

## Peer Review File

**Manuscript Title:** NAC guides a ribosomal multienzyme complex for nascent protein processing

### Reviewer Comments & Author Rebuttals

#### Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

Shan, Ban and Coworkers present a beautiful story on the molecular basis of how NAC coordinates N-terminal acetylation by the NatA N-terminal acetyltransferase complex. The authors combine biochemical, cell biological and structural cryo-EM data on ribosome-nascent chain (RNC) complexes with NAC/NatA/E, NAC/NatA/E/Met-AP1 and NAC/NatA/E/Met-AP1/HYPK to generate a compelling model for how NAC contributes to the recruitment of both Met-AP1 and NatA/E and how it relieves the intrinsic NatA inhibitory activity of HYPK. Although the proteins bound to the RNC are generally of significantly lower resolution than the RNC, the docked high-resolution structures and complexes (predicted by AlphaFold) fit the cryo-EM density well and are validated by biochemical and cellular studies. Overall, the studies are very clearly described and presented and provide important new insights into the coordination of co-translational N-terminal acetylation by NatA. I believe that the study is very suitable for publication in Nature pending that the authors address the following relatively minor issues:

1. Page 5, line 15; “as previously suggested in biochemical studies<sup>21</sup>.” Should also reference 20, which made the primary biochemical observation.
2. Page 5, lines 24-25; the authors should elaborate a bit on the differences with the yeast system as this would be of significant interest to the field.
3. Page 6, lines 11-14; in the sentence “These results indicate that high affinity NatA interaction with the ribosome is predominantly mediated through the flexibly disposed NAC UBA domain, but that the electrostatic contacts with ribosomal RNA optimally position the enzyme for nascent chain

interactions.” While the data for the former clause is shown, it is unclear what data the latter clause is based on. I may have missed the data, but if the authors don’t show that the electrostatic contacts with ribosomal RNA optimally position the enzyme for nascent chain interactions, this clause should be deleted.

4. Page 6, line 16; change “Figs 3” to “Fig. 3”

5. Figure S9-C; the legend should include text to understand how to interpret the plot.

6. The authors should comment about whether or not the nascent chain is observed in the exit tunnel, and if so to what resolution. It appears to be visible in red in panel 3A.

7. The authors should comment in the methods section on what percentage of their particles of the various complexes contained all the added components compared to partially occupied ribosomes.

8. Since it was not mentioned, I am assuming crosslinking was not employed in the cryo-EM analysis.

Referee #2 (Remarks to the Author):

As the nascent polypeptide chain emerges from the ribosomal tunnel, it is subject to a number of co-translational modifications, including N-terminal excision and acetylation, which are essential in higher eukaryotes. Despite decades of research, the interplay between the different enzymes that mediate these processes at the tunnel exit is not well-understood. The study of Lentzsch et al reports complementary biochemical, structural and in vivo approaches from three independent laboratories showing that the nascent polypeptide-associated complex (NAC) coordinates the actions of N-acetyltransferase Nat A and the methionine aminopeptidase MetAP1. The experiments are well-performed and technically solid, the results are nicely presented, and the conclusions are very exciting, fundamentally important and will be of general interest to the readers of Nature. Nevertheless, I have a series of major and minor comments that I hope will improve the manuscript further.

Major points.

1. Page 3, line 25. If I understand correctly from the methods, the biochemical and structural studies are performed with human factors and Rabbit RNCs i.e. this is a heterologous complex in all cases. Given the conservation of humans and rabbit ribosomes, I find this acceptable. In fact, it is quite common to use the more efficient active rabbit system compared to the human one. However, I don't find it acceptable to suppress this information and not to make it clear at the onset of the study (I only noticed this on page 6, line 30 that rabbit RNCs were mentioned whereas on page 3 it is not made clear - or were human RNCs used here but I could not find anything about human translation system in the methods). If indeed heterologous complexes were used everywhere, then this should be clearly stated as a limitation of the study and a few sentences provided to explain why this is valid i.e. the high conservation between the human and rabbit factors and ribosomes – assuming this is the case.
2. In addition to heterologous Human factor-rabbit ribosome complexes (see point 1 above), the structures are made with complexes that contain inactive factors. On page 4, line 25 it is stated that structures were solved of the “ternary complex comprising RNCrpl4, NAR and NatA/E” and later “...a quaternary complex that includes MetAP1”...in fact, from the methods it appears that the authors used a catalytically inactive NatA with Naa10 E24Q/Y26F mutations and MetAP1 with a D220N mutation. I don't see this as a problem, but it needs to be made clear in the text and is again a limitation of the study in that it's not the wildtype proteins that were used. In fact, it is unclear why the mutations were used? Are the wildtype proteins dissociating after performing their activity? Maybe the rationale of using these mutants could be explained in the main text too.
3. In relation to point 2, if inactive mutants were used in the study to trap pre-cleavage or pre-acetylation states, then maybe one would expect to see the substrate in the active sites? Despite the authors using RNCs, I was surprised that there was little mention of the nascent chains...were they observed in the tunnel at all? In Figure 1A it is mentioned that the position of the nascent chain in the

PET is indicated in dark grey but its unclear whether density is actually observed in any of the structures. In Figure 3A there is a surface representation for a nascent chain which implies that something has been modelled – what exactly and to what degree of confidence, which residues? In Figure 1B the legend implies that the solid pink arrow is the path observed for the nascent chain where the dashed link arrow is hypothetical...however, apparently no density or model is shown to indicate that the solid pink path can be shown with any degree of confidence. In fact, its not really made clear that all structures used the RpL4 RNC...for example, on page 7, line 30 it is simply written “RNC bearing a nascent chain of 105aa in length”...why not state that it is in the RpL4 nascent chain rather than the reader having to search for this information? I am presuming that the authors don’t actually visualize any nascent chain outside of the exit tunnel – nevertheless, this should be stated if that is the case...in this respect, the authors write that the SNAP25 nascent chain folds into a rigid helix, leading the reader to wonder if the authors tried using this nascent chain for structure determination? And if not, why not?

4. The resolution of the complexes is relatively modest 3.2-3.9Å and local resolution 6-10Å much worse for the factors. I understand that ligands at the exit tunnel are generally flexible and therefore the resolution suffers. However, the authors also know this in advance, which does lead one to wonder why such modestly sized datasets were collected i.e. all under 10,000 micrographs. With such an experienced structural lab with huge resources, I would have expected much larger datasets to be collected in order to increase the final particle numbers in the final reconstructions or allow for further subsorting for stable classes...this could have considerably improved the local resolution, which may have provided more confidence for the models being generated based on fitting on alpha-fold models and therefore more confidence in the interpretation. Obviously, this would be a lot of work to start again but one does wonder if density for some components could be improved by additional approaches using the current datasets, such as combining classes from different datasets or masked classification using all classes containing density for the factors etc. It would seem reasonable and not to time-consuming to illustrate that the authors have attempted to get everything out of their datasets. I would also strongly urge the authors to deposit the raw data in the EMPIAR database.

5. The interplay with SRP is mentioned. It is a shame that no experimental data is shown here as to how SRP interplays with NAC in the context of MetAP1 and NatA. The authors have all the proteins and assays ready and a few additional experiments would nicely increase the completeness of the manuscript significantly.

#### Minor points

1. The presentation of the structural results is confusing in the beginning. The authors determine structures of the ternary and quaternary complex and then show only images of the quaternary complex in Figures 1 and 2. The FRET data in Figure 1 is related to NatA and not MetAP1 so why not just present the ternary complex structure first to complement this data? I understand that the information from the ternary complex is also present in the quaternary complex but the ternary complex is a separate result

and, in my opinion, needs to be also presented in the main figures too. In fact, it's the higher resolution complex with the slightly better statistics.

2. On page 5, line 25, the authors mention that the binding modes for mammalian NatA/E here are strikingly different to that for yeast without NAC. Are the authors trying to say that if NAC was present in the yeast complex, then the binding would be the same? Or what is the point here? This statement is at best ambiguous or at worse misleading.

3. The authors like to use lots of abbreviations in this paper for RRL, NTA, NME, RNC, PET etc etc...for someone not in the field, it can be quite confusing, especially NTA and NME. Perhaps its better to simply write some of them out and reserve the abbreviations for the factors. Also to check really how many times they are used in the main text...is it really saving so much space...space usage does not appear to be a problem with this manuscript.

### Referee #3 (Remarks to the Author):

In mammalian cells, the N-termini of many nascent chains are processed. In the present study, the authors show that the nascent chain-associated complex NAC orchestrates this processing on the polypeptide exit site of the ribosome. The two subunits of NAC, alpha and beta, provide multiple binding sites to recruit the methionine aminopeptidase MetAP1 and the N-acetyl transferase NatA to the ribosome. They further unravel the role of HYPK, a competitive inhibitor of NAC which has structural similarity to NAC. The authors propose that NAC displaces HYPK from ribosomes upon exposure of nascent chains on the exit site to initiate the processing of the N-terminus of the nascent peptide.

The study is exciting and considerably increases our understanding of how NAC facilitates the complex processing events of nascent chains by different modifying enzymes on the ribosome exit site. In a recent study by the same authors, the role of NAC in recruitment of MetAP1 and SRP was shown. Apparently, NAC uses the same UBA domain of subunit alpha for SRP binding and for NatA binding. The structures shown here support the proposed role of NAC in enzyme recruitment, however, convincing evidence for a sequential hand-over by NAC of the nascent chains from one enzyme to the next, as proposed in the model in Fig. 5, is not provided. Still, this is an exciting study of excellent quality which is of interest for a very broad readership.

### Major points

1. The multiple interactions of NAC to different enzymes are compatible with the model presented in Fig. 5 according to which NAC serves as part of a dynamic clockwork. However, alternatively, NAC might serve as a passive multi-tool on the exit site that like a Swiss army knife facilitates the processing by different binding partners. In the present study, structural information on the three states of Fig. 5 (pre-3, pre-4 and post-4) are not provided. Since the authors defined the chain lengths that populate these different stages (less than 68, 68-88, and larger than 88), it should be possible to capture the structural arrangement of NAC and its cofactors during these stages. This should allow to clarify whether NAC is rather a passive recruitment factor that uses its spider arms to increase the affinity of enzymes to the ribosome, or whether NAC indeed directs N-terminal protein processing (as the title claims) in a coordinated sequence of events. Either outcome would be interesting but the study was even stronger if this issue could be addressed.
2. Along the same lines: the authors use *C. elegans* as a model to demonstrate convincingly the relevance of NAC in vivo. This model should allow to test whether NAC mutants lacking individual binding sites for MetAP1 and NatA (thus the C-terminus of beta and the UBA domain of alpha) can cross-complement each other if they are co-expressed simultaneously. This would again allow to distinguish a passive multi-tool recruiter from a dynamic clockwork device.

3. The data shown suggest that nascent chains can be acetylated by NatA even in the absence of NACalpha, particularly if HYPK is absent. This is evident from Fig. 2H or Fig. 4D. Does the acetylation here still occur co-translationally? The assay shown in Fig. 3D should allow to follow the acetylation of chains of different lengths also under conditions in the absence or presence of the UBA domain of NACalpha and/or of HYPK.

4. The UBA domain apparently binds as well to SRP as to NatA. Can both factors bind simultaneously and what is the physiological relevance of the mutual interactions to the same arm of the NAC complex. A comprehensive analysis is certainly beyond the scope of this manuscript, but the authors should shortly discuss this aspect.

## **Author Rebuttals to Initial Comments:**

Dear editor,

Thank you for your time and efforts in handling this manuscript. We also thank the reviewers for positive comments, helpful suggestions and for suggesting interesting additional experiments. Below are our point-by-point responses (blue) to reviewers' comments (black). We think that the manuscript is significantly improved and hope that it is now acceptable for publication.

## **Referee #1 (Remarks to the Author):**

Shan, Ban and Coworkers present a beautiful story on the molecular basis of how NAC coordinates N-terminal acetylation by the NatA N-terminal acetyltransferase complex. The authors combine biochemical, cell biological and structural cryo-EM data on ribosome-nascent chain (RNC) complexes with NAC/NatA/E, NAC/NatA/E/Met-AP1 and NAC/NatA/E/Met-AP1/HYPK to generate a compelling model for how NAC contributes to the recruitment of both Met-AP1 and NatA/E and how it relieves the intrinsic NatA inhibitory activity of HYPK. Although the proteins bound to the RNC are generally of significantly lower resolution than the RNC, the docked high-resolution structures and complexes (predicted by AlphaFold) fit the cryo-EM density well and are validated by biochemical and cellular studies. Overall, the studies are very clearly described and presented and provide important new insights into the coordination of co-translational N-terminal acetylation by NatA. I believe that the study is very suitable for publication in Nature pending that the authors address the following relatively minor issues:

We thank the reviewer for their positive comments and have made the changes proposed in the text as detailed below.

1. Page 5, line 15; "as previously suggested in biochemical studies<sup>21</sup>." Should also reference 20, which made the primary biochemical observation.

We apologize for this omission and have added Magin, R. et al 2017.

2. Page 5, lines 24-25; the authors should elaborate a bit on the differences with the yeast system as this would be of significant interest to the field.

Due to the page limitation in Nature, we have extended this discussion in the legend of Figure S11 as follows: *“While the N-terminal helical domain of Naa15 contacts the ribosome near the exit tunnel in both mammalian and yeast systems, all other structural elements that mediate NatA recruitment and positioning on the ribosome are distinct in the two organisms. Firstly, ribosome-bound NAC captures and helps position NatA/E in mammals, whereas in yeast, the rRNA extension Es27 is proposed to act as a protein recruitment hub for NatA in place of NAC. Secondly, the second catalytic subunit Naa50 mediates an additional contact with the rRNA extension Es7a on the yeast ribosome, whereas Naa50 is not involved in ribosome contact and hence does not contribute to the ribosome affinity of NatA/E in the mammalian system. Thirdly, the location of the NatA/E complex at the ribosome exit site is completely different in the two organisms. Finally, the ribosome binding site of mammalian NAC overlaps with that of yeast NatA/E, suggesting that yeast NAC antagonizes rather than facilitates the ribosome recruitment of NatA/E. These differences, together with the absence of the NatA regulator HYPK in S. cerevisiae, suggest that the ribosome recruitment mechanisms for protein biogenesis factors are distinct between yeast and higher eukaryotic organisms.”*

3. Page 6, lines 11-14; in the sentence “These results indicate that high affinity NatA interaction with the ribosome is predominantly mediated through the flexibly disposed NAC UBA domain, but that the electrostatic contacts with ribosomal RNA optimally position the enzyme for nascent chain interactions.” While the data for the former clause is shown, it is unclear what data the latter clause is based on. I may have missed the data, but if the authors don’t show that the electrostatic contacts with ribosomal RNA optimally position the enzyme for nascent chain interactions, this clause should be deleted.

What we mean is that the NAC UBA is flexibly linked to its globular domain, as we previously showed (Jomaa, Hsieh et al, Science 2022), so the UBA contact would bring NatA/E to the ribosome but would not position NatA/E for nascent protein processing. The direct interaction of Naa15 with rRNA near the polypeptide tunnel exit serves this role. In addition, there is a reproducible 3-4 fold decrease in NatA/E binding affinity upon mutation of the positively charged helix on Naa15 that contact rRNA, indicating that these contacts are established in the E-S ground state complex. We changed this clause to read *“... the electrostatic contacts of the positively charged helix in NatA with ribosomal RNA near the polypeptide tunnel exit may optimally position the enzyme for nascent chain interactions.”*

4. Page 6, line 16; change “Figs 3” to “Fig. 3”

Done.

5. Figure S9-C; the legend should include text to understand how to interpret the plot.

We have updated the figure and the legend to clarify the AlphaFold prediction plots.

6. The authors should comment about whether or not the nascent chain is observed in the exit tunnel, and if so to what resolution. It appears to be visible in red in panel 3A.

We observe density for nascent chains in maps of stalled translating ribosomes with a P-site tRNA. The nascent chain density extends throughout the polypeptide tunnel in all three homogeneously refined maps resolved at approximately 4-7 Å. In each reconstruction, the C terminus of the nascent chain connects to the 3' CCA motif of the P-site tRNA and is sufficiently well ordered to model the amino acid residues adjacent to the tRNA. As the resolution of the nascent chain density decreases towards the tunnel exit, we tentatively modeled it in the tubular density, also guided by lowpass-filtered maps. There is no clear density for the nascent chain outside the tunnel, likely because it is conformationally dynamic. For all three structures, we modeled the nascent polypeptides within the tunnel as poly-UNK chains. We have updated the main text and the methods section to clarify that.

7. The authors should comment in the methods section on what percentage of their particles of the various complexes contained all the added components compared to partially occupied ribosomes.

We have updated the methods section accordingly.

8. Since it was not mentioned, I am assuming crosslinking was not employed in the cryo-EM analysis.

We have not crosslinked the samples used for cryo-EM.

#### **Referee #2 (Remarks to the Author):**

As the nascent polypeptide chain emerges from the ribosomal tunnel, it is subject to a number of co-translational modifications, including N-terminal excision and acetylation, which are essential in higher eukaryotes. Despite decades of research, the interplay between the different enzymes that mediate these processes at the tunnel exit is not well-understood. The study of Lentzsch et al reports complementary biochemical, structural and in vivo approaches from three independent laboratories

showing that the nascent polypeptide-associated complex (NAC) coordinates the actions of N-acetyltransferase Nat A and the methionine aminopeptidase MetAP1. The experiments are well-performed and technically solid, the results are nicely presented, and the conclusions are very exciting, fundamentally important and will be of general interest to the readers of Nature. Nevertheless, I have a series of major and minor comments that I hope will improve the manuscript further.

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#### Major points.

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We have clarified that our RNCs were generated from rabbit reticulocyte lysate on page 3.

2. In addition to heterologous Human factor-rabbit ribosome complexes (see point 1 above), the structures are made with complexes that contain inactive factors. On page 4, line 25 it is stated that structures were solved of the “ternary complex comprising RNCrpl4, NAR and NatA/E” and later “...a quaternary complex that includes MetAP1”...in fact, from the methods it appears that the authors used a catalytically inactive NatA with Naa10 E24Q/Y26F mutations and MetAP1 with a D220N mutation. I don't see this as a problem, but it needs to be made clear in the text and is again a limitation of the study in that it's not the wildtype proteins that were used. In fact, it is unclear why the mutations were used? Are the wildtype proteins dissociating after performing their activity? Maybe the rationale of using these mutants could be explained in the main text too.

We used catalytically inactive mutants of NatA and MetAP1 in order to capture the pre-chemistry steps in our biochemical and structural studies. As chemistry for both enzymes are rapid (within the timescale of mixing), we were concerned that using wildtype enzymes will generate the post-chemistry complexes. We have clarified this on page 3 and page 5.

3. In relation to point 2, if inactive mutants were used in the study to trap pre-cleavage or pre-acetylation states, then maybe one would expect to see the substrate in the active sites? Despite the authors using RNCs, I was surprised that there was little mention of the nascent chains...were they observed in the tunnel at all? In Figure 1A it is mentioned that the position of the nascent chain in the PET is indicated in dark grey but its unclear whether density is actually observed in any of the structures. In Figure 3A there is a surface representation for a nascent chain which implies that something has been modelled – what exactly and to what degree of confidence, which residues? In Figure 1B the legend implies that the solid pink arrow is the path observed for the nascent chain where the dashed link arrow is hypothetical...however, apparently no density or model is shown to indicate that the solid pink path can be shown with any degree of confidence. In fact, its not really made clear that all structures used the RpL4 RNC...for example, on page 7, line 30 it is simply written “RNC bearing a nascent chain of 105aa in length” ...why not state that it is in the RpL4 nascent chain rather than the reader having to search for this information? I am presuming that the authors don’t actually visualize any nascent chain outside of the exit tunnel – nevertheless, this should be stated if that is the case...in this respect, the authors write that the SNAP25 nascent chain folds into a rigid helix, leading the reader to wonder if the authors tried using this nascent chain for structure determination? And if not, why not?

Please see our response to comment 6 by referee #1. We made our best efforts to capture the pre-cleavage state of the cotranslational enzyme complex, including the use of catalytically deficient enzyme mutants and using a nascent chain (SNAP25) that folds into a rigid helix. Nevertheless, the density for the nascent chain was not visible outside the polypeptide exit tunnel, likely due to its conformational dynamics.

4. The resolution of the complexes is relatively modest 3.2-3.9Å and local resolution 6-10Å much worse for the factors. I understand that ligands at the exit tunnel are generally flexible and therefore the resolution suffers. However, the authors also know this in advance, which does lead one to wonder why such modestly sized datasets were collected i.e. all under 10,000 micrographs. With such an experienced structural lab with huge resources, I would have expected much larger datasets to be collected in order to increase the final particle numbers in the final reconstructions or allow for further subsorting for stable classes...this could have considerably improved the local resolution, which may have provided more confidence for the models being generated based on fitting on alpha-fold models and therefore more confidence in the interpretation. Obviously, this would be a lot of work to start again but one does wonder if density for some components could be improved by additional approaches using the current datasets, such as combining classes from different datasets or masked classification using all classes containing density for the factors etc. It would seem reasonable and not too time-consuming to illustrate that the authors have attempted to get everything out of their datasets. I would also strongly urge the authors to deposit the raw data in the EMPIAR database.

Over the course of the project, we have prepared and imaged a large number of cryo-EM samples that differed in composition or grid preparation methods – with 63,821 micrographs in total – to characterise the structures of the ternary and quaternary complexes. The two key datasets were collected on ternary complex samples with RNCs bearing the preprolactin and the RpL4 nascent chains, both at a nascent chain length of 53 amino acids. As only a modest set of RNC particles with NAC-NatA/E was found in the

RNC<sub>pPL</sub> dataset, we focused on the RNC<sub>RpL4</sub> dataset, which was described in the manuscript. Below, we describe an additional data processing approach to address this comment, which resulted in a higher resolution reconstruction for the bound NatA/E (termed the 80S-NAC-NatA/E structure, shown in new supplementary figure S8) and which corroborated the architecture, docked molecular models, and the interactions of NatA/E described for the RNC<sub>RpL4</sub>-NAC-NatA/E complex earlier.

We have reprocessed the data and expanded the particle set to try and obtain a reconstruction of ribosome-bound NAC-NatA/E at higher resolution. As we see NAC-NatA/E bound not only to translating ribosomes, we opted to pool ribosome particles with these factors regardless of whether they contain a nascent chain or not. Briefly, we started by selecting ribosomes with NatA/E density in the RNC<sub>RpL4</sub> dataset through focused 3D classification, then unbinned the data and performed another homogeneous refinement (figure S8A). This resulted in 197,483 particle images, from which we attempted to select particles that are homogeneous with respect to NAC-NatA/E conformation. By performing 3D classification with a focused mask covering the NatA/E core [NatA/E without the basic helix of Naa15], we obtained ten classes that correspond to distinct conformations of NAC-NatA/E on the ribosome. We have reconstructed maps for every class and compared them. While the NAC $\beta$  anchor and the basic helix of Naa15 are stably positioned on the surface of the large ribosomal subunit and in turn resolve as well as the adjacent segments of the ribosome for all classes, the globular domain of NAC and the bulk of NatA/E show a greater degree of flexibility.

In the new figure S8B-E, we present the map reconstructed from the particles of the better resolved 3D class. The obtained map has an overall resolution of 3.05 Å (figure S8A,B), while the resolution of the NAC-NatA/E sub-volume is 4.35 Å (figure S8C) with the local resolution ranging between 4 and 6 Å for the factors (figure S8E). While the architecture of the LSU-NAC-NatA/E complex in the new map matches that of the ternary complex presented earlier (figure S8D), the secondary structure and some bulky side-chains are better resolved in the new map.

5. The interplay with SRP is mentioned. It is a shame that no experimental data is shown here as to how SRP interplays with NAC in the context of MetAP1 and NatA. The authors have all the proteins and assays ready and a few additional experiments would nicely increase the completeness of the manuscript significantly.

While exploring the interplay between SRP, NAC and the enzymes is an exciting direction, it will require a long list of experiments with unclear outcomes that are beyond the scope of the current manuscript. Once SRP is introduced, we will need to examine nascent chains belong to different biogenesis pathways (ER targeting versus cytosolic processing/folding). We will also need to resolve the multiple potential mechanisms that contribute to the antagonism between the different factors, including whether the MetAP or NatA reaction is more affected by SRP, and if so, whether this effect arises from the clash of MetAP1 with the SRP GTPase domain, the competition of SRP and NatA for the NAC UBA, or the effect of different nascent chains and factors on the position of the NAC globular domain. We do not want to

show a cursory experiment on SRP-NatA/MetAP interplay without delving into the underlying mechanisms, which will be a shame for the field.

Referee 3 also raised this issue but suggested that we elaborate more in writing instead of performing additional experiments. We have followed their advice and commented on the potential interplay of these nascent chain processing enzymes with SRP in the discussion (p10).

#### Minor points

1. The presentation of the structural results is confusing in the beginning. The authors determine structures of the ternary and quaternary complex and then show only images of the quaternary complex in Figures 1 and 2. The FRET data in Figure 1 is related to NatA and not MetAP1 so why not just present the ternary complex structure first to complement this data? I understand that the information from the ternary complex is also present in the quaternary complex but the ternary complex is a separate result and, in my opinion, needs to be also presented in the main figures too. In fact, it's the higher resolution complex with the slightly better statistics.

As there are only minor structural differences between the models of NAC-NatA/E of the ternary and quaternary complexes, and Nature strictly limits the number of main figures, we were not able to present the architecture of the ternary complex and of quaternary complex in main text. Nevertheless, while in main figures we present the overall architecture for the quaternary complex only, the detailed density panels C-F shown in figure 2 correspond to the maps obtained for the ternary complex as it is indeed the better resolved structure. We have updated the figure legend to clarify that. Furthermore, we also refer in the main text to the even better resolved map of the ternary complex provided as part of this revision in a new supplementary figure S8.

2. On page 5, line 25, the authors mention that the binding modes for mammalian NatA/E here are strikingly different to that for yeast without NAC. Are the authors trying to say that if NAC was present in the yeast complex, then the binding would be the same? Or what is the point here? This statement is at best ambiguous or at worse misleading.

Please see our response to comment 2 by referee #1, who raised a similar question. We have described in detail the differences between the yeast and mammalian cotranslational NatA/E complexes on the ribosome in the legend to Figure S11.

We are not sure about the exact reasons behind the observed differences, as the role of yeast NAC in NatA/E recruitment has not been studied. On the one hand, the previous yeast structure was generated from a native pulldown; if NAC were an integral component of the yeast cotranslational nascent chain processing complex, it would likely have been captured. On the other hand, we cannot exclude the possibility that NAC association on yeast ribosome was more flexible and was therefore lost during sample preparation. Based on the major differences between the yeast vs mammalian complex structures and the observation that HYPK was also not present in *S. cerevisiae*, the evidence leans more towards fundamental differences in the enzyme recruitment mechanism between *S. cerevisiae* and higher eukaryotic organisms. However, this remains a speculation.

3. The authors like to use lots of abbreviations in this paper for RRL, NTA, NME, RNC, PET etc etc...for someone not in the field, it can be quite confusing, especially NTA and NME. Perhaps its better to simply write some of them out and reserve the abbreviations for the factors. Also to check really how many times they are used in the main text...is it really saving so much space...space usage does not appear to be a problem with this manuscript.

We replaced all the NTA and NME abbreviations.

Referee #3 (Remarks to the Author):

In mammalian cells, the N-termini of many nascent chains are processed. In the present study, the authors show that the nascent chain-associated complex NAC orchestrates this processing on the polypeptide exit site of the ribosome. The two subunits of NAC, alpha and beta, provide multiple binding sites to recruit the methionine aminopeptidase MetAP1 and the N-acetyl transferase NatA to the ribosome. They further unravel the role of HYPK, a competitive inhibitor of NAC which has structural similarity to NAC. The authors propose that NAC displaces HYPK from ribosomes upon exposure of nascent chains on the exit site to initiate the processing of the N-terminus of the nascent peptide.

We thank the reviewer for the positive comments. We would like to clarify that we did not show NAC displacing the entire HYPK from NatA. Instead, we showed that NAC and HYPK cobind on NatA, and NAC displaces an intervening linker in HYPK that positions the inhibitory N-terminus in the NatA active site, thereby allosterically activating the NatA-HYPK complex.

The study is exciting and considerably increases our understanding of how NAC facilitates the complex processing events of nascent chains by different modifying enzymes on the ribosome exit site. In a recent study by the same authors, the role of NAC in recruitment of MetAP1 and SRP was shown. Apparently, NAC uses the same UBA domain of subunit alpha for SRP binding and for NatA binding. The

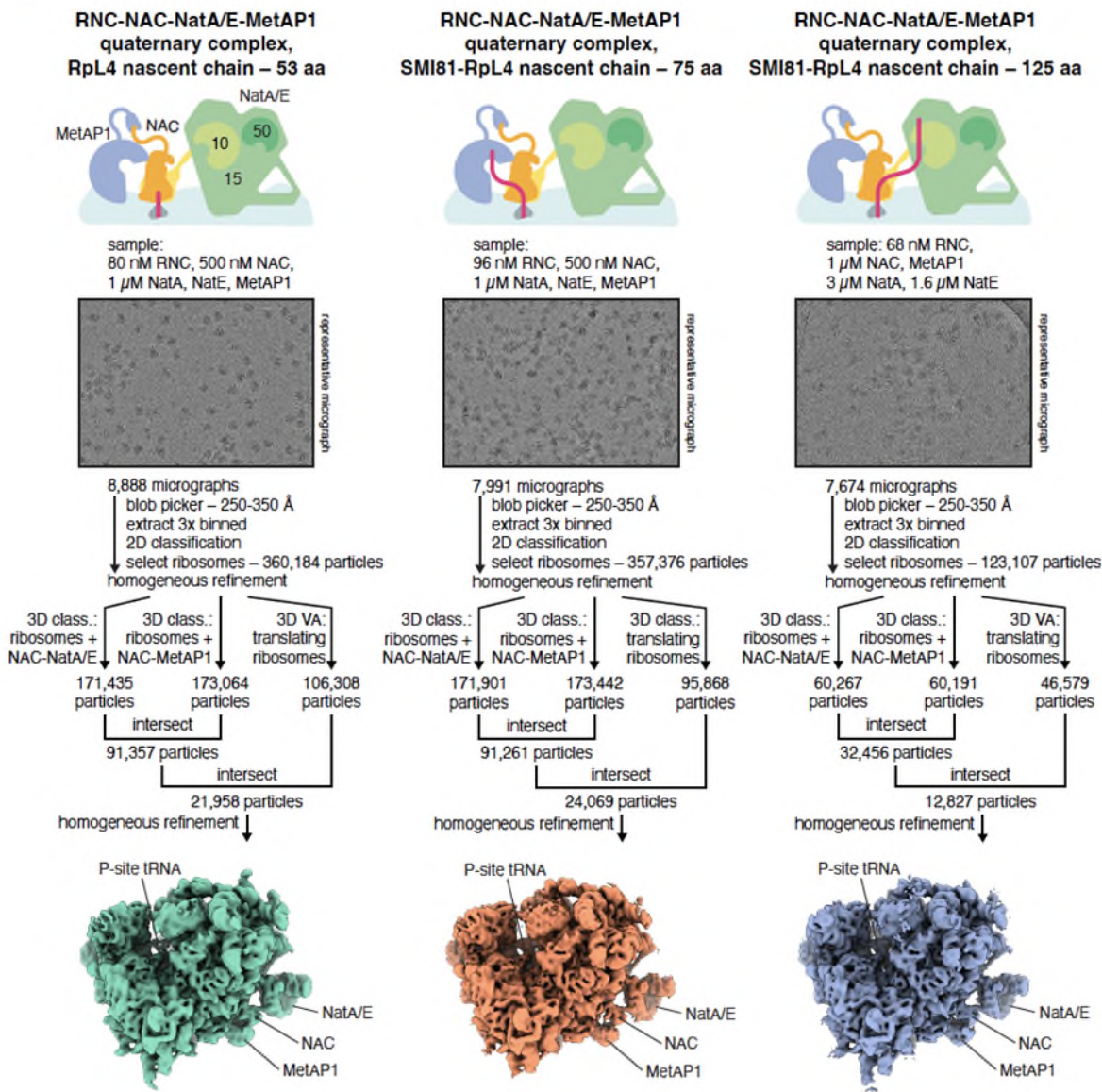
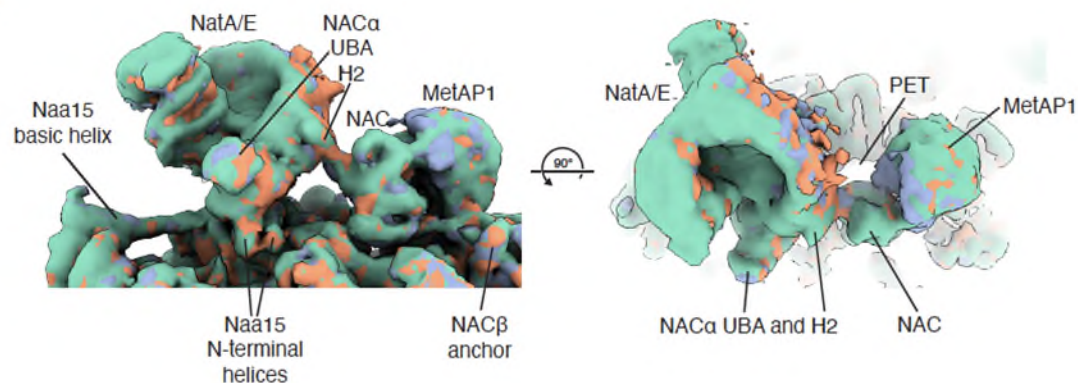
structures shown here support the proposed role of NAC in enzyme recruitment, however, convincing evidence for a sequential hand-over by NAC of the nascent chains from one enzyme to the next, as proposed in the model in Fig. 5, is not provided. Still, this is an exciting study of excellent quality which is of interest for a very broad readership.

#### Major points

1. The multiple interactions of NAC to different enzymes are compatible with the model presented in Fig. 5 according to which NAC serves as part of a dynamic clockwork. However, alternatively, NAC might serve as a passive multi-tool on the exit site that like a Swiss army knife facilitates the processing by different binding partners. In the present study, structural information on the three states of Fig. 5 (pre-3, pre-4 and post-4) are not provided. Since the authors defined the chain lengths that populate these different stages (less than 68, 68-88, and larger than 88), it should be possible to capture the structural arrangement of NAC and its cofactors during these stages. This should allow to clarify whether NAC is rather a passive recruitment factor that uses its spider arms to increase the affinity of enzymes to the ribosome, or whether NAC indeed directs N-terminal protein processing (as the title claims) in a coordinated sequence of events. Either outcome would be interesting but the study was even stronger if this issue could be addressed.

We thank the reviewer for this question. Our model is indeed that NAC positions different enzymes for optimal nascent chain processing. Nevertheless, this positioning is not entirely passive since it will depend, for example, on the sequence of the nascent chain. As was shown in our earlier publication, the signal sequence on an ER-destined protein can destabilize the docking of the globular domain of NAC at the ribosome exit site, which would in turn prevent productive docking of MetAP1 and perhaps NatA (Jomaa et al, Science 2022; Gamedinger et al, Science 2024). Furthermore, as shown here, NAC removes the inhibitory contact of HYPK, enabling activation of NatA on the ribosome. Finally, as shown below, we have performed complementation experiments suggested by the reviewer and found that WT NAC is more efficient than a mixture of NAC variants that only mediate MetAP1 or NatA recruitment, indicating that sequential nascent chain processing is indeed completed within the same pre-assembled multi-enzyme complex organized by NAC.

To test further the role of NAC in this process, we have prepared cryo-EM samples of quaternary complexes for all states shown in figure 5, assembled with RNCs bearing nascent chains of 75 and 125 amino acids and collected datasets on these samples. The two new datasets were processed following the workflow initially presented for the data collected on the quaternary complex with the 53 amino acid nascent chain; the procedure is outlined in the figure below (A). We obtained reconstructions of the translating ribosomes with NatA/E-NAC-MetAP1 bound at the exit tunnel and compared those with the map of the quaternary complex with the 53 amino acid nascent chain we report in the manuscript. The overall configuration of the complexes is the same with NAC globular domain positioning NatA/E and MetAP1 at the opening of the ribosomal tunnel (A). Superposition of the three maps (B) does not reveal notable changes of the conformation of NAC or the enzymes. These results suggest that the multienzyme complex organized around the globular domain of NAC is prepositioned throughout translation of the nascent chain up to 125 amino acids, allowing the nascent chain to access the enzyme active site as they reach the necessary length.

**A****B**

Finally, we clarify that the sequence of events in figure 5 is in large part derived from biochemical data and logic. Removal of the initiator methionine by MetAP1 is required for acetylation by NatA, and our data now show that these sequential reactions also occur in the corresponding order during elongation of the nascent chain (step 3 before step 4). We know that both enzymes are positioned near the ribosome exit site starting at early stages of translation (step 2 before 3) as we described above. Although not structurally shown, it is also reasonable that the long flexible arms of NAC initiate contact with both enzymes before they dock near the exit tunnel (step 1 before 2). This sequence of events, together with cartoon depictions of the structural information for NAC, MetAP1 and NatA, are depicted in Figure 5.

2. Along the same lines: the authors use *C. elegans* as a model to demonstrate convincingly the relevance of NAC *in vivo*. This model should allow to test whether NAC mutants lacking individual binding sites for MetAP1 and NatA (thus the C-terminus of beta and the UBA domain of alpha) can cross-complement each other if they are co-expressed simultaneously. This would again allow to distinguish a passive multi-tool recruiter from a dynamic clockwork device.

We thank the reviewer for the great suggestion. After some contemplation, we decided that these experiments would not be feasible *in vivo* for multiple reasons: (1) expressing these mutants *in vivo* would lead to a mixture of NAC heterodimers with different composition (WT $\alpha$ /WT $\beta$ , WT $\alpha$ /mutant $\beta$ , mutant $\alpha$ /WT $\beta$ ; mutant $\alpha$ /mutant $\beta$ ); (2) *In vivo*, MetAP2, which is NAC independent, provides an alternative pathway for iMet excision; (3) additional ribosome-associated factors, such as EBP1 and SRP, will respond to the NAC mutations and complicate the interpretation of results.

We therefore performed the suggested experiment *in vitro*, in which we can precisely control the composition of mutant NAC heterodimers and measure whether the combination of the two NAC mutants complement one another in sequential nascent chain processing. As shown in the new Figures 3E-3G, the results showed that the combination of NAC mutants deficient in recruiting MetAP1 or NatA failed to complement one another and gave an overall acetylation reaction that is significantly slower than that with wildtype NAC. These data indicate that NAC plays a more active role in organizing the sequential enzymatic reactions than the simplest model in which it only recruits the enzymes for their respective reactions.

3. The data shown suggest that nascent chains can be acetylated by NatA even in the absence of NAC $\alpha$ , particularly if HYPK is absent. This is evident from Fig. 2H or Fig. 4D. Does the acetylation here still occur co-translationally? The assay shown in Fig. 3D should allow to follow the acetylation of chains

of different lengths also under conditions in the absence or presence of the UBA domain of NACalpha and/or of HYPK.

It is unclear whether the small amount of acetylation product in the *in vivo* experiments in the absence of NAC is co- or post-translational, as these are blots for the mature protein and not the tRNA-linked nascent chain. In the reconstituted reaction using stalled RNC substrates (which is by definition cotranslational), NAC is strictly required for reaction of the NatA•HYPK complex (Fig 4B green line, Fig 4C, UBA mutants) but not required when NatA is free (Fig. 4B, blue line). Quantitative proteomics data (Kulak et al, Nature Methods 2014) suggest that in human cells, NatA is in small excess over HYPK (copy numbers 609891 vs, 380554, respectively). Therefore it is plausible that a population of HYPK-free NatA *in vivo* may mediate a low level of post-translational acetylation. This reaction will be much slower than the cotranslational reaction, though, given the low affinity of NatA for peptide substrates ( $K_d$ 's in the mM range) without ribosome contact.

Because the assay in figure 3D uses translationally stalled RNCs, they will not be able to report on post-translational acetylation.

Finally, as describe above in response to comment 1 from this referee, we have obtained and compared cryoEM structures of RNC-NAC-MetAP1-NatA complexes at different nascent chain lengths and have not observed major structural differences. The simplest model is that the nascent chain will sample its conformational space, likely guided by NAC, for the pre-positioned enzymes during translation, and will be processed when it reaches sufficient length required to access the respective enzyme active sites.

4. The UBA domain apparently binds as well to SRP as to NatA. Can both factors bind simultaneously and what is the physiological relevance of the mutual interactions to the same arm of the NAC complex. A comprehensive analysis is certainly beyond the scope of this manuscript, but the authors should shortly discuss this aspect.

Please see our response above to comment 5 by referee #1. We have expanded on this point in the discussion (p10).

**Reviewer Reports on the First Revision:**

Referees' comments:

Referee #1 (Remarks to the Author):

The authors did a nice job addressing my concerns and I feel that the manuscript is now appropriate for publication.

Referee #2 (Remarks to the Author):

The authors have satisfactorily addressed my comments and concerns. I congratulate them on a great piece of work that will be of great interest to the scientific community.

Referee #3 (Remarks to the Author):

The authors adequately addressed all points raised on the initial submission. This is an excellent study of very high general relevance. I fully support its publication in Nature in its present form.