessary to transition from the "native-like" tRNA structure bound to KEOPS to the "distorted" tRNA structure bound to KEOPS, but I have missed a legend explaining how the movie was generated. Based on my assumption, any transient tRNA conformation on the path from one structure to the other is a hypothesis without direct experimental evidence. Therefore, I think that the author's statement is not true, and I recommend that it is completely removed from the manuscript.

Supplementary Figure 4:
I appreciate that the authors clarified that the lanes labelled "G" and "A" are sequencing lanes to identify the position of G26 and its modification. Sequencing gels are difficult to run, and as typical there are unspecific reverse transcription stops making it difficult to clearly read a sequence. Having said this, I am wondering if possibly lanes G and A are switched? That would be more consistent with my interpretation of the sequencing gel.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns and the manuscript is ready for publication.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Chuquimarca, Beenstock et al. perform an extensive characterization of the assembly of the KEOPS complex, building on the excellent earlier work of the Sicheri lab in this area. In a sense, this study brings together their other findings and rationalizes a number of mysterious earlier observations via thorough interpretation of the cryoEM structures of the complex in two conformations and extensive biochemical validation presented in the current manuscript. The insights are such that the authors were able to perform some engineering of enzyme complex and substrate to be able to restore catalysis. This was an enjoyable paper to read and I have only minor suggestions for improvements.

We thank this reviewer their supportive comments.

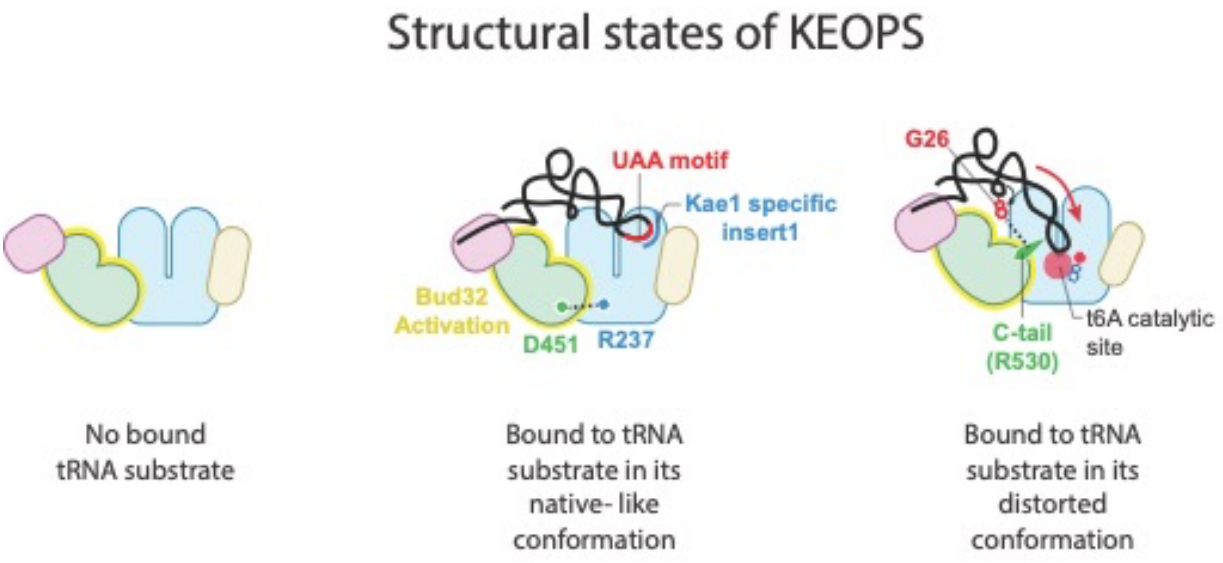
Major-ish points

1. The authors have generated perhaps too much compelling data and therefore have relied heavily on the supplement for including additional data. This breaks up the reading experience. Perhaps it's a necessary evil, but if possible the authors should try to move as much to the main text as possible. One avenue could be to amalgamate Figures 3 and 4 to create room for another figure. Minimally, the other useful panels to include in the main text would be the cartoons to guide the reader understanding of the assembly of the complex and the conformation changes observed.

We have reorganized the figure set to address the reviewer's point. We have also accepted the reviewer's suggestion to amalgamate Figures 3 and 4 of our previous submission (new Figure 2 in the current submission).

We now take full advantage of the figure limit that *Nature Communication* allows for the main document. In the current submission we have ten main figures, which reduces the number of supplementary figures from seven in the previous submission to three in the current one.

We accept the suggestion to make use of cartoons to guide reader understanding. To this end, we have added a cartoon schematic depicting the different states discussed in the manuscript as a graphical abstract:



We have also moved a schematic summarizing expected and novel contacts of KEOPS subunits with tRNA substrate from **Supplementary Figure 3c** and **d** to the main figure set as new **Figure 3**.

2. The SEC-MALLS shows the SEC part but not the MALLS. There are summary data (i.e. MW estimates) but not the MALLS itself.

Sorry for the omission. We have modified the figure (now **Supplementary Figure 1d**) to include the missing data.

3. The methods are incomplete in places. The methods should be detailed, not refer solely to previous literature. There is no description of the antibiotics used in expression, whether the proteins were co-expressed in E. coli or whether the complexes were assembled through mixing. Details of the complex assembly, the concentration of components (ratios if mixed), conditions, and whether 2uM of each component were added to enzymatic assays or a 2uM concentration of purified complex, etc. If individually purified, what were the final concentrations and how were they measured?

We have made the following changes to the methods section to address the reviewer's request:

- We have expanded the description of Trm1 expression and purification in the methods section entitled *Protein expression and purification*
- We have added a description of the antibiotics used for the expression of all proteins and have clarified that the proteins were expressed individually in the methods section entitled *Protein expression and purification*
- We have added a description of the antibiotics used for the expression of tRNAs in the methods section entitled *T7 in vitro transcription and purification of tRNA*

- We have added a description of how complexes were assembled in the methods sections entitled *In vitro t6A assays and HPLC analysis of tRNA modifications*, *Fluorescence polarization assays*, *ADP Glo™ assay*, *Size exclusion chromatography - multi angle light scattering analysis (SEC-MALS),* and *Electron microscopy data collection*

Minor
4. I believe the authors will be required to include maps for each structure as supplemental figures under current Nature Comm guidelines, and there's only one currently in Figure S4. It would be nice to see what the maps look like regardless.

We now included cryo-EM density maps superimposed on all atomic models displayed in the revised manuscript (**Figures 1b**, **c**, **d** and **e**, **Figures 2a**, **b** and **c, Figures 3b** and **c**, **Figures 8a** and **b**, **Figure 9a**).

5. Abstract. I understand the word limit but there needs to be definition of KEOPS, ANN and GAMOS in the abstract for accessibility to a broader readership.

We have now defined all acronyms on first usage

6. Line 89- please elucidate why Bud32 is a pseudokinase rather than a kinase

We have added the following text to the introduction to address this point (Page 4, lines 94-96)

> "Unlike most members of the eukaryotic protein kinase superfamily, Bud32 functions as an ATPase rather than a protein kinase and can therefore be classified as a pseudo kinase".

7. Line 121- further detail of the mutant is warranted in the main text, rather than the methods.

We now provide additional details for the mutations in Bud32 and Pcc1 used to improve sample quality for Cryo EM, including a schematic in **Fig. S1c**. (Page 5 lines 127-132):

> "Therefore, to address monomer-dimer heterogeneity we used a previously described Pcc1 mutant (denoted $Pcc1^{Mut}$, **Fig. S1c** and methods) that cannot dimerize KEOPS but still supports tRNA modification activity[45,46]. To promote tRNA binding to KEOPS, we employed a glutamate to arginine substitution at

position 152 (E152R) in Bud32 (**Fig. S1c**) that enhances tRNA-binding affinity but impairs tRNA modification function[40]."

We further provide details in the Methods section (Page 18 lines 503-509):

"The *Pyrococcus furiosus* Pcc1^{Mut} protein expression construct was generated by cloning into the pGEX2T vector two Pcc1 encoding sequences in tandem, connected by 7 repeats of the dipeptide glycine-serine. The second Pcc1 sequence encoded the double mutations Ala75Tyr and Val79Arg, which prevent Kae1 binding as previously described[29,45]. Thus, the Pcc1^{Mut} construct produces a heterodimer Pcc1 complex, with one Pcc1^{WT} protomer with a 7XGS sequence at its C-terminus linking to one Pcc1 $^{Ala75Tyr + Val79Arg}$ protomer."

8. Line 130- 4 fold by what assay?

The assay in question is a competitive displacement assay where we monitor the ability of unlabelled tRNA to competitively displace a fluorescently labeled CCA-3' containing oligonucleotide that binds specificality to the Cgi121 subunit of KEOPS. The revised text now reads (Page 5 lines 136-138):

"KEOPS reconstituted with Pcc1^{Mut} and Bud32^{E152R} displayed ~4-fold enhanced tRNA binding compared to KEOPS WT in a competitive displacement assay (**Fig. S1e**)"

9. Line 224- PKA is the usual comparator for a protein kinase structure. Is there a reason for choosing phosphorylase kinase?

We agree that PKA is a usual comparator for protein kinases, however Bud32 harbors a very minimalistic kinase domain. We therefore chose to use phosphorylase kinase as a point of reference because it is one of few protein kinases bound to a *bona fide* peptide substrate in its active site and its structure is simpler compared to PKA in that it lacks the extraneous secondary structure elements that are unique to the AGC family kinases. If this reviewer feels it necessary that we use PKA as our comparator, we will revise the figure accordingly.

Some typos – rationale (line 108), grooves (line 155), early on the authors have used backslash rather than forward slash for listing names of KEOPS proteins across species – is this intended?

We have corrected the typos. We thank this reviewer for pointing these out.

Reviewer #2 (Remarks to the Author):

In this manuscript, the authors describe the first structure of the entire KEOPS complex including tRNA which is essential for modifying the anticodon loop of tRNAs across archaea and eukaryotes. The authors report three cryo-EM structures: the apo KEOPS structure, KEOPS with a tRNA bound in its canonical conformation, and KEOPS bound to a distorted tRNA. Furthermore, they characterize the interactions with KEOPS and with tRNA through detailed mutagenesis studies including tRNA binding, modification activity and ATPase activity of the KEOPS subunit Bud32. The main conclusion of the authors is that Bud32 plays a role in recognizing tRNA and in KEOPS regulation, and they imply a role of Bud32 in reorganizing the tRNA structure to facilitate modification by Kae1. Given the importance and complexity of KEOPS in modifying tRNAs and in causing a severe, inherited diseases, it is of high interest to gain insight into KEOPS structure and function. Therefore, this manuscript has the potential for high impact.

We appreciate this reviewer statement of the importance of uncovering the basis of tRNA modification by KEOPS.

While the structures shared in this manuscript are generally interesting and the large number of biochemical experiments are conducted in an appropriate manner, the manuscript is not suitable for publication in Nature Communication since the main conclusions are not supported by the experiments.

To address the concern that the main conclusions of the manuscript are not supported by the experiments, we have carried out a major re-write to make clearer the distinctions between fact and hypotheses. We also present additional experimental data and analysis to support any conclusions drawn, as detailed below in response to specific reviewer comments below.

MAIN ISSUES

1. The main issue is that the KEOPS-tRNA structures have low resolution (3.5 – 3.9 Angstrom with the tRNA having only a resolution of about 5.5 Angstrom) limiting interpretation.

We agree with the reviewer that higher resolution structures of the KEOPS-tRNA complex would enable a deeper understanding of tRNA modification by this complex. To help address this concern, we have extended the resolution of the structure of KEOPS bound to distorted tRNA from 3.92Å to 3.59 Å. Furthermore, where appropriate, we have qualified our text to better account for the limitation of interpretations arising from the intermediate resolution of the tRNA structure.

Examples of text changes to better qualify our interpretations:

Line 167-169:

> Due to its apparent flexible mode of binding, density for much of the tRNA in both conformations remained limited to ~5-6 Å (**Fig. S2f**), with improved resolution at interfaces with the core protein complex (see below).

Lines 178-181:

> Although we could not unambiguously model the position of the tRNA bases and some of the side chains due to low resolvability (see **Table 2** for the Q scores[51] of key residues in this study), these could be inferred in part from the published crystal structures of KEOPS proteins and of $tRNA^{Lys}_{UUU}$ in isolation

In the first conformation, the tRNA exhibits a native-like fold, similar to its crystal structure (PDB 7KJU) (**Fig. 2a** left side and **b,** RMSD 1.36 Å). Correspondence between the observed experimental density and the known crystal structure of the tRNA gave higher confidence in the model's correctness. In the second conformation, the anticodon domain of the tRNA exhibits an unexpected, distorted conformation (RMSD 4.02 Å relative to the native fold) (**Fig. 2a** right side, and **Fig. 1d-e** for a comparison between native like and distorted conformations). Due to the limited resolution and the absence of a pre-existing atomic structure, we could not model the tRNA structure with full confidence and thus interpretations based on this structure were made with caution.

2. Moreover, the biochemical experiments do not support the main conclusions derived from the structure. Importantly, there is no direct evidence that Bud32 alters the tRNA conformation as claimed in the abstract. In particular, the summarizing statement on page 11, line 318 is not supported by data: "these results suggest that a key role of the tail region of Bud32 is to promote conformational changes in tRNA structure by inducing the flip of the G26 base, which in turn causes an extension of the anticodon domain and disordering of the anticodon loop. These effects collectively facilitate t6A modification by KEOPS."

We accept this reviewer's point and in the revised manuscript we have toned down the implied cause and effect relationship between Bud32 ATPase activity, the contact between the Bud32 tail and flipped base of G26, and large conformational changes observed in the tRNA. In particular, we have removed the statement mentioned by the reviewer from the revised version.

3. Based on the electron densities, it is convincing that the tRNA exists in at least two different conformations. However, the structure with the distorted tRNA conformation has a resolution of only 3.9A allowing us to hypothesize the rough backbone conformation of the tRNA and possibly the flipped-out conformation of G26, but not more. It is important to note that the tRNA itself has a resolution of only 5.5 Angstrom (Fig. S2F) further limiting the interpretation of this structure.

To help address the issue of limited resolution of the reported structures, as noted above, we have improved the resolution of the structure of KEOPS bound to distorted tRNA from 3.92Å to 3.59 Å. Notably, the improved resolution allows us to more clearly visualize a contact between the flipped base which we infer corresponds to position G26 and the R530 sidechain in the tail of Bud32 as elaborated in response to in point 5 below.

4. It must be emphasized that the anticodon loop is disordered in the structure of the distorted tRNA as density is missing. Therefore, no conclusions can be drawn about the anticodon loop; in particular, it is not justified to suggest that this conformation allows the anticodon loop to reach into the active site of Kae1 when it is obviously not in the active site (**Fig 3**).

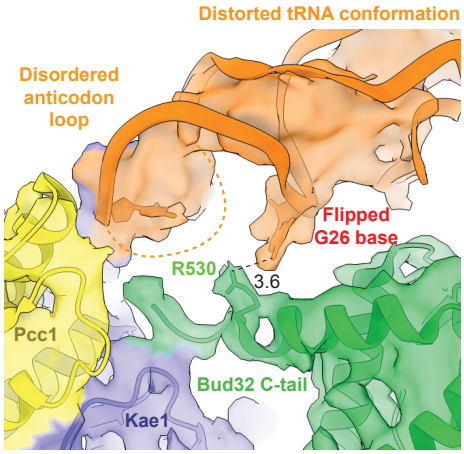
We agree with this reviewer that the anticodon loop is disordered in one of the two structures. We make explicit note of this point in Page 7 lines 203-211:

"In the KEOPS-distorted tRNA structure however, 7 nucleotides within the anticodon loop (U33 to C39, inclusive) encompassing A37 are disordered. Thus, A37 (although not visible due to disorder) in principle would have the ability to more closely approach the active site. Indeed, modeling reveals A37 could approach within 5.5 angstroms of the inferred position necessary for its modification. Together with additional flexibility of the tRNA structure evident in the 3D variability analysis (see **Supplementary Movie 1**), we reason that the observed disorder of the anticodon loop in the distorted state could possibly position the A37 base into the active site for modification, hinting that this may be an integral step in the tRNA modification reaction."

We think that it is reasonable to state that since the anticodon loop is disordered in the distorted state structure, the disorder would enhance the potential of A37 to access the active site of Kae1 relative to the complete inaccessibility of A37 in the ordered state structure. We note that in the absence of a conformation change in the tRNA, the buried and inaccessible nature of A37 base would make it impossible for it to be modified by KEOPS.

5. There is no good evidence for the proposed interaction of G26 of the tRNA with R530 in Bud32. In figure 4D, we see that the proposed electron density for R530 is in contact with the anticodon loop of the tRNA. The flipped-out density for G26 does not contact the protein density at all. The proposed distance between R530 and G26 is not stated. <u>While I agree that G26 seems to be flipped out of the tRNA, I cannot see any evidence for the involvement of Bud32 in this conformational change.</u>

We have carried out further refinement of our cryo-EM maps by increasing the number of particles from ~43,000 to ~83,000. This has allowed us to increase the resolution of the structure of KEOPS bound to distorted tRNA from 3.92 Å to 3.59 Å, allowing us to better visualize a 3.6 Angstrom interaction between the side chain of R530 in the Bud32 C-tail and the G26 flipped base (see improved map in revised **Figure 9A** below of the current submission). We have also toned down our inference of a cause-and-effect relationship by removing statements indicating that Arg530 'causes' G26 to flip out from its buried state in the tRNA's native-like conformation. Instead, we simply state that the Arg530 sidechain interacts with the G26 base in its flipped-out state.



6. There is no biochemical evidence that G26 is important for KEOPS function (Fig. 5).
   -Mutating G26 does not change tRNA binding to KEOPS,
   -It does not affect the ATPase activity of Bud32, and
   -It enhances (!) the modification activity of KEOPS.
   The authors claim that tRNA G26U "rescues" the R530D mutation in Bud32 with respect to tRNA modification (Figs. 5G), but this effect is simply explained by the higher activity of G26U: comparing Fig. 5F and G shows that regardless of the Bud32 mutation, the presence of G26U in tRNA leads to 0.01-0.02 in normalized levels of t6A in tRNA.

We have generated new experimental data that strengthen our conclusion that G26 is important for KEOPS function and that the function of G26 is correlated in some way to its ability to flip and to contact the Arg530 sidechain in the tail region of Bud32. This physical contact is more clearly evident in the improved resolution cryo-EM density maps (see above).

We previously showed that substituting G26 to A, U or G had no effect on tRNA binding (**Figure 4a**) or on Bud32 ATPase activity (**Figure 4b**). Furthermore, the G26A (similarly sized base) substitution had no effect on t6A activity while the G26C and G26U (large for small sized base) substitutions caused ~2-fold and ~3-fold enhancement of t6A activity (**Figure 4c**). These results led us to hypothesize that substitutions at G26 that would likely hinder local base pairing potential and/or base stacking in the native-like conformation of tRNA may have the beneficial effect of enhancing the t6A modification by KEOPS.

In new experiments we now show that all three substitutions do not perturb the overall tRNA fold as assessed by circular dichroism analysis (**New Data Figure 4d**), and thus any effect of the substitutions on tRNA structure must be subtle.

We also showed that the post transcriptional modification of G26 to m^2_2G26 caused a decrease in t6A modification (**Figure 4e**). Since the m^2_2G26 modification is appreciated to stabilize G26 native base paring by restricting spurious interactions, we hypothesized that t6A modification by KEOPS is reduced by over stabilization of local tRNA structure at the D-arm.

As the flipped G26 base contacts Arg530 in the tail of Bud32 (**Figure 9A** shows the direct contact in an improved map), we hypothesized that the function of the G26 base and Arg530 in the Bud32 tail might be functionally related/connected.

We then show that mutation of Arg530 in the Bud32 tail to Asp (R530D) has no effect on tRNA binding (**Figure 9d**), or on Bud32 ATPase activity (**Figure 9e**), but caused a complete loss of t6A modification activity (**Figure 9f**). In new experiments we also show that mutating the kinase active site (Asp451Ala denoted Kdead) to abolish phospho-transfer activity, causes the same complete loss of t6A modification activity (**New Data in Figure 9d-f**). Importantly, both the R530D and Kinase dead mutants of Bud32 are equally dead over a wide range of enzyme concentrations (**New Data Figure 9g**) and time points (**New Data Figure 9h**).

Supported by previous and new experiments (**figure 9h**), we now show that combination of the t6A activity enhancing substitution of G26U in tRNA can partially overcome the Bud32 R530D mutation but has no effect on the equally incapacitated Bud32 Kinase Dead mutation (**Figure 9h**). These results provide suggestive evidence that G26 in tRNA and R530 in the Bud32 tail are mechanistically linked in a way that G26 and the Bud32 ATPase function are not. In the absence of additional data we do not commit to a specific model of how they may be linked.

We hope that the added data and the more cautious explanation addresses the concerns of this reviewer.

7. The authors further characterize a previously reported importance of nucleotides in the tRNA D arm for tRNA modification by KEOPS (Fig. 6). In particular, their data reveal that mutation of G24 severely impairs tRNA binding to KEOPS. This is interesting, but difficult to explain based on the reported structure as the D arm is not in contact with the KEOPS proteins. Other mutations in the D arm also disrupt Bud32 ATPase activity and tRNA modification. These effects are most easily explained by an altered tRNA conformation based on the mutations which may include alternative base-pairing.

We agree with the reviewer that the effects of substitutions on t6A and Bud32 ATPase activity may not be related to a direct contact between the tRNA and KEOPS proteins although in our new analysis of KEOPS-tRNA dynamics, we see a close approach between the two (**Supplementary Movie 1**). We agree with this reviewer that more likely, the observed effects are related to effects on the local structure and dynamics of the tRNA. Consistent with a subtle effect on local structure, new CD analysis indicates that the overall tertiary structure of the tRNA is not perturbed by the mutations (**Figure 4d**). This leads us hypothesize that the ability of the D-arm to undergo a local conformational change (observed in comparing the two tRNA-KEOPS co-structures) are mechanistically important for the t6A modification reaction.

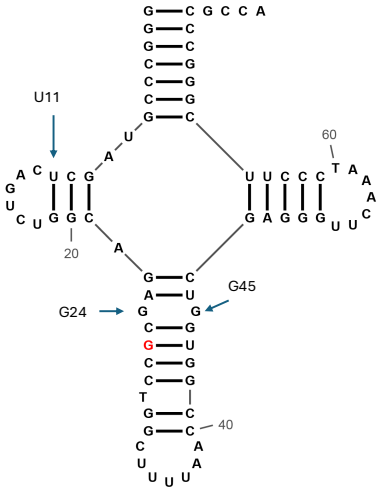
8. The authors must
   a) include mutations of G24 to A to maintain its size and stacking ability, they must
   b) predict the base-pairing of the mutated tRNA sequence, and they must
   c) check the structure of the mutated tRNA alone before drawing any conclusions regarding the recognition by KEOPS.
   Given that the importance of C10 and U11 was previously reported, this manuscript does not add significant additional insight into tRNA recognition by KEOPS.

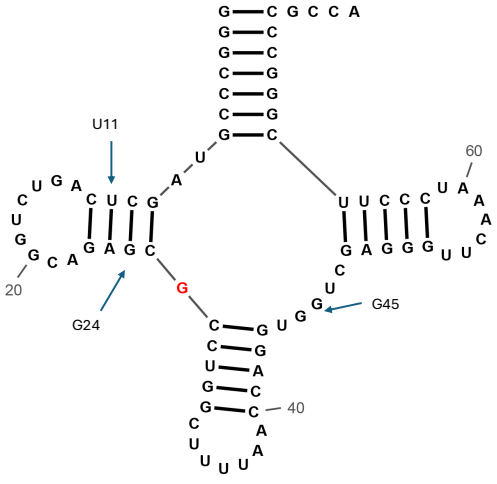
In light of these helpful recommendations, we have performed the following experiments.

a) We generated the requested G24A mutant and tested it for

- Bud32 ATPase activity: Bud32 ATPase activation by tRNA addition to KEOPS was greatly decreased (almost to basal levels).
- KEOPS t6A activity: t6A activity of KEOPS was completely abolished.
- KEOPS tRNA binding activity: tRNA binding was not adversely affected.

b) We have performed 2D and 3D structure predictions. However, we found the results inconclusive. For example, in the crystal structure of $tRNA^{Lys}$, G24 base pairs with the bases of U11 and G45. However, 2D prediction software programs (for example the minimum energy fold from the Vienna webserver and the tRNAScan SE server) did not successfully predict these base pairing interactions (as shown below):





Therefore, we chose not to include 2D prediction data in the revised manuscript.

c) We have performed Circular Dichroism experiments (as described in DOI: 10.1093/nar/gkae179) and the results show that the tRNA fold is not outwardly disrupted by the G24A mutation, which agrees with the tRNA binding experiment. We have also tried to crystalize the tRNA mutants to visualize the effect of mutation on local structure but were not able to obtain diffracting crystals.

Overall the results indicate that tRNA with a G24A mutation is still bound by KEOPS, but it loses two of its most central features as a KEOPS substrate; namely the ability to activate Bud32 and to accept the t6A modification. We therefore conclude that position 24 is part of the conserved D-arm motif that acts as a determinant for t6A modification of a tRNA by KEOPS.

9. In the current version of the manuscript, the authors imply that the sequence of the D arm is a recognition element for KEOPS (Fig. 7). This claim is not supported by structural or biochemical data. While it is interesting that they can convert tRNA Ala into a KEOPS substrate by mutating the anticodon (!) triplet, this is no proof for the importance of the D arm sequence for KEOPS.

We agree with the reviewer's point that our data does not unequivocally show that the D-arm is a recognition element for KEOPS, since our two structures did not reveal direct contacts between the D-arm and KEOPS. However, we now provide a movie highlighting the dynamic structure of the tRNA when bound to KEOPS. The movie also shows that KEOPS forms a transient contact with the tRNA in the D-arm region (**Supplementary Movie 1**).

We also show that this D-arm motif is highly conserved in KEOPS substrates (just like the UAA motif) suggesting that it is functionally important.

To address this issue further, we have made new attempts to engineer a non-substrate tRNA into a substrate of KEOPS. In new experiments, we have progressively swapped regions of a substrate tRNA into a non-substrate tRNA focusing on progressively larger portions of regions encompassing the conserved D-arm motif.

The results displayed in new **Figure 6 c, d** and **e** show that we were highly successful in reconstituting the ability of the tRNA to activate Bud32 ATPase activity (almost equal to the level of activation induced by a natural substrate) but were not able to reconstitute the ability of the tRNA to be t6A modified by KEOPS.

The results show that a D-arm motif is undoubtably important for Bud32 activation and that there may be additional factors at play that we do not understand that are needed for the t6A reaction. We accept the reviewer's point and rather than calling it a substrate recognition element, we now call it a substrate determinant since it is not required for recognition per see but is required for t6A modification after the tRNA is bound (i.e. after it is recognized by KEOPS).

10. In their model (Fig. 7C), the authors imply that Bud32 activity is induced at the same time as a tRNA conformational change (step 3), but as explained above they do not provide any evidence for this mechanistic link. As a side note, G26 is shown in the wrong position in the schematic tRNA structure in Fig. 7C.

We agree with the reviewer that we cannot order the reaction events with certainty. As such we have removed the ordered/temporal pathway schematic and summarize our findings in the discussion as follows:

"Structural and functional analysis are consistent with four key steps in the catalytic cycle of KEOPS:
1. Substrate recruitment. The tRNA is recruited by the binding of Cgi121 to its CCA tail. tRNA substrate is then fully engaged by a dynamic set of secondary interactions with Bud32, Kae1 and Pcc1 (depicted in **Fig. 7**).
2. Activation of Bud32 ATPase activity. Bud32 activation is a requirement for tRNA modification by Kae1. Since Bud32 activation depends on features unique to KEOPS substrates, this dependency might serve to ensure that Kae1 will only be active when a correct substrate is bound. We now show that this key step depends on three factors. Firstly, the interaction between the tRNA anticodon and conserved residues in the Kae1-specific-insert I. These contacts are most evident when the tRNA is in a native-like conformation. Secondly, the positioning of Arg237 of Kae1 in the active site of Bud32, where it facilitates ATP hydrolysis. This could explain why tRNA binding to the Cgi121-Bud32 complex in the absence of Kae1 does not potentiate ATPase activity[42]. Based on its positioning, we reason that Arg237 could exert its effect by stabilization of the γ–phosphate of ATP or the hydroxy anion (OH^-) intermediate generated by deprotonation of water by the catalytic base Asp467 of Bud32. Since Arg237 is visualized in the same position in Kae1-Bud32 structures without tRNA (PDB 3EN9 for example[47]), we speculate that Bud32 activation may arise from a change in conformational dynamics of its bilobal architecture, as this represents a point of regulation for other protein kinases[64]. Importantly, mutation of the equivalent Arg residue in human Kae1 (namely Arg247Gln in OSGEP) is causative for GAMOS[16]. Thus, our findings suggest the basis for this pathogenicity is due to a loss in the ability of OSGEP/Kae1 to activate PRPK/Bud32 in response to tRNA binding. Thirdly, a conserved C10-U11-G24-C25 D-arm motif that is unique to KEOPS substrate tRNAs.
3. Reordering of the tRNA anticodon loop, liberating A37 to be t^6A modified. This step correlates with a base flip at the inferred position of G26. Structural and mutational analysis point to a potential role for Arg530 in the C-terminal tail of Bud32 in interacting with the flipped-out base. We hypothesize that when G26 interacts with Arg530, it is released from its native interactions within the tRNA, which assists to distort the anticodon domain, enabling A37 to access the active site of Kae1. Pcc1 appears to play a role in this step by interacting with the anticodon loop since it is dispensable for the activation of Bud32 but is needed for t^6A modification. It is unclear if this step happens in parallel to Bud32 ATPase activation or after it.
4. The tRNA is modified followed by release from KEOPS, allowing commencement of a new catalytic cycle.

An expanded understanding of the mechanisms that underpin the catalytic cycle KEOPS awaits the determination of additional and higher-resolution structures of the holo-enzyme tRNA substrate complex. ”

Additional comments:

• Why did the authors not include a substrate analog of TC-AMP to bind to Kae1 and an ATP analog to bind to Bud32 in their structures for cryo-EM?

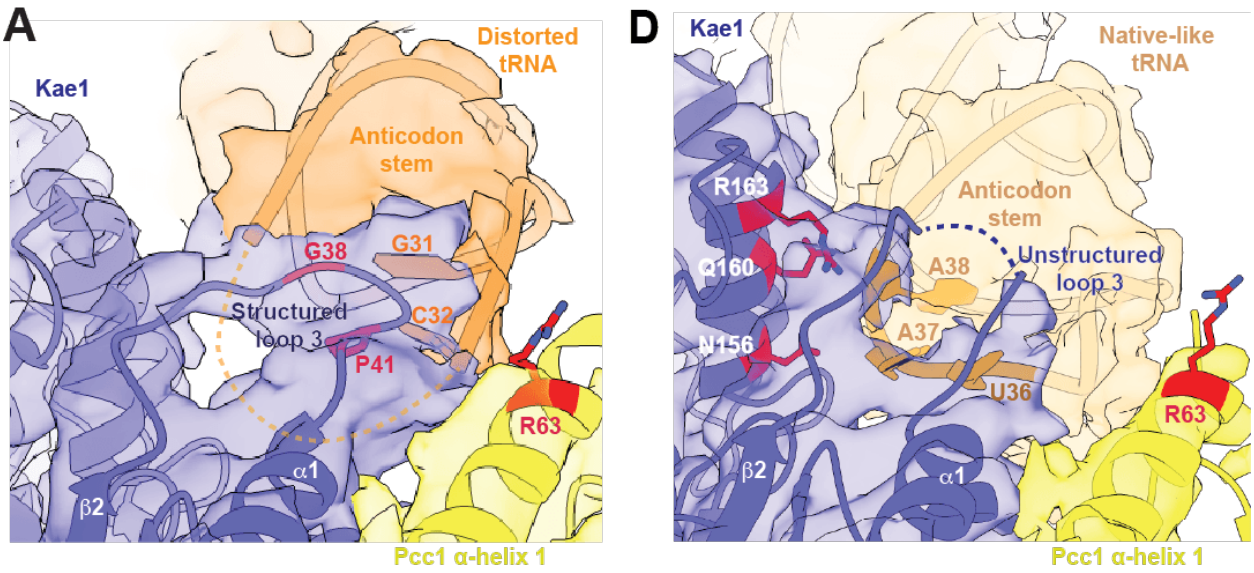
We agree with this reviewer that including substrate analogs of Kae1 and Bud32 are logical additions to structural determination attempts. In fact, we tried a variety of ATP analogs including AMP-PNP and ATP-gamma-S to improve the resolution of our maps and to gain further insight into KEOPS function, but they did not materially improve the resolution of our maps. In fact, in some cases we obtained greatly diminished maps. Unfortunately, a TC-AMP mimetic (DOI: 10.1093/nar/gkab026) is not commercially available. We looked into having the TC-AMP mimetic synthesized by a contract research organization but the cost was prohibitive.

• The authors state that a new contact is formed between C32 and G31 in the anticodon loop and loop 3 of Kae1. However, this contact is not evident in Fig 5D, and the comparative structure of the undistorted tRNA is not shown.

We have updated our figure set with higher resolution structures and have added a side-by-side comparison of the distorted tRNA co-structure with the native-like tRNA co-structure. These figures show that loop 3 of Kae1 is structured and juxtaposed to G31 and C32 of the tRNA in the distorted conformation and not in the native like conformation. The resolution does not allow one to visualize direct contacts. We have clarified this point in the main text (Page 11, lines 317-321)

> “In both tRNA bound structures, the tRNA anticodon loop is oriented towards the active site of Kae1 and Pcc1. In the distorted tRNA conformation, the G31-C32 dinucleotide of tRNA is juxtaposed to loop 3 of Kae1 (residues 23-43, between β-sheet 2 and α-helix 1) and the C’ terminus of Helix 1 of Pcc1 (**Fig. 8a)**. Local resolution did not allow to visualize direct contacts.”

Figure 8



• In the methods sections, the authors must provide equations which were used to determine dissociation constants and IC50 values.

IC50 values were calculated using the log(inhibitor) vs response equation below:
Y= Bottom+ (Top-Bottom)/(1+10^(X-LogIC50)). Bottom= maximally inhibited response,
Top= maximal response.

We have added the missing information to the methods section as directed.

• The description of the fluorescence polarization assays states that it was “done in triplicates”. Were these triplicates done on different days with different preparations? The reported IC50 data are highly variable raising concerns. For example, Fig. 5A and B report IC50 for the wild-type system (Bud32 or tRNA which should be the same) as 1.53 +/- 0.5 microM and 3.0 +/- 0.81 microM. These data suggest that the authors are overestimating the precision of their data which must be addressed.

The experiments in question were carried out over the course of multiple years using different preparations of protein, tRNA, solutions and fluorescent probe. This likely contributed to the observed variability across experiments. Despite this, the IC50 values across experiments were not dramatically different (within 4-fold) ranging from 0.75, 1.38, 1.39, 1.42, 1.53 and 3 μM for the displacement of the CCA probe from KEOPS WT with tRNA Lys WT. The variability does highlight the importance of testing mutant proteins and tRNAs at the same time under identical conditions if one wants to compare relative differences.

Therefore we only compared IC50 values within each experiment and not across different experiments. For example, we conclude that $tRNA^{Lys}$ G26A binds with higher affinity to KEOPS than $tRNA^{Lys}$ G26U, since the two tRNA samples were ran in the same set of experiments (**Figure 4a).** We cannot comment on the relative binding affinities of these two tRNA samples compared to $tRNA^{Lys}$ C25A which was run in a completely separate experiment (**Figure 5d**).

In all experiments in the manuscript, each experimental triplicate represents three technical replicates that were run on the same day. We have clarified this in the current submission.

• In Fig 2C and D: why are the variants R163E and Q160D labelled with an asterisk?

We have removed the asterisks in question. We thank this reviewer for pointing this out.

• Why is figure 5H labelled to include tRNAMet whereas the figure legend refers to tRNALys?

We will unify the nomenclature in the figure legend and the figure. It should read tRNA Lys WT and tRNA Lys G26 dimethylated. We thank this reviewer for pointing this out.

• In Figure 7B, why is the peak for t6A shifted for Ala36-UUA38 compared to Lys WT?

This mistake occurred when we created the figure from the raw chromatograms. One can see that the whole chromatograph is misaligned such that all the peaks including unmodified A (prominent peak at ~20 minutes retention time) as well as t6A are shifted from their expected position. We have correctly aligned the chromatograms in the revised figure and thank this reviewer for pointing this out.

• In the supplementary material, the author should show the position of Bud32 E152R which was used to render the KEOPS complex inactive and explain the effect of this mutation.

We have included the figure in question as a supplementary figure 1c. We do not have an obvious answer as to why the mutation causes a loss of activity but the Glutamate residue lies suggestively close to Arg530 in the tail of Bud32, which we show is essential for t6A function.

• For Fig. S6B, the authors should clarify in the figure legends what is shown in the lanes labelled as “G” and “A”.

“G” and “A” (**figure 3Sb** in the current submission) refers to transcription blockages in the primer extension analysis indicative of the presence of guanine and adenine. The following clarification has been added to the figure legend:

> **b** Primer extension analysis of m^2_2G-modified and non-modified $tRNA^{Lys}$. Red box indicates quantitative blockage of reverse transcriptase arising from complete m^2_2G modification at G26. G and A refer to transcription blockages at guanine and adenine sites correspondingly.

We thank the reviewer pointing out this issue.

Reviewer #3 (Remarks to the Author):

In this work the authors report three novel cryo-EM structures of KEOPS complex (two with distinct tRNA conformations and one apo). Based on these structures and considerable biochemical follow up on the mutants based on the structures, the authors propose a catalytic cycle of this enzyme complex. The resulting cycle has a number of key features: (1) Bud32 ATPase regulation through Kae1 tRNA binding via Arg237 positioning (2) Order to disorder transition in the anticodon loop on the tRNA associated with a G26 base flip. Although the apo structure is very similar to the previous structures in the field (the ones that authors used to build their models, combination of PDBs 3enh and 3eno) the structure in the presence of tRNA together with resolution of multiple conformations and associated biochemistry, all resulting in a mechanistic model, certainly seems to be an advance for the field worth publishing. I think the work should be published with minor text/figure revisions addressing the following concerns.

We thank this reviewer their supportive comments.

The main concern is the fact that cryo-EM map resolutions are quite varied within and across the structures (based on examination of provided maps). Many of the key arguments about the specific interactions mentioned in the manuscript are not unambiguously supported by the experimental density. Furthermore, there seems to be slight "streaking" likely due to some degree of preferred orientations in the sample and likely resulting in the differential resolutions in different reconstruction directions. Examining the maps, there are side chain densities missing and extra densities present next to some residues. Often it seems that specific residue interaction arguments are made based on a roughly ~5A map resolution for those regions. Due to the rigorous biochemical experimental follow up, I do not think this invalidates the paper's findings but the fact that many atomic interactions are based on map regions with resolution considerably worse than reported should be explicitly addressed and disclosed to the readers. For example, in the 3' CCA interaction, side chain of a key, bulky interacting residue, Phe21 is not resolved at all in the distorted structure and barely resolved in the ordered tRNA structure. Side chain densities for Arg163 and and Arg237 are also quite ambiguous. Arg530 placement is also ambiguous with even the backbone not being very well defined. Additionally, the exact structure of the distorted tRNA is also ambiguous. Although there is a clear density for where flipped G26 base would be, the phosphate-sugar backbone for bases 11-18, 22-25, 41-45 is completely out of the cryo-EM density making overall positioning of G26 questionable, even if one low pass filters the map.

Here are concrete things that I think can make the manuscript better in this regard:
(1) When discussing specific interactions preface the discussion with a comment on the quality of the map in that region.
(2) Report 3D FSC plots for all the reconstructions to indicate resolution anisotropy
(3) Report per chain average Q-scores for all models and when discussing specific

residues include that residue Q-score in the main text to provide a quantitative assessment of the map resolution in that region.

We fully accept this reviewer's criticism and have revised the manuscript adhering to the above recommendations.

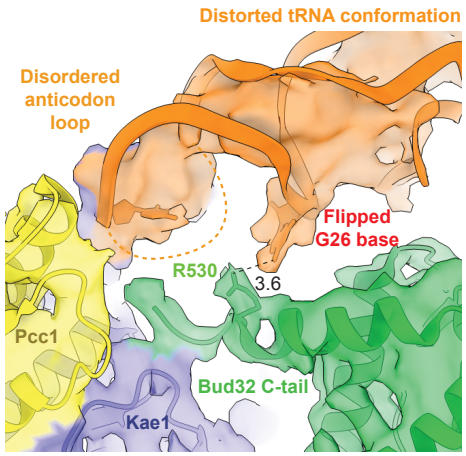
1. We have removed the comparison of the X-ray crystal and cryo EM structures of the Cgi121-CCA tail interaction from this submission (**Supplementary Figure 3a** in the previous submission). We accept that the resolution of the cryo EM structures do not allow atomic level description of the interactions between the CCA tail and Cgi121. We rely on the detail from our previous publication instead (Beenstock et al. Nat Comm 2020).
2. We have added throughout the main text prefaces commenting on map quality for each region discussed. In particular, we have added the following preface when discussing Arg163 and Arg237 of Kae1:

> "In its native-like conformation, the conserved 36-UAA-38 motif of tRNA[52-54], is positioned to contact a conserved region in Kae1 termed the Kae1-specific-insert (**Fig 8d**). This insert distinguishes Kae1-family enzymes from other members of the functionally diverse ASKHA-fold family[50,51]. Although the local resolution limits definitive side chain and base positioning, Asn156, Gln160 and Arg163 in the Kae1-specific-insert are well placed to interact with A37 (the modification site of the tRNA itself) and A38 (**Fig. 8d**)."

3. To assess resolution anisotropy, we have performed anisotropy analysis on all reconstructions using CryoSPARC. The output is displayed in **Supplementary Figure 3**.
4. In **Supplementary Table 1**, we now report per chain average Q-scores for residues and nucleotides involved in interactions that we comment on in the manuscript. Many of the Q-scores in question deviate from ideal values, consistent with the relatively low resolution of our maps. We make note of **Supplementary Table 1** as follows:

> "Although we could not unambiguously model the position of all tRNA bases and some protein side chains due to low map resolvability limited by the resolution (see **Supplementary Table 1** for the Q scores[51] of key residues in this study), these could be inferred in part from the published crystal structures of KEOPS proteins and of $tRNA^{Lys}_{UUU}$ in isolation.".

5. We have continued to improve the resolution of our cryo-EM maps with further rounds of data processing. As a prime example of the improvement of our maps, the interaction of the A37 flipped base with the side chain of Arg530 in the tail of Bud32 is now more clearly defined as is the backbone positioning of most of the Bud32 tail.
6. We acknowledge that in some of the displayed figures that the model does not lie fully in the map density. This is due in part to the choice of map contour levels.



Beyond the above, below are some more general comments addressing which I think can make the manuscript better:

(1) Although authors get dimer/monomer equilibrium they use a mutant to focus on the monomer structure. Could it be that the physiologically relevant structure is the dimer? Could authors provide/cite some evidence that it is not and structure of the monomer is enough?

The role of KEOPS dimerization is intriguing and not fully understood. In vitro, dimerization is dispensable for t6A modification activity as shown in our manuscript (**Fig. S1g**) and previously (DOI: 10.1093/nar/gkw542). In vivo, the fifth KEOPS subunit Gon7/Pcc2 prevents dimerization as it binds to the dimerization surface of Pcc1 (DOI: 10.1093/nar/gkw542, 10.1093/nar/gkv155, 10.1038/s41467-023-36210-y). There is some evidence by other groups that when KEOPS can dimerize, it results in lower-than-normal KEOPS protein levels in vivo, indicating that dimerization could lead to KEOPS degradation. However, the basis for this is not understood (DOI: 10.1038/s41467-019-11951-x). Interestingly, a Pcc1-Pcc1 mutant that prevents dimerization (similar to the Pcc1^{Mut} used in this study) cannot rescue a Pcc1 null mutant phenotype in yeast (DOI: 10.1016/j.molcel.2008.10.002). While this result suggests that Pcc1 mediated dimerization is important for in vivo function, the mutations in question would also perturb Gon7 interaction, which is also essential. Due to the complexity of the literature, we chose not to discuss this in the current paper to not over burden the readers.

(2) The idea that tRNA binding on Kae1 activates Bud32 via repositioning of the Arg237 is cool. However, the protein structures seem to be nearly identical between the apo and

tRNA bound complexes and although the authors place Arg237 side chain in a very different conformation in apo vs tRNA bound, I am not sure that is warranted based on the experimental density. But beyond that, can the authors provide some sort of mechanism to how this allosteric activation of Bud32 happens considering effectively identical Bud-Kae structures?

This is an excellent question that piqued our interest. Based on the similarity of the tRNA bound and unbound structures, our working hypothesis is that tRNA binding to KEOPS may affect the conformational dynamics of the Bud32 kinase domain, which in turn influences its catalytic function. We inferred this possibility based on prior work on the unrelated Eph receptor kinases (RTKs) (PMID: 16977320). In response to the suggestion of the reviewer, we now speculate in the discussion section that something similar might underlie the regulation of Bud32 ATPase activity by tRNA binding to KEOPS.

> Since Arg237 is visualized in the same position in Kae1-Bud32 structures without tRNA (PDB 3EN9 for example[47]), we speculate that Bud32 activation may arise from a change in conformational dynamics of its bilobal architecture, as this represents a point of regulation for other protein kinases[61]

(3) Can the authors comment on how Asn156Ala, Gln160Asp, and Arg163Glu mutants would enhance the tRNA binding given their model, as that's what they see experimentally but it doesn't quite make sense structurally?

The main message from this tRNA binding experiment is that the mutations in KEOPS subunits leave binding to tRNA largely intact. However, as this reviewer points out, tRNA binding affinity was enhanced to some degree (2.5 to 5-fold) as a result of the Asn156Ala, Gln160Asp, and Arg163Glu mutations in Kae1. We agree that this is an interesting question and the short answer is that we do not know why. One possibility is that the mutations, which are predicted to inhibit tRNA binding to one tRNA conformation, could favor tRNA binding in a different tRNA conformation. We would like to pursue structures of these tighter KEOPS-tRNA complexes in the event they further inform on protein function but this is a major undertaking and thus beyond the scope of the present study.

(4) Reporting specific distance from A37 to the TC-AMP in the distorted tRNA structure is beyond what the experimental evidence shows. That whole region is completely disordered (ie, completely missing density) in the distorted tRNA structure and coupled to the fact that the phosphate-sugar backbone for bases 11-18, 22-25, 41-45 is completely out of the cryo-EM density makes overall placement of that half of tRNA questionable. This is on top of the fact that TC-AMP is not present in any of these structures and its placement is inferred from the previous structures.

Therefore reporting the exact distance between the base and TC-AMP in the tRNA distorted structure is certainly an overinterpretation of the data which will likely be missed by readers without structural biology background and will instead be interpreted as hard

truth. Much more explicit language about what actually can be clearly resolved in the structures reported here and what is inferred should be used.

We have taken this suggestion to heart when we re-wrote the manuscript so that we don't mislead non-structural biologists. The main point we are trying to make here is that in the native conformation A37 is definitely out of reach for modification, while in the distorted conformation A37 *could* be poised for modification due to the disordered nature in the encompassing tRNA anticodon loop. The revised text now reads:

> "The KEOPS native-like tRNA structure revealed that although the anticodon loop is directed towards the active site of Kae1 (**Fig. 2c**) A37 is not suitably positioned for t^6A modification (**Fig. 2c inset**). In the t^6A modification reaction, N^6 of A37 is modified by TC-AMP positioned in the active site of Kae1 (**Fig. 1a**)[48]. Using the published structure of the Kae1 family enzyme TsaD bound to a TC-AMP mimetic molecule (PDB 6Z81), we modeled the expected position of TC-AMP in the active site of Kae1 in the KEOPS-tRNA structures. Based on this modeling, in the native-like state, N^6 of A37 lies ~17.2 Å away from its site of attack on TC-AMP, thereby ruling out t^6A modification (**Fig. 2c inset**, predicted site of attack in TC-AMP inferred by the catalytic model proposed for the prototypic carbamylating enzyme TobZ[49]). In the KEOPS-distorted tRNA structure however, 7 nucleotides within the anticodon loop (U33 to C39, inclusive) encompassing A37 are disordered, indicating that this region has higher flexibility in this conformation. Thus, A37 (although not visible due to disorder) in principle would have the ability to more closely approach the active site of Kae1. Although, we cannot discern the precise position of A37, modeling reveals that it could approach within 5.5 angstroms of the inferred position necessary for its modification."