nature portfolio

Peer Review File

Phosphate-dependent nuclear export via a non-classical NES class recognized by exportin Msn5

Corresponding Author: Dr Yuh Min Chook

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)
Summary:
In this beautiful study, the authors determined the structure of the yeast Msn5 karyopherin family member of nuclear exporters in complex with RanGTP and the NES of the Pho4 cargo by employing cryoEM, kinetics, in vitro and in vivo studies and genetics. Fung et al. documented that Msn5 interacts with an NES structure that is completely distinct from the well characterized short, leucine rich motifs which interact with Export1/Crm1 protein nuclear exporters. In contrast, the authors determined that the Pho4 NES is an extended 35 aa IDR sequence that anchors to the concave surface of Msn5 via 2 phosphorylated serines (as well as additional aa interactions) which are phosphorylated and regulated by nuclear Pho80/Pho85. These studies likely define a new paradigm as other know Msn5 cargo possess aa stretches with rather similar characteristics. The authors further demonstrate that free Msn5 is autoinhibited.

Critique:
The discoveries and conclusions are exceedingly well documented and well-presented both in the text and in the figures. The results are highly significant as they provide a paradigm shift for understanding how cells regulate protein nuclear export. However, a few improvements are recommended.

1. Mig1 referencing. Previously, employing genetics and imaging, the Johnston lab (De Vit and Johnston, 1999) determined that Msn5 cargo, Mig1, possesses a large aa region, distinct from the leucine-rich NES domain for Crm1, that is regulated by Snf1 kinase-dependent phosphorylation. This work should be cited in the main text rather than the supplementary materials.
2. In vivo findings and Fig. 4. The authors elected to employ RNA sequencing of “downstream genes” to document the in vivo significance of Msn5 sites identified to be important for transcription factor Pho4 and Mig1 binding. The resulting data uncover “trends” expected for defective nuclear export of these transcription factors, but the data are not completely convincing. Perhaps, imaging of tagged Mig1 in wild-type and mutant Msn5 cells under glucose vs. glycerol carbon sources (via De Vit and Johnson, 1999) would provide a direct in vivo assessment of the Msn5 NES binding sites.
Minor:
1. IDR is not defined.
2. Pg. 16-17 indicated that there is a “lack of knowledge” of kinases that phosphorylate Msn5 cargo is not entirely correct as Mig1 is phosphorylated by Snf1.
3. Pg. 23, line 383: change leading sentence of “conclusions” as Pho4 is not the first identified protein nuclear export cargo that differs from the XPO1-recognized NESs

Reviewer #2

(Remarks to the Author)

Reviewers comments: Manuscript “Phosphate-dependent nuclear export via a novel NES class recognized by exportin Msn5. Ho Yee Joyce Fung1,2, Sanraj R. Mittal3, Ashley B Niesman1,2, Jenny Jiou1,4, Binita Shakya1,5, Takuya Yoshizawa1,6, Ahmet E Cansizoglu1,7, Michael P. Rout3, Yuh Min Chook1”

Msn5/Kap142 is an unusual exportin with the ability to both import and export cargo (Yoshida and Blobel, 2001) that can be

either RNA or protein. Prior work in the field has delineated substantial structural information on how the Msn5 exportin homolog Exportin-5 interacts with tRNA cargo, however, little information exists on how this family of exportins interacts with protein cargo although it required for export of a number of cargo such as phosphorylated Pho4 (Kaffman et al, 1998 based on poor interaction with the Pho4SA mutant that has 5 alanines at serine sites found to be phosphorylated by Pho80-Pho85. Serines 100, 114, 128, 152, 223 consensus for four sites is Ser-Pro-X-Ile/Leu), Ste5 (Mahanty et al 1999; Huh et al, 2016), Far1 and Far1/Cdc24 complex (Blondel et al, 1999; Shimada et al, 2000), Crz1 (Boustany and Cyert, 2002), RPA (Yoshida and Blobel, 2006) and HO endonuclease (Bakhrat et al 2008), to name a few. In this study, the authors have done cryoEM studies on the interaction of the Pho4 transcription factor with Msn5. The O'Shea lab previously identified Pho4 as being exported by Msn5 after it was phosphorylated. In the Chook lab study the authors find that the traditional hydrophobic nuclear export signal NES used by the Exportin-1/XPO1 system is not used by Msn5. Rather a novel phosphorylated 35-residue NES tinteracts with the concave surface of Msn5 through two Pho4 phosphoserines and do so through two basic patches in Msn5. The authors conclude that they have identified a novel previously unknown mechanism of phosphate-specific recognition. They discover that unliganded Msn5 is autoinhibited and find that this provide a model for the life cycle of Msn5 unliganded and liganded to Pho4/Ran-binding and from this information propose a mechanism for Pho4 release in the cytoplasm. Overall this is a readable manuscript that clearly presents findings. The list of findings suggest significant advancement in our knowledge of how exportins in the Msn5 class recognize protein cargo. This is a carefully documented study that will be important to the karyopherin field and deserves to be accepted after the following issues are addressed.

1). Lines 55-56 This sentence needs to include what the specific phosphorylation sites are that are needed for binding to Msn5. They Kaffman O'Shea paper cites Pho4SA mutant that harbors 5 sites and reference their prior paper that showed these 5 sites are phosphorylated by Pho80-Pho85. They did not, in either paper, delineate which of these 5 sites (listed in the prior paper in the reference notes) are actually required for binding to Pho4. This discussion topic needs more information with accurate wording. This is crucial to avoid confusion and emphasize that you find only two of the phosphorylated sites are required for binding.

2). Line 50-51 "which transports various transcription factors… ref4. This statement is innaccurate should include other proteins

3). Line 68-69 Sentence is unclear. Where is sentence explaining that exportins generally bind IDRs? It is needed before sentence commenting on attributes of Msn5.

4). Line 51 The authors Fung and Chook cite Ref4 LINE 657 as review for the karyopherin field: Wing, C.E., Fung, H.Y.J., and Chook, Y.M. (2022). Karyopherin-mediated nucleocytoplasmic transport. Nat Rev Mol Cell Biol 23, 307-328. 10.1038/s41580-021-00446-7.
THE REFERENCE IS INCORRECT ACCORDING TO PUBMED WHICH ONLY LISTS FUNG AND CHOOK AS AUTHORS. This needs to be corrected and a review that does not include the authors of this manuscript needs to also be included. I suggest the authors also correct their review.

Their information on Msn5 from the Fung and Chook review of 2022 lacks references and is insufficiently detailed to be a good reference for Msn5/Exportin-5.
"This is true for other exportins and biportins except for the yeast exportin MSN5, which exports many phosphorylated proteins by binding to a still undefined phospho-
NES that is located in the IDRs of its cargoes. For example, transcription factor PHO4 must be phosphorylated at two specific sites within a 150-residue-long IDR inter-domain linker53. Sixteen other MSN5 cargoes, all involved in either environmental response or cell cycle regulation, contain IDR segments that drive export only when phosphorylated. No common sequence/structural features have been identified for this potential phospho- NES (Supplementary Table 2). Although the human homologue of MSN5 (XPO5) is known to export RNAs, its one putative protein cargo is the phosphorylated RNA editing enzyme ADAR1, suggesting that the recognition of a phospho-NES may be evolutionarily conserved54,55 "

5). 54. Fritz J et al. RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. Mol. Cell Biol 29, 1487– 1497 (2009). [PubMed: 19124606]
55. Sakurai M et al. ADAR1 controls apoptosis of stressed cells by inhibiting Staufen1-mediated mRNA decay. Nat. Struct. Mol. Biol 24, 534–543 (2017). [PubMed: 28436945] These refs 54 and 55 are Incorrect references for the paragraph on Msn5 in the Fung and Chook 2022 review down loaded through Pubmed This is invalid. Correct references are required.

6). Lines 85-85 The authors describe PHO4 in terms of an intrinsically disordered domain and reference 24,25

Ref24 Ogawa, N., and Oshima, Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in Saccharomyces cerevisiae. Mol Cell Biol 10, 2224-2236. 10.1128/mcb.10.5.2224-2236.1990.
Ref25 Shimizu, T., Toumoto, A., Ihara, K., Shimizu, M., Kyogoku, Y., Ogawa, N., Oshima, Y., and Hakoshima, T. (1997). Crystal structure of PHO4 bHLH domain- DNA complex: flanking base recognition. EMBO J 16, 4689-4697. 10.1093/emboj/16.15.4689. Although the literature is full of recent work on intrinsic disorder regions, very popular right now, there is no mention of IDR intrinsic disorder region and its relation to function of Pho4 in Ref 24 or Ref25 based on perusal of the articles and computer searching for disorder, IDR, intrinsic. This is another invalid sentence. The authors would need to provide evidence of the link between an IDR and PHO4 function they are referring through either through correct referencing or their own analysis that would be included in this manuscript.

7). This reviewer is raising concern based on inaccurate referencing and incorrect statements including in their own review in Nature Reviews. To be considered further, as this reviewer does not have so much time, the journal Nature Communications needs to have staff check each sentence and reference cited by Fung and Chook in this manuscript and verify that the sentence is a correct statement that matches what is in each reference they cite. These inaccuracies warrant correction.

8). Materials and Methods: Although some parts of materials and methods are sufficiently documented, other parts of Materials and Methods are incomplete. The NIH requires that protocols be written with sufficient detail that another person would be able to replicate what is described. Currently the methods are not quite there yet.Complete methods are required. examples lines 431-432: information missing on PCR and blunt end ligation. lines 490-491: No method included for site-directed mutagenesis and how they confirmed the identity of the mutations after the manipulation. Lines 439-440: length of time with EmulsiFlex-C5 and temperature for lysis is missing. Temp for column chromatography and length of time is missing. Number of cells used for preps and volumes also missing. Lines 488-489: Information on the cDNA library used to clone PHO4 and how the cDNAs were isolated is missing. Oligonucleotides used for PCR and sequencing and cloning need to be listed as well as evidence of what clones were sequenced to confirm they are correct and if only checked by restriction sites then that should be listed.

9). Supplemental materials and methods on Analysis of Pho4 density/map features in the additional cryo-EM maps of the Msn5-RanGTP-pPho41-200 complex
discusses assignment of images to state1, state2, state3. The method used to do this assignment needs to be included along with software . They should also mention whether there are other shapes not described by states 1-2, 2-1, 2-2, 3-1, 3-2- in Extended Table 1.

10). Figure 1 and Figure 2 would be important additions to the field. However the color scheme makes it hard to figure out what is what. Perhaps the grey Msn5 could be lightened and the yellow for Pho4 and green/blue cyan for Ran could be brightened. If possible additional views with rotation could be added perhaps in supplemental with more discernment of the concave binding pocket in Msn5 that is bound by Pho4. The third structure with the numbered heat domains is confusing because in addition to yellow and cyan there is also navy blue and an orange red and not clear what that signifies. A KEY listing the numbers, with color and what each group of numbers signifies would help.

11). The discussion of the cryo-EM in the text mentions a 3.0 angstrom cryo-EM structure for Msn5-RanGTP-Pho4(1-200) and a 4.9 angstrom map of Msn5-RanGTP-Pho4(full length) trimeric complexes. This information should be in the abstract along with mention that the two key Pho4 phosphorylation sites for binding and the two sites that contribute minimally. I suggest that the authors include a reference to: which explains that 3.5 angstrom is considered high resolution for cryo-EM and that 4+ angstrom can be used to get meaningful information to make it clear to people who are better versed in classic crystallography.One example from 2011 is Adv Protein Chem Structural Biol manuscript; available in PMC 2013 Jul 2.
Published in final edited form as: 2011; 82: 1-35.
PMCID: PMC3698602 NIHMSID: NIHMS480946
PMID: 21501817 ATOMIC RESOLUTION CRYO ELECTRON MICROSCOPY OF MACROMOLECULAR COMPLEXES Z. Hong Zhou

12). Figure 5 A structure comparison with Exportin-5 is beneficial, pointing out approximate areas that might be involved in protein cargo contact together with the known areas involved in RNA contact. The dark orange vs light orange is hard to distinguish. The exportin-5::premiRNA structure is much more compact than the Msn5::Pho4 structure where pho4 binds horizontally as well as vertically. Is this because of more extensive vertical contacts with the RNA and more extensive horizontal contacts with pho4? would there be more extensive vertical contacts with full length Pho4?

13). Repeat point for Figure 2. This author is very enthusiastic about Figure 2 However the colors are not bright enough to see readily and could be improved. Also it is hard to visualize the Msn5 NES binding to the central concave portion of Msn5 in Figure 1, it could be improved to stand out more and also have another view

14). Lines 272-280. This reviewer is enthusiastic about this part of the manuscript and extended table 2. However, the authors ought to include Pho4 in the table referencing themselves as “this study”. In addition, the authors only show the proteins that conform to their proposed consensus. I think they need to at a minimum list the remainder of the 16 they examined in the table legend with the references to those papers and let us know whether those papers had done a detailed enough analysis to narrow down potential interaction site.

15). Lines 281-289: It would be clearer if the authors stated the stretch of Pho4 that is binding to the basic patches and also explain whether different cargoes would be predicted to bind to the same basic patches or to different ones on Msn5. It seems either possibility is feasible.

16). Lines 162-175 would benefit from having a table that has the various affinities of the Pho4 and Msn5 mutants and is combined with the discussion in Extended results “Comparison of pPho4 S114A and S128A mutants and the Msn5 mutants at their respective binding sites “As described in the main text, we mutated the Pho4 serines that are phosphorylated (Pho4 S114A, Pho41-200 S128A and Pho41-200 S114/128AA) and the phosphate binding sites on Msn5 (Msn5 HRYAAA and Msn5 RRAA). The 5-fold affinity decrease of the Msn5 HRYAAA mutant (KD 230[160,350 nM) compared to Msn5 WT matches the 6-fold decrease seen for pPho4 S114A vs. pPho4 WT. However, the 50-fold affinity decrease of Msn5 RRAA

(KD 2.2[1.0,6.1] µM) vs the 3-fold decrease of pPho4 S128A is surprising as Msn5 R393 and R458 contact only the phosphate group of pS128 in the structure (Fig. 2c, left panel). It is possible that the loss of the phosphate group in pPho4 S128A is less detrimental because Msn5 R393 and R458 may still interact with the alanine at position 128 or its neighboring residues. Alternatively, in the absence of pS128, pS114 may bind at the Msn5 RR site (see above). " are important and a shortened version ought to be in the main text with a table of affinities".

17). Line 144: the sentence …three 90° turns of the zig-zagging chain (Fig. 2a and b)… is an apt description that helps one visualize the Msn5-Pho4 interaction and might be repeated earlier in the text when the structure is first described.

18). The authors discuss the different conformers (states) of the Msn5/Pho4/Ran complexes. Can they speculate on the relationship of those conformers to the life-cycle of the complex viz association in cytosol and nucleus and release after export. Do they have any favored models?

139. 19). The authors cite as " interesting" that : "Interestingly, phosphomimic mutations do not mimic Pho4 phosphorylation: Pho41-200
140. 158 S114/128DD and Pho41-200 S114/128EE bind Msn5 weakly, like unphosphorylated Pho41-
141. 159 200, with KDs ~2-3 µM (Extended Data Fig. 6a)."

This reviewer thinks the word "Surprisingly" rather than Interestingly is better suited for the sentence because it is not expected at all that the phosphomimetic mutations would reduce binding, the expectation is that they should mimic phosphorylation. What is the effect of these amino acid substitutions on the structure of Pho4 and does this differ from the phosphorylated Pho4 structure and explain the reduced binding? Extended data figure 6 supports what has been written but some summary statement about why is important to the results section description. one might consider the impact of the phosphomimetic EE mutations on this structure described by the14 non-phospho mutations later in the results section and how this region would be impacted by the EE mutations.

19). The sentence
Lines 161-162 Phospho-serines pS114 and pS128 were previously reported to be critical for
Msn5-mediated nuclear export Ref5. is inaccurate, because reference 5, Komeili, A., and O'Shea, E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science 284, 977-980. 10.1126/science.284.5416.977 IS THE WRONG REFERENCE FOR THEIR STATEMENT AND THEIR STATEMENT IS INCORRECT. The correct reference is REF7 Kaffman, A., Rank, N.M., O'Neill, E.M., Huang, L.S., and O'Shea, E.K. (1998). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature 396, 482-486. 10.1038/24898 . However, in Ref 7, the Kaffman and O'Shea paper did not delineate which of 5 phosphorylated sites were critical for binding to Msn5. I suggest the authors revisit the excellent Kaffman and O'Shea paper and properly describe what they showed. In my opinion it is the work in this Fung et al manuscript that is the first delineation of the individual phosphorylated residues required for binding to Msn5 and a strong description should be made.

20). Figure 4 RNA-seq analysis is an excellent addition to support their findings. However, it is standard to include tables of the raw data with full lists of genes and fold effects in supplementhis information should be added to the manuscript.

21). Extended Figure 7 growth data. It is quite amazing that the growth curves of overexpressed WT and HRY RR AAA AA point mutant Msn5 are nearly superimposable. How can this be if the HRYRR AAAAA point mutant is not binding cargo as efficiently? Does this mean that the growth impairment is independent of binding to cargo? This is a confusing finding. Is the mutant a dominant negative? Was it tested in a wild type background?

22). The authors analyze 14 other non-phospho-serine residues within the Pho4 core residues for their role in binding to Msn5. Lines 233-234 "Mutating all the non-phospho-serine Msn5-interacting residues in region 2 (113YpSPLIHT119 to GpSPGGSG; R2mut) decreased Msn5-binding by 40-fold…" AND the other mutants discussed therein. It is not clear how these mutations affect the overall structure and stability of Pho4 and whether this might impact binding to Msn5. A discussion of this caveat is needed in addition to their conclusion sentence "In summary, many hydrophobic and small polar side chains in each of Pho4 NES regions 2, 3 and 4 contribute substantially to total binding energy. They are as important as phosphoserines pS114 and pS128 for high-affinity Msn5-binding. Importantly, though, many Pho4-Msn5 contacts also involve the Pho4 main chain. "

23.) Fig6 and Extended Fig10 of structure of unliganded Msn5 provides additional important information. In Fig6 it would be better to have the pink Msn5 also be pink with Pho4 and RanGTP in the trimeric complex so one can more easily pick it out and compare. A superimposed picture of unliganded and liganded Msn5 (minus Ran and pho4) would be very helpful even if certain parts of liganded Msn5 may not be so well defined because of occlusion by binding partners.

24). The final data in Fig 6E regarding the h17loop and RanBP and allosteric switch is interesting. Figure 6D legend states: "CRM1 and pPho4 are not shown and the docked Ran-RanBP1 is aquamarine and purple, respectively." Whereas the results text states lines 367-371 Alignment of RanGTP- RanBP1 from the XPO1-bound structure (3M1I51) with the RanGTP of Msn5-RanGTP- pPho41-200 reveals a steric clash between the Msn5 h17loop-extended h17B helix with the superimposed Ran tail and RanBP1 (Fig. 6d)." This reviewer was unable to visualize the steric clash being described and suggest improvements in the figure.

25). LINES 377-381 on a model for Msn5 function are important and would benefit from a cartoon model summary at the end of the figures that describes the overall model described in these lines and also points out the Pho4 binding pocket and

pho4 binding site with key residues cartooned in.

26). The figure legends are overall quite explanatory but what is needed is knowledge of the concentrations of proteins used in all of the experiments. This information needs to b added either in legends or some kind of paragraph describing each figure in extended methods.

Reviewer #3

(Remarks to the Author)
The manuscript studied the phosphate-dependent nuclear export mechanism of the transcription factor Pho4, facilitated by the yeast exportin Msn5. The authors resolved multiple Msn5 structures, including the Msn5 apo state and Msn5-Ran-Pho4 complex states, revealing a novel 35-residue NES motif that differs from the canonical NES motif recognized by XPO1. The study highlighted the critical role of phosphorylation at S114 and S128 in regulating the interaction between Pho4 and Msn5, enhancing our understanding of the mechanisms governing nuclear transport and cellular signaling pathways. These structural findings were also corroborated by biochemical and cellular experiments on mutants. Overall, I believe the innovation of the paper is strong, and the new scientific discovery serves as a valuable addition to the existing nuclear localization mechanism. It can be considered for publication with several modifications.

Comments

1. Since I cannot find Extended Data Movie 1, the structural variability details of Msn5 remain unclear to me. I find this to be an intriguing point. Given that the structural resolution of Msn5 proteins in most structural states is high enough for modeling, it is recommended to construct their atomic structure models and subsequently compare the different structural states based on these models. This would aid in understanding the complete molecular mechanism of Msn5.

2. I did not find the data processing flowchart, which is essential. This figure should encompass all classifications for the various structural states.

3. The model-map matching qualities should be provided. In addition to Fig. 2c, for the significant domains or motifs in different structures, particularly in the local regions where side chain conformation or residue distances are discussed, it would be beneficial to display the fitting qualities of the structural model to the density map.

4.I believe many readers will be curious about how similar the structures of the Msn5 monomer and the Msn5-Ran-Pho4 complex are compared to the corresponding structures predicted by AlphaFold2 or AlphaFold3.

5.The authors did not mention the structure of the Msn5-Ran complex. Has this been included in the Msn5-Ran-Pho4 dataset? Has there been any effort to resolve this structure? By comparing it to the Msn5 monomer and the Msn5-Ran-Pho4 ternary complex structure, it seems that a more complete understanding of the Msn5-Pho4 interaction process could be elucidated.

6.The authors note that the Msn5 protein itself can form an autoinhibited state and cannot bind to Pho4. It is only upon binding to Ran that the Msn5 protein can interact with Pho4. I think the structural basis for this process is crucial. However, the authors primarily focus on analyzing the Ran binding site, rather than the Pho4 binding sites. I believe that a more in-depth investigation of the differences at the Pho4 binding site in these structures is required to clarify the regulatory mechanism by which Msn5 interacts with Pho4.

7.The authors mention that Msn5 recognizes a specific NES motif on the Pho4 protein. This unique NES sequence and recognition mechanism significantly differ from those of common NESs. This is very interesting; however, there is a lack of in-depth discussion. What important biological implications might arise from this unique NES sequence and recognition mechanism during the evolutionary process? A thorough discussion on this would enhance the depth of the research presented in this study.

8.This study presents a substantial amount of experimental results but lacks a systematic summary. After a more comprehensive and in-depth analysis of the various structural models in this work, it is recommended that the author include a flowchart illustrating all the structural changes involved in Msn5's recognition of the Pho4 protein. This should clearly convey information in a well-organized and logical manner, encompassing self-inhibition, phosphorylation, conformational changes, Ran regulation, and more.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have appropriately addressed all my previous criticisms/comments. I also note that authors also did a commendable and very thorough job addressing the comments of the other two reviewers. I look forward to the publication of this important research in Nature Communications.

Reviewer #2

(Remarks to the Author)
Reviewer 2 is happy with the authors response to the review. They have improved the paper which is a significant advance to the field and also carefully documented. The paper should be accepted for publication.

Two minor points:

Comment on: [[We have revised the paragraph to make it clear that NESs refer to linear sequence elements that are most often found in IDRs. The new lines 63-65 now reads:
“Exportins are thought to recognize their protein cargoes by binding to linear sequence elements known as nuclear export signals (NESs) that occur within intrinsically disordered regions (IDRs) of cargoes or to the folded domains of cargoes4,28,29.”]].

I believe it is more prudent to write that NESs [“often”- single word addition to sentence] occur within IDRs... The authors discuss that there are also folded NESs [“or to the folded domains of cargoes “] which presumably are unlikely to be within IDRs.

In supplemental the Sup Table 2 goes to Sup Table 4 and skips Sup Table 3. Confusion here. Sup Table 3 is on a separate file. Perhaps authors should stick in Sup Table 3 here in addition to having separate file or insert a sentence saying Sup Table 3 is a separate file.

Supplementary Table 4 has typos. In the third column next to Ste5 the sequence of ref 9 appears to be jumbled up with incorrect numbering. Mig1 which appears alphabetically later in the table has ref 5 which suggests the reference order has to be corrected.

Reviewer #3

(Remarks to the Author)
The author has added a substantial amount of analysis and comments in the revised manuscript, significantly enhancing the rigor, scientific significance, and quality of the article. I believe the current version is ready for publication.

Reviewer #1 (Remarks to the Author):

REVIEWER #1's COMMENTS

Summary:
In this beautiful study, the authors determined the structure of the yeast Msn5 karyopherin family member of nuclear exporters in complex with RanGTP and the NES of the Pho4 cargo by employing cryoEM, kinetics, in vitro and in vivo studies and genetics. Fung et al. documented that Msn5 interacts with an NES structure that is completely distinct from the well characterized short, leucine rich motifs which interact with Export1/Crm1 protein nuclear exporters. In contrast, the authors determined that the Pho4 NES is an extended 35 aa IDR sequence that anchors to the concave surface of Msn5 via 2 phosphorylated serines (as well as additional aa interactions) which are phosphorylated and regulated by nuclear Pho80/Pho85. These studies likely define a new paradigm as other know Msn5 cargo possess aa stretches with rather similar characteristics. The authors further demonstrate that free Msn5 is autoinhibited.

Critique:
The discoveries and conclusions are exceedingly well documented and well-presented both in the text and in the figures. The results are highly significant as they provide a paradigm shift for understanding how cells regulate protein nuclear export. However, a few improvements are recommended.

AUTHORS' RESPONSE

We thank the reviewer for these encouraging comments and for suggestions to improve our work.

REVIEWER #1's COMMENT

1. Mig1 referencing. Previously, employing genetics and imaging, the Johnston lab (De Vit and Johnston, 1999) determined that Msn5 cargo, Mig1, possesses a large aa region, distinct from the leucine-rich NES domain for Crm1, that is regulated by Snf1 kinase-dependent phosphorylation. This work should be cited in the main text rather than the supplementary materials.

AUTHORS' RESPONSE

We apologize for missing the citation for Mig1 in the main text.

The De Vit and Johnston, 1999 reference is now added to the sentence on lines 236-239:
"When this same analysis is applied to another cargo of Msn5, the Mig1 transcription factor[9], we see the same general trend towards overexpression in both the empty vector and mutant strains (Fig. 4b and e)."

The same paper is also cited in the Introduction when all published Msn5 cargoes were mentioned (lines 50-54):
"At least 18 different proteins have been reported to be exported by Msn5, including transcription factors and other proteins involved in response to nutrients and stress, and proteins involved in cell cycle maintenance[7-24]."

REVIEWER #1's COMMENT

2. In vivo findings and Fig. 4. The authors elected to employ RNA sequencing of "downstream genes" to document the in vivo significance of Msn5 sites identified to be important for transcription factor Pho4 and Mig1 binding. The resulting data uncover "trends" expected for defective nuclear export of these transcription factors, but the data are not completely convincing. Perhaps, imaging of tagged Mig1 in wild-type and mutant Msn5 cells under glucose vs. glycerol carbon sources (via De Vit and Johnson, 1999) would provide a direct in vivo assessment of the Msn5 NES binding sites.

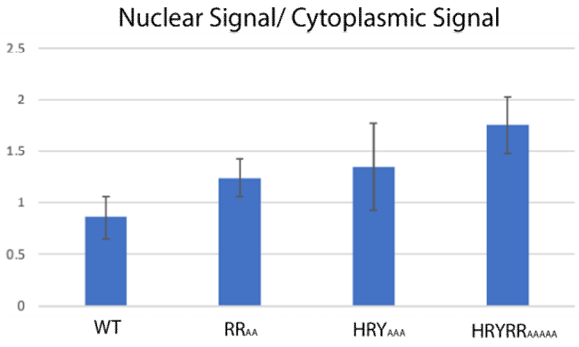
AUTHORS' RESPONSE

We agree that these are the obvious experiments and indeed we have attempted this and several variations on this experiment.

In particular, we attempted to use Pho4 with a C-terminal GFP tag, overexpressed on a plasmid, as a reporter, and then a second round of plasmid-based overexpression carrying 1) the WT Msn5, 2) the single phosphoserine binding pocket mutant Msn5 RR_{AA}, 3) the single phosphoserine binding pocket mutant Msn5 HRY_{AA}, or 4) the double phosphoserine binding pocket mutant Msn5 $HRYRR_{AAAAA.}$ Additionally, we tried endogenously tagging Pho4 to use as a reporter with only the Msn5 constructs being overexpressed on a plasmid.

We certainly saw results that were overall entirely consistent with the RNAseq and other data presented in this paper, but because of the low and variable levels of expression of both Msn5 and of the tagged cargo, something to which this system strangely among all transport factors is particularly sensitive, we were not able to get statistically reproducible results that we felt would be acceptable for this publication.

Nevertheless, we show in this reviewer-only graph, that in a double blinded experiment where images for strains carrying the Pho4-GFP reporter and one of each of the four different Msn5 constructs were collected by one researcher, and then the percentage of cells with nuclear GFP signal (indicating impaired cargo-transporter binding) was determined by another researcher, the percentage of nuclear signal is highest among double binding pocket Msn5 mutants, followed by the single binding pocket mutants, and finally by the WT Msn5.



Instead, we will included a sentence in the main text briefly describing our efforts and why we ultimately chose not to include said data in this report (lines 214-224):
"To examine the functional relevance of these *in vitro* findings *in vivo*, we first attempted plasmid overexpression of the Pho4-GFP fluorescent nuclear export reporter while also expressing either WT Msn5 or Msn5 $HRYRR_{AAAAA}$, a Msn5 construct with both phosphate-binding HRY and RR

hotspots mutated. Unfortunately, dramatic variability in expression levels of both the reporter cargo and the exportin precluded reliable quantification of the nuclear-cytoplasmic localization results. Consistent with previous reports that Msn5 overexpression results in a slower growth phenotype[50-52], we found that overexpression of both WT and HRYRR$_{AAAAA}$ Msn5 proteins were equally deleterious to cell growth (Supplementary Fig. 10). We therefore took the alternative approach to grow the two Msn5 strains to mid-log growth phase and extract their total RNA for mRNA sequencing (Fig. 4, Supplementary Table 3)."

REVIEWER #1's COMMENT

Minor:
1. IDR is not defined.

AUTHORS' RESPONSE

Thank you for catching this mistake. We have defined IDR at its first mention in the Introduction section (lines 63-65):
"Exportins are thought to recognize their protein cargoes by binding to linear sequence elements known as nuclear export signals (NESs) that occur within intrinsically disordered regions (IDRs) of cargoes or to the folded domains of cargoes[4,28,29]."

REVIEWER #1's COMMENT

Minor:
2. Pg. 16-17 indicated that there is a "lack of knowledge" of kinases that phosphorylate Msn5 cargo is not entirely correct as Mig1 is phosphorylated by Snf1.

AUTHORS' RESPONSE

We have removed this statement and revised the paragraph (lines 307-312) to:
"We examined sequences within the regions of the other Msn5 cargoes that had been reported to be important for nuclear export[7-24]. Nine of the 17 Msn5 cargoes contain sequences that seem to fit the criteria noted above (Supplementary Table 4). Future studies using appropriate kinases and various mapped cargo fragments will be needed to test these putative NESs for Msn5-binding and nuclear export."

REVIEWER #1's COMMENT

Minor:

3. Pg. 23, line 383: change leading sentence of "conclusions" as Pho4 is not the first identified protein nuclear export cargo that differs from the XPO1-recognized NESs

AUTHORS' RESPONSE

We revised the 1st sentence of Conclusion in lines 458-460 to:
"We have demonstrated that the 35-residue phosphorylated NES of Pho4 represents a novel class of linear NESs, distinct from the previously characterized XPO1-recognized NESs, and elucidated the mechanism by which it is recognized by Msn5."

Reviewer #2 (Remarks to the Author):

REVIEWER #2's COMMENTS

Manuscript "Phosphate-dependent nuclear export via a novel NES class recognized by exportin Msn5. Ho Yee Joyce Fung1,2, Sanraj R. Mittal3, Ashley B Niesman1,2, Jenny Jiou1,4, Binita Shakya1,5, Takuya Yoshizawa1,6, Ahmet E Cansizoglu1,7, Michael P. Rout3, Yuh Min Chook1"

Msn5/Kap142 is an unusual exportin with the ability to both import and export cargo (Yoshida and Blobel, 2001) that can be either RNA or protein. Prior work in the field has delineated substantial structural information on how the Msn5 exportin homolog Exportin-5 interacts with tRNA cargo, however, little information exists on how this family of exportins interacts with protein cargo although it required for export of a number of cargo such as phosphorylated Pho4 (Kaffman et al, 1998 based on poor interaction with the Pho4SA mutant that has 5 alanines at serine sites found to be phosphorylated by Pho80-Pho85. Serines 100, 114, 128, 152, 223 consensus for four sites is Ser-Pro-X-Ile/Leu), Ste5 (Mahanty et al 1999; Huh et al, 2016), Far1 and Far1/Cdc24 complex (Blondel et al, 1999; Shimada et al, 2000), Crz1 (Boustany and Cyert, 2002), RPA (Yoshida and Blobel, 2006) and HO endonuclease (Bakhrat et al 2008), to name a few.

In this study, the authors have done cryoEM studies on the interaction of the Pho4 transcription factor with Msn5. The O'Shea lab previously identified Pho4 as being exported by Msn5 after it was phosphorylated. In the Chook lab study the authors find that the traditional hydrophobic nuclear export signal NES used by the Exportin-1/XPO1 system is not used by Msn5. Rather a novel phosphorylated 35-residue NES tinteracts with the concave surface of Msn5 through two Pho4 phosphoserines and do so through two basic patches in Msn5. The authors conclude that they have identified a novel previously unknown mechanism of phosphate-specific recognition. They discover that unliganded Msn5 is autoinhibited and find that this provide a model for the life cycle of Msn5 unliganded and liganded to Pho4/Ran-binding and from this information propose a mechanism for Pho4 release in the cytoplasm. Overall this is a readable manuscript that clearly presents findings. The list of findings suggest significant advancement in our knowledge of how exportins in the Msn5 class recognize protein cargo. This is a carefully documented study that will be important to the karyopherin field and deserves to be accepted after the following issues are addressed.

AUTHORS' RESPONSE

We thank the reviewer for pointing out the potential "significant advancement" that our work could provide. We also thank the reviewer for raising issues that when addressed would improve our manuscript. However, we would like to point out two corrections to the reviewer's comments above: 1) The role of Msn5 as an importin is uncertain even though an early study by Yoshida and Blobel reported that Msn5 imports the replication factor RPA. Subsequent studies have reported that RPA is imported by Kap95 and not Msn5 (Belanger et al, 2011, DNA Cell Biol). 2) The Msn5 exportin homolog Exportin-5 or XPO5 was structurally characterized, both in its unliganded state and bound to pre-miRNA, but not to tRNA.

REVIEWER #2's COMMENT

1). Lines 55-56 This sentence needs to include what the specific phosphorylation sites are that are needed for binding to Msn5. They Kaffman O'Shea paper cites Pho4SA mutant that harbors 5 sites and reference their prior paper that showed these 5 sites are phosphorylated by Pho80-Pho85. They did not, in either paper, delineate which of these 5 sites (listed in the prior paper in

the reference notes) are actually required for binding to Pho4. This discussion topic needs more information with accurate wording. This is crucial to avoid confusion and emphasize that you find only two of the phosphorylated sites are required for binding.

AUTHORS' RESPONSE

We think the sentences that caused concern spanned lines 53-56 in the original manuscript was "Msn5 recognizes phosphorylated cargoes for nuclear export, such as the phosphate-sensing transcription factor *Sc* Pho4[5]. Pho4, regulated by phosphorylation from the Cyclin-CDK Pho80-Pho85 kinase complex, requires phosphorylation at specific sites for binding Msn5 and subsequent nuclear export[5-9]."

The reviewer suggested that we state in the Introduction the specific Pho4 phosphorylation sites that are needed for Msn5-binding. However, we prefer to keep this 2nd paragraph of our Introduction section more general, with fewer details, for the general readership of Nature Communication. Therefore, we kept the two sentences as is. We include information on specific Pho4 phosphorylation sites later, in the Results section.

We thank the reviewer for examining/questioning our referencing. Upon close examination, we think the Kaffman paper (1998, Nature, old REF 7) rather than the Komeili (1999, Science old REF 5) paper is more suitable citation for "Msn5 recognizes phosphorylated cargoes for nuclear export, such as the phosphate-sensing transcription factor *Sc* Pho4". We now cite the 1998 Kaffman paper (new REF 7).

The next sentence "Pho4, regulated by phosphorylation from the Cyclin-CDK Pho80-Pho85 kinase complex, requires phosphorylation at specific sites for binding Msn5 and subsequent nuclear export" is about Pho4 being phosphorylated by Pho80-Pho85 for Msn5-mediated nuclear export. Because the sentence covers multiple points, from the Pho80-Pho85 kinase, to Pho4 phosphorylation and Msn5 exporting phosphorylated Pho4, it is best referenced by citing multiple papers – starting with the discovery that Pho80-Pho85 phosphorylates Pho4 (Kaffman, 1994 old REF 6/new REF 25), and the discovery that the phosphorylation controls Pho4 localization (O'Neill, 1996 old REF 8/ new REF 27), identification of Msn5 as the exporter of phosphorylated Pho4 (Kaffman, 1998 old REF 7/new REF 7), and lastly, dissection of the five Pho4 phosphoserines to determine which ones direct export, import and transcription (Komeili, 1999 old REF 5/ new REF 26).

The Komeili 1999 paper (new REF 26) definitively showed that S114 and S128 must be phosphorylated for nuclear export and Msn5 interaction (Fig 1A and B, respectively; more in response to point 19). Therefore, we believe that our wording and the new citations are indeed accurate. We removed the old REF 9 (Springer 2003) because it connected differential phosphorylation to downstream gene expression, information which is not directly related to Msn5 export and binding.

We thank the reviewer for pushing us to cite more accurately. We also thank the reviewer for encouraging us to emphasize that we had found two of the five Pho4 phosphorylation sites drive Msn5-binding. However, we cannot claim this discovery because Erin O'Shea's group had already made that discovery in 1999.

REVIEWER #2's COMMENT

2). Line 50-51 "which transports various transcription factors… ref4. This statement is innaccurate should include other proteins

AUTHORS' RESPONSE

We have revised that sentence to the following new sentences and included 18 citations for original discovery of 18 different Msn5 cargoes (lines 50-54):
"At least 18 different proteins have been reported to be exported by Msn5, including transcription factors and other proteins involved in response to nutrients and stress, and proteins involved in cell cycle maintenance[7-24]."

REVIEWER #2's COMMENT

3). Line 68-69 Sentence is unclear. Where is sentence explaining that exportins generally bind IDRs? It is needed before sentence commenting on attributes of Msn5.

AUTHORS' RESPONSE

We have revised the paragraph to make it clear that NESs refer to linear sequence elements that are most often found in IDRs. The new lines 63-65 now reads:
"Exportins are thought to recognize their protein cargoes by binding to linear sequence elements known as nuclear export signals (NESs) that occur within intrinsically disordered regions (IDRs) of cargoes or to the folded domains of cargoes[4,28,29]."

REVIEWER #2's COMMENT

4). Line 51 The authors Fung and Chook cite Ref4 LINE 657 as review for the karyopherin field: Wing, C.E., Fung, H.Y.J., and Chook, Y.M. (2022). Karyopherin-mediated nucleocytoplasmic transport. Nat Rev Mol Cell Biol 23, 307-328. 10.1038/s41580-021-00446-7. THE REFERENCE IS INCORRECT ACCORDING TO PUBMED WHICH ONLY LISTS FUNG AND CHOOK AS AUTHORS. This needs to be corrected and a review that does not include the authors of this manuscript needs to also be included. I suggest the authors also correct their review.

Their information on Msn5 from the Fung and Chook review of 2022 lacks references and is insufficiently detailed to be a good reference for Msn5/Exportin-5.
"This is true for other exportins and biportins except for the yeast exportin MSN5, which exports many phosphorylated proteins by binding to a still undefined phospho-NES that is located in the IDRs of its cargoes. For example, transcription factor PHO4 must be phosphorylated at two specific sites within a 150-residue-long IDR inter-domain linker53. Sixteen other MSN5 cargoes, all involved in either environmental response or cell cycle regulation, contain IDR segments that drive export only when phosphorylated. No common sequence/structural features have been identified for this potential phospho- NES (Supplementary Table 2). Although the human homologue of MSN5 (XPO5) is known to export RNAs, its one putative protein cargo is the phosphorylated RNA editing enzyme ADAR1, suggesting that the recognition of a phospho-NES may be evolutionarily conserved54,55 "

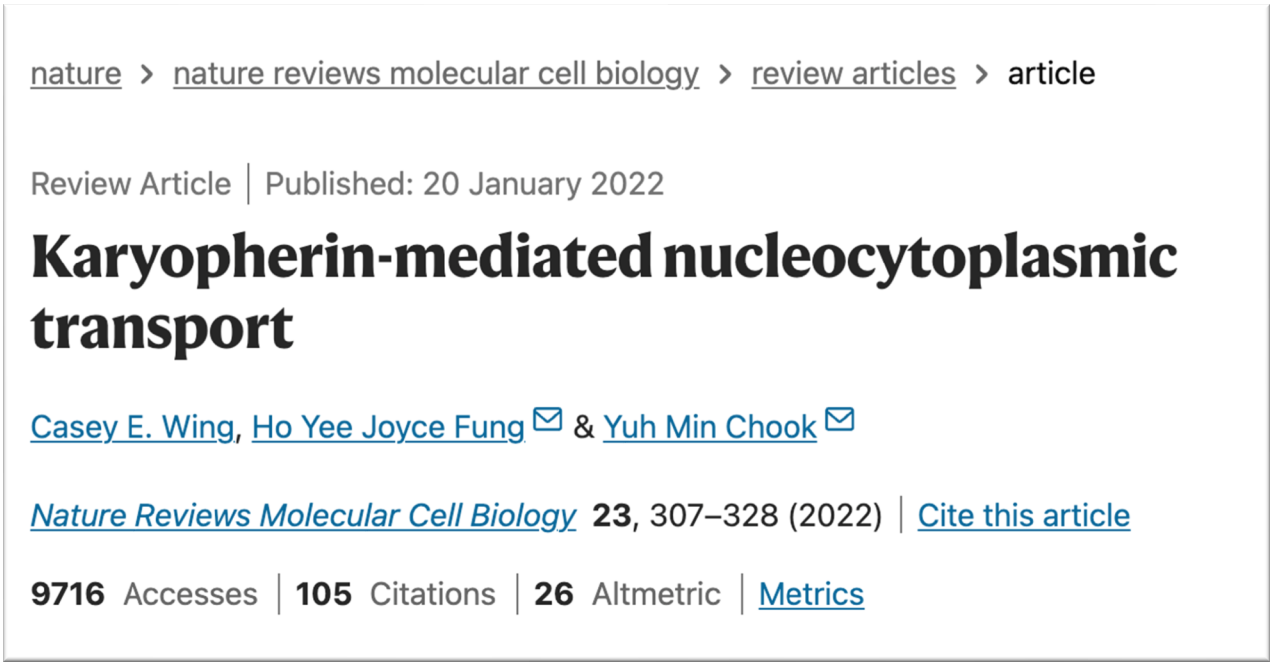
5). 54. Fritz J et al. RNA-regulated interaction of transportin-1 and exportin-5 with the double-stranded RNA-binding domain regulates nucleocytoplasmic shuttling of ADAR1. Mol. Cell Biol 29, 1487– 1497 (2009). [PubMed: 19124606]
55. Sakurai M et al. ADAR1 controls apoptosis of stressed cells by inhibiting Staufen1-mediated mRNA decay. Nat. Struct. Mol. Biol 24, 534–543 (2017). [PubMed: 28436945] These refs 54 and 55 are Incorrect references for the paragraph on Msn5 in the Fung and Chook 2022 review down loaded through Pubmed This is invalid. Correct references are required.

AUTHORS' RESPONSE

We are unclear how the confusion arose that we had referenced our own review article incorrectly. We checked Pubmed https://pubmed.ncbi.nlm.nih.gov/35058649/, which shows the review that we cited is indeed
"Nat Rev Mol Cell Biol. 2022 May;23(5):307-328. doi: 10.1038/s41580-021-00446-7. Epub 2022 Jan 20.
Karyopherin-mediated nucleocytoplasmic transport
Casey E Wing[1], Ho Yee Joyce Fung[2], Yuh Min Chook[3]

- PMID: 35058649
- PMCID: PMC10101760
- DOI: 10.1038/s41580-021-00446-7"

The three authors of this citation, including Fung and Chook who are the 1st and senior authors of the current manuscript, wrote this review article together. Below is a screenshot of the paper in the journal url https://www.nature.com/articles/s41580-021-00446-7



We have decided to be more thorough and reference all the review articles that have been published on general (rather than disease-based) mechanisms of Karyopherins in the last five years. These include 1) our Wing et al 2022 review, 2) Yang, Y. *et al.* Nuclear transport proteins: structure, function, and disease relevance. *Signal Transduct Target Ther* **8**, 425 (2023). https://doi.org:10.1038/s41392-023-01649-4, and 3) More than a zip code: global modulation of

cellular function by nuclear localization signals. Chang CC, Hsia KC. FEBS J. 2021 Oct;288(19):5569-5585. doi: 10.1111/febs.15659. Epub 2020 Dec 19. PMID: 33296547

As for the Nat Rev Mol Cell Biol. Review article that was published in 2022, the journal had a firm limit on the number and ages of references cited, which meant that we could not cite many primary research papers especially the older ones. The manuscript was also rigorously reviewed by two other experts in the field and the editor.

REVIEWER #2's COMMENT

6). Lines 85-85 The authors describe PHO4 in terms of an intrinsically disordered domain and reference 24,25

Ref24 Ogawa, N., and Oshima, Y. (1990). Functional domains of a positive regulatory protein, PHO4, for transcriptional control of the phosphatase regulon in Saccharomyces cerevisiae. Mol Cell Biol 10, 2224-2236. 10.1128/mcb.10.5.2224-2236.1990.
Ref25 Shimizu, T., Toumoto, A., Ihara, K., Shimizu, M., Kyogoku, Y., Ogawa, N., Oshima, Y., and Hakoshima, T. (1997). Crystal structure of PHO4 bHLH domain- DNA complex: flanking base recognition. EMBO J 16, 4689-4697. 10.1093/emboj/16.15.4689. Although the literature is full of recent work on intrinsic disorder regions, very popular right now, there is no mention of IDR intrinsic disorder region and its relation to function of Pho4 in Ref 24 or Ref25 based on perusal of the articles and computer searching for disorder, IDR, intrinsic. This is another invalid sentence. The authors would need to provide evidence of the link between an IDR and PHO4 function they are referring through either through correct referencing or their own analysis that would be included in this manuscript.

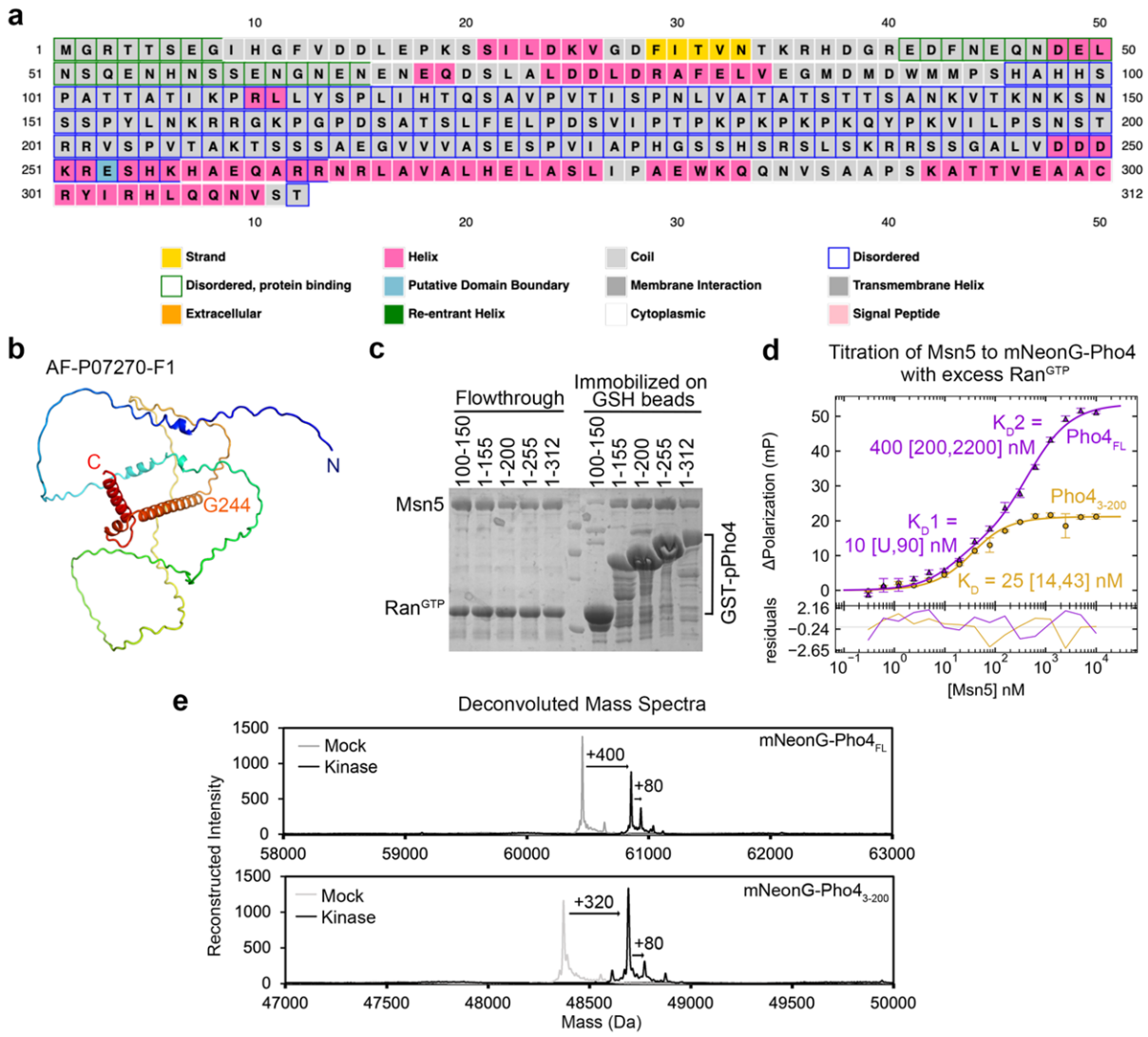
AUTHORS' RESPONSE

The sentence of concern in the original manuscript was: "The 312-residue Pho4 consists of a 240-residue IDR followed by a C-terminal 84 helix-loop-helix domain[24,25]."

We had referenced the Ogawa, N., and Oshima, Y. (1990) paper here because it was the first to map the functional regions/domains of the Pho4 protein. We referenced the Shimizu et al (1997) paper because it was the first structural analysis of Pho4, of its only folded C-terminal bHLH domain, thus a good reference for the sentence and for the Pho4 domain organization schematic in Figure 1a. We are not aware of papers that addressed the intrinsically disordered (also sometimes referred to as "unfolded") nature of the rest of Pho4, but there are many web tools that predict that the rest of Pho4 adopts no tertiary structure, hence is intrinsically disordered. In general, intrinsic disorder is a more accurate biophysical/structural designation than unfolded.

A new way to visualize that Pho4 is mostly intrinsically disordered is the AlphaFold model in the Uniprot entry for Pho4. We now show both the results of the PSIPRED secondary structure and intrinsic disorder prediction and the Pho4 AlphaFold model in the new Supplementary Fig. 1a and 1b. These results show clearly that residues 1-247 of Pho4 form an intrinsically disordered region of the protein.

New Supplementary Fig. 1 is shown below:



Supplementary Fig. 1. Biochemical analysis of Pho4 binding to Msn5. (a-b) Secondary structure prediction by PSIPRED **(a)** and the AlphaFold model of Pho4 **(b)** (rainbow, blue to red from N- to C-terminus) predict that residues 1-243 to be intrinsically disordered. **(c)** GST-Pho4 constructs of different lengths were phosphorylated and then immobilized on GSH beads, incubated with Msn5 and Ran^{GTP} and washed extensively before the unbound (flowthrough) and bounds proteins were visualized by SDS-PAGE and Coomassie staining. **(d)** FP titration of Msn5 into 20 nM mNeonG-Pho4 (FL or 3-200) in the presence of 30 µM Ran^{GTP}**.** Data points are averages of triplicate measurements with error bars representing standard deviation. $Pho4_{FL}$ data is fitted using two-site (independent) binding model and assuming mNeonG-$Pho4_{FL}$ is at 10 nM dimer concentration, whereas $Pho4_{3-200}$ data is fitted with 1-site binding model. Fitting residues are plotted below. Dissociation constants (K_D) are reported with 95% confidence interval in brackets. U means unable to be determined. **(e)** Deconvoluted mass spectra from intact mass analysis of mNeonG-Pho4 proteins used in **(d)**. mNeonG-$Pho4_{FL}$ contains at least 5 phosphates and mNeonG-$Pho4_{3-200}$ has 4 phosphates.

REVIEWER #2's COMMENT

7). This reviewer is raising concern based on inaccurate referencing and incorrect statements including in their own review in Nature Reviews. To be considered further, as this reviewer does not have so much time, the journal Nature Communications needs to have staff check each

sentence and reference cited by Fung and Chook in this manuscript and verify that the sentence is a correct statement that matches what is in each reference they cite. These inaccuracies warrant correction.

AUTHORS' RESPONSE

The Wing et al review article was also rigorously reviewed by two other experts in the field and the editor of Nature Review Molecular Cell Biology. The journal had a firm limit on the number and ages of references cited, which meant that we could not cite many older primary research papers. We are happy to receive instructions from the Nature Review Molecular Cell Biology and Nature Communications editors.

REVIEWER #2's COMMENT

8). Materials and Methods: Although some parts of materials and methods are sufficiently documented, other parts of Materials and Methods are incomplete. The NIH requires that protocols be written with sufficient detail that another person would be able to replicate what is described. Currently the methods are not quite there yet.Complete methods are required. examples lines 431-432: information missing on PCR and blunt end ligation. lines 490-491: No method included for site-directed mutagenesis and how they confirmed the identity of the mutations after the manipulation. Lines 439-440: length of time with EmulsiFlex-C5 and temperature for lysis is missing. Temp for column chromatography and length of time is missing. Number of cells used for preps and volumes also missing. Lines 488-489: Information on the cDNA library used to clone PHO4 and how the cDNAs were isolated is missing. Oligonucleotides used for PCR and sequencing and cloning need to be listed as well as evidence of what clones were sequenced to confirm they are correct and if only checked by restriction sites then that should be listed.

AUTHORS' RESPONSE

We have added the information requested by the reviewer to our revised methods. In general, we have added more details to the methods. Changes have been made throughout the long methods section and is thus not specially highlighted here. Primers used in this study are now listed in the new Supplementary Table 5.

REVIEWER #2's COMMENT

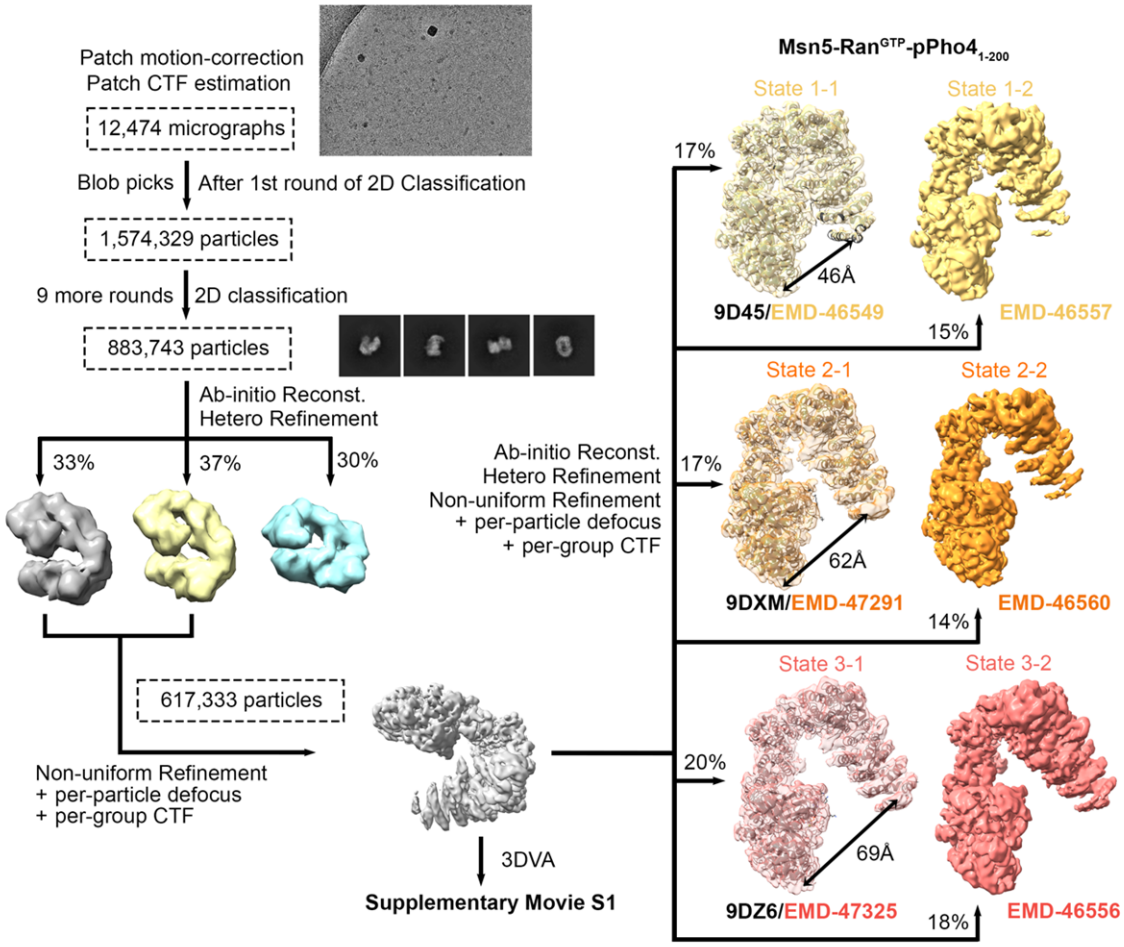
9). Supplemental materials and methods on Analysis of Pho4 density/map features in the additional cryo-EM maps of the Msn5-RanGTP-pPho41-200 complex discusses assignment of images to state1, state2, state3. The method used to do this assignment needs to be included along with software. They should also mention whether there are other shapes not described by states 1-2, 2-1, 2-2, 3-1, 3-2- in Extended Table 1.

AUTHORS' RESPONSE

The Supplementary Table is not a suitable place to describe potential all the conformations of the 6 reconstructions. Instead, we provide this information in the methods section (lines 681-689): "After additional 9 extensive rounds of 2D classification, 883,743 particles were used to obtain 3 *ab initio* models, which were subsequently submitted to heterogeneous refinement. ~30% of the particles partitioned into a map that looks like Msn5 alone. The rest of the particles, a total of 617,333 particles can be further refined using non-uniform refinement to generate a 2.97 Å

resolution map which showed clearly density for Ran^{GTP} but not Pho4. These particles were then further used to generate 6 new *ab initio* models which were then submitted to heterogenous refinement and non-uniform refinement with per-particle defocus and per-group CTF optimization."

Furthermore, as suggested by Reviewer #3, we added visual flowcharts of our data processing strategies to the new Supplementary Fig. 2, 4 and 14, which summarizes the details described in the methods. Supplementary Fig. 2 is shown here as an example:



Supplementary Fig. 2. Summary of data processing for Msn5-Ran^{GTP}-pPho4$_{1-200}$. Details of data processing are described in methods. In brief, particles from the Msn5-Ran^{GTP}-pPho4$_{1-200}$ cryo-EM dataset (representative micrograph shown) were picked by blob picker and 2D classified; representative 2D classes are shown. *Ab-initio* reconstruction with 3 classes showed populations of Ran^{GTP} bound Msn5 (gray and yellow) and unliganded Msn5 (blue). Non-uniform refinement of the 617,333 particles yielded a reconstruction that clearly has the Ran^{GTP} bound, but with no obvious Pho4 density. These particles were analyzed by 3D variability analysis in cryoSPARC (Supplementary Movie S1 is a representative movie showing one of the flexible movements of the complex). The particles were further separated into 6 classes that were then grouped into 3 states based on their Msn5 solenoid conformations, as indicated by the distances between the termini. Particle distributions are indicated as percentages. States 1 (States 1-1 and 1-2; yellow) Msn5 solenoids are the most compact, and States 3 (States 3-1 and 3-2; coral) the least compact. States 2 (States 2-1 and 2-2; orange) Msn5 solenoids intermediate in compactness. States 1-1, 2-1 and 3-1 maps are shown as transparent surfaces with their associated PDB models as gray cartoons (EMDB and PDB codes in bold). States 1-2, 2-2 and 3-2 maps are drawn as opaque surfaces and labeled by their EMBD codes. All the Msn5 maps are aligned at their Ran^{GTP} binding regions.

We have also included new detailed information to the Methods section on the map analysis software used to assign states, continuing from the quote above (lines 689-697):

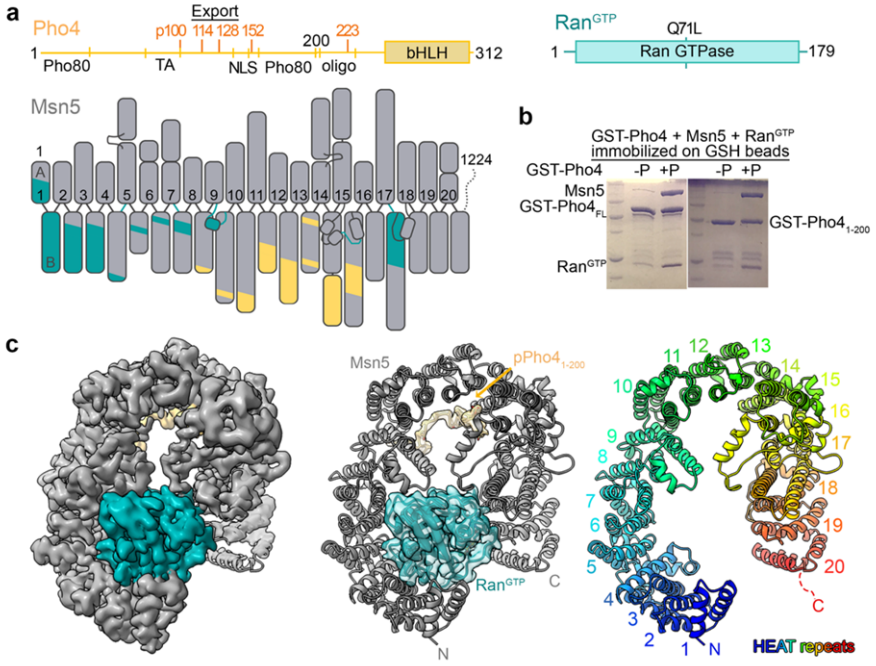
"These 6 reconstructions were classified into different states based on visual inspection and map alignment in ChimeraX[84].
Msn5-Ran^{GTP}-pPho4$_{FL}$ was processed similarly and had 1,104,150 particles after a single round of 2D classification. After 7 additional rounds of 2D classification, 395,996 particles were classified into 6 classes, only 2 of which with most particles looked like good particles and were submitted to non-uniform refinement without per-particle defocus and per-group CTF optimization to yield two maps of ~ 5 Å resolution. The resulting 2 reconstructions were classified into different states based on visual inspection and map alignment in ChimeraX with the 6 maps obtained for Msn5-Ran^{GTP}-pPho4$_{1-200}$."

REVIEWER #2's COMMENT

10). Figure 1 and Figure 2 would be important additions to the field. However the color scheme makes it hard to figure out what is what. Perhaps the grey Msn5 could be lightened and the yellow for Pho4 and green/blue cyan for Ran could be brightened. If possible additional views with rotation could be added perhaps in supplemental with more discernment of the concave binding pocket in Msn5 that is bound by Pho4. The third structure with the numbered heat domains is confusing because in addition to yellow and cyan there is also navy blue and an orange red and not clear what that signifies. A KEY listing the numbers, with color and what each group of numbers signifies would help.

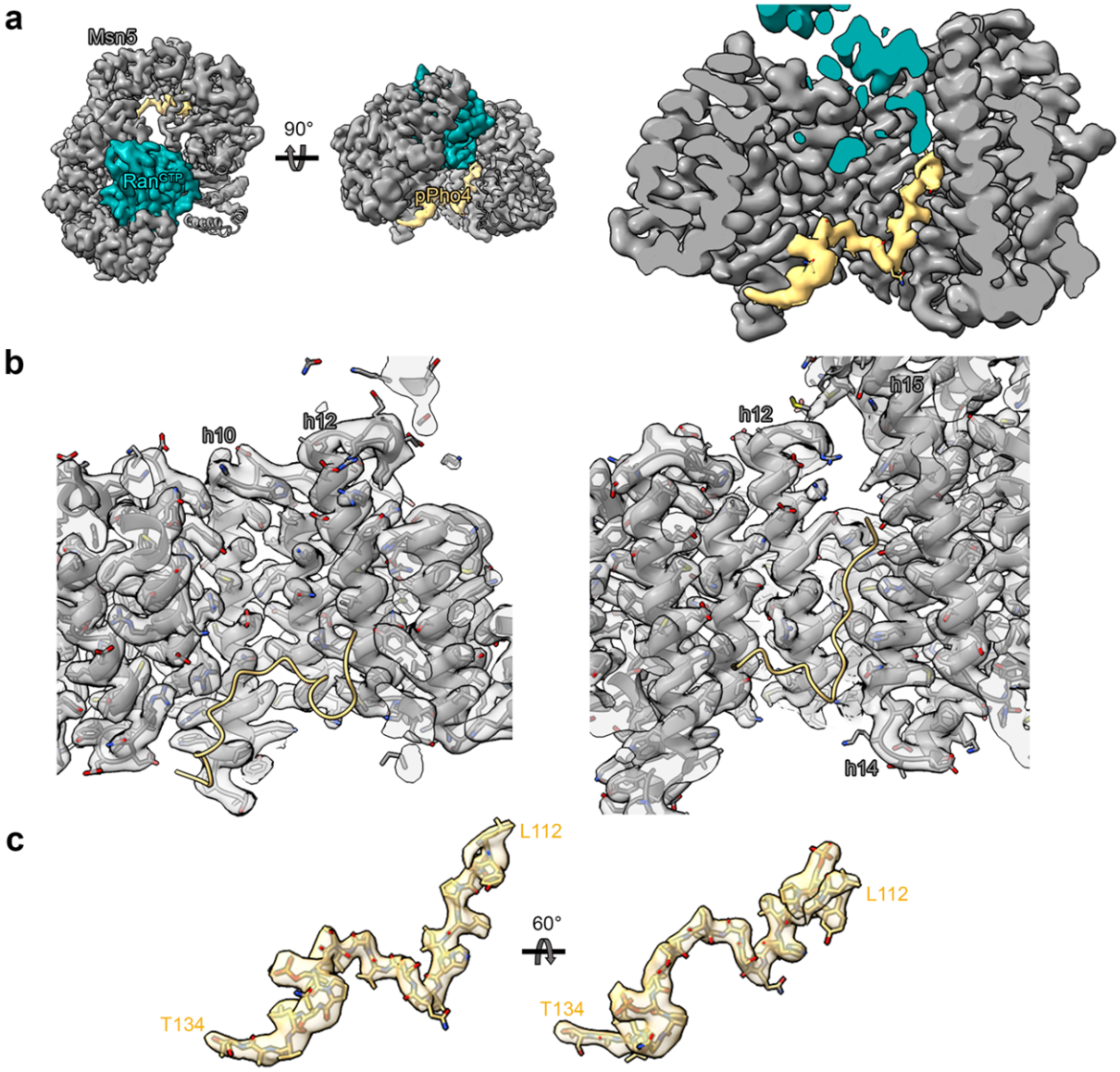
AUTHORS' RESPONSE

We have tried many color combinations over the several years that we have worked on the manuscript, and arrived at the one that all authors agree is best for clarity. Instead of changing the colors, we have changed the drawing styles of the three Fig. 1c panels to improve clarity:



The third structure (rightmost panel of Fig 1c) is colored using the standard rainbow color convention of blue for N-terminus proceeding to red for C-terminus, which is stated in the legends: “Msn5 shown alone in rainbow colors with HEAT repeats labeled”.

We also included additional views in a new Supplementary Fig. 8, as suggested by the reviewer:



Supplementary Fig. 8. Map quality of the Pho4-binding interface of Msn5. (a) Top (left) and front (middle) views of the Msn5 (gray)-Ran^{GTP} (dark cyan)-pPho4$_{1-200}$ (yellow) State 1-1 map, colored as the overlayed final model. The rightmost panel is an enlarged front view (same as in the middle panel), zoomed-in and sliced in the middle to view the map features of the bound pPho4 (yellow). **(b)** Views of the left and right sides of the pPho4-binding concave surface of Msn5, showing the map quality. Msn5 is drawn as gray cartoon with side chains shown as sticks. The pPho4 peptide is shown as cartoon, with the map omitted. **(c)** Map quality of the Pho4 peptide, overlayed with the peptide shown as sticks, in two views. The last and first residues modelled are labeled.

Pho4 does not bind in a concave pocket. Rather, it binds to a large and open surface on the concave side of the Msn5 solenoid. We hope this is better presented with our revised and new additional figures.

REVIEWER #2's COMMENT

11). The discussion of the cryo-EM in the text mentions a 3.0 angstrom cryo-EM structure for Msn5-RanGTP-Pho4(1-200) and a 4.9 angstrom map of Msn5-RanGTP-Pho4(full length) trimeric complexes. This information should be in the abstract along with mention that the two key Pho4 phosphorylation sites for binding and the two sites that contribute minimally. I suggest that the authors include a reference to: which explains that 3.5 angstrom is considered high resolution for cryo-EM and that 4+ angstrom can be used to get meaningful information to make it clear to people who are better versed in classic crystallography. One example from 2011 is Adv Protein Chem Structural Biol manuscript; available in PMC 2013 Jul 2. Published in final edited form as: 2011; 82: 1-35. PMCID: PMC3698602 NIHMSID: NIHMS480946 PMID: 21501817 ATOMIC RESOLUTION CRYO ELECTRON MICROSCOPY OF MACROMOLECULAR COMPLEXES Z. Hong Zhou

AUTHORS' RESPONSE

We thank the reviewer for this suggestion to highlight the important findings in this paper in the summary. However, due to the word limit of the summary, we have instead added that we have a "high resolution" cryo-EM structure.

The reviewer is correct in saying that although the 4.9 Å map is not a high-resolution by modern standards of cryo-EM, it can still provide important insights. We agree and therefore highlight this information in the first two sentences of the Results section titled "**Cryo-EM maps of pPho4$_{1-200}$- and pPho4$_{FL}$- bound Msn5 reveal Msn5 flexibility**" in lines 106-109:
"We purified ternary complexes of Msn5-Ran^{GTP}-pPho4$_{1-200}$ and Msn5-Ran^{GTP}-pPho4$_{FL}$ for cryo-EM structure determination. We solved three 3.0-3.2 Å resolution structures of the former and obtained 4.9 Å resolution maps of the latter (Fig. 1c, Table 1, Supplementary Fig. 2-4 and Table 1)."

We also show the 4.9 Å map in a main figure, Fig. 2d, as the information it provides is supported by mutagenic/binding studies. We describe the critical information learned from the low-resolution map in lines 138-145:
"The bound Pho4 residues 112-134 form a large and sprawling 1256.9 Å^2 interface with Msn5. This Pho4 segment is likely the persistently bound core portion of the Pho4 NES. The same Pho4 segment is resolved in the most compact Msn5-Ran^{GTP}-pPho4$_{FL}$ map, despite the lower resolution map (Fig. 2d and the State 1 map shown in Supplementary Fig. 4). This map shows additional Pho4 features beyond the N-terminal most of Pho4 residues modeled (L112) in the Msn5-Ran^{GTP}-pPho4$_{1-200}$ map, that is adjacent to a basic patch at the B helices of Msn5 repeats h16-h18 (Fig. 2d)."

REVIEWER #2's COMMENT

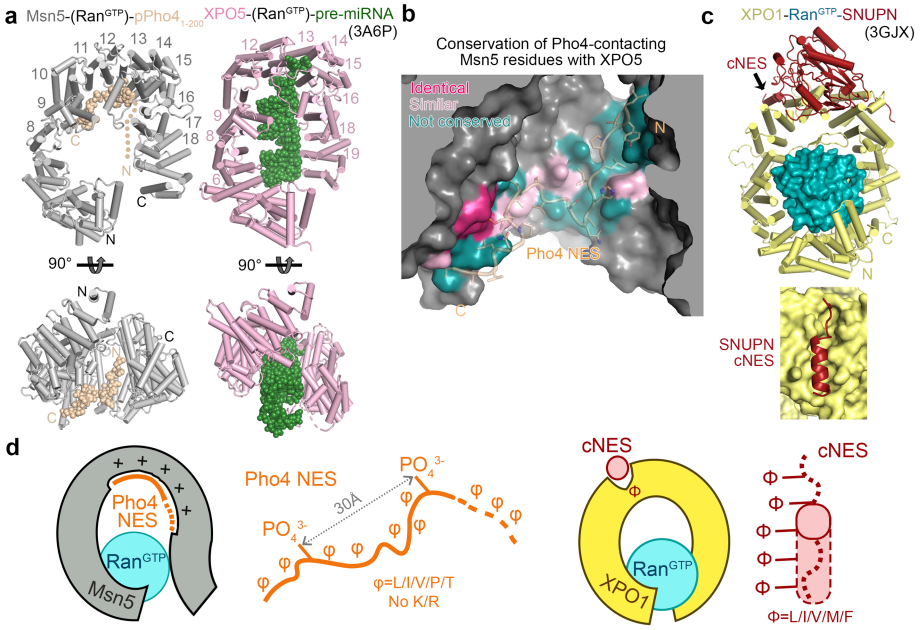
12). Figure 5 A structure comparison with Exportin-5 is beneficial, pointing out approximate areas that might be involved in protein cargo contact together with the known areas involved in RNA contact. The dark orange vs light orange is hard to distinguish. The exportin-5::premiRNA

structure is much more compact than the Msn5::Pho4 structure where pho4 binds horizontally as well as vertically. Is this because of more extensive vertical contacts with the RNA and more extensive horizontal contacts with pho4? would there be more extensive vertical contacts with full length Pho4?

AUTHORS' RESPONSE

Importins and exportins are well known for their conformational flexibility, adopting different conformations when bound to different ligands. The same is true of Msn5 and XPO5, where a particular ligand stabilizes a specific conformation or even an ensemble of conformations in the case of Msn5-Pho4 interaction. The maps of Msn5 bound to full length Pho4 (Fig 2d and Supplementary Fig. 6e) are like those for Msn5 bound to $Pho4_{1\text{-}200}$. An additional NES region (labeled "Extra density (NES Region 1)") is observed in the map for the bound FL Pho4 (Fig. 2d). This extra bound NES region binds "horizontally" rather than "vertically" in the views that we show.

Following the reviewer's suggestion, we have now changed the colors in Fig. 5b from the old and less obvious dark/light orange to the more strikingly different magenta/light pink combination.



XPO5 makes extensive contacts with the pre-miRNA through both the vertical and horizontal interfaces. We do not elaborate on these interactions because they were already described in the original publication, and they are not the focus of our study. The goal of Fig. 5 is to display the Msn5/XPO5 conservation, with the focus on the Pho4 binding site, hence analysis of the Pho4 binding Msn5 residues rather than the pre-miRNA XPO5 ones.

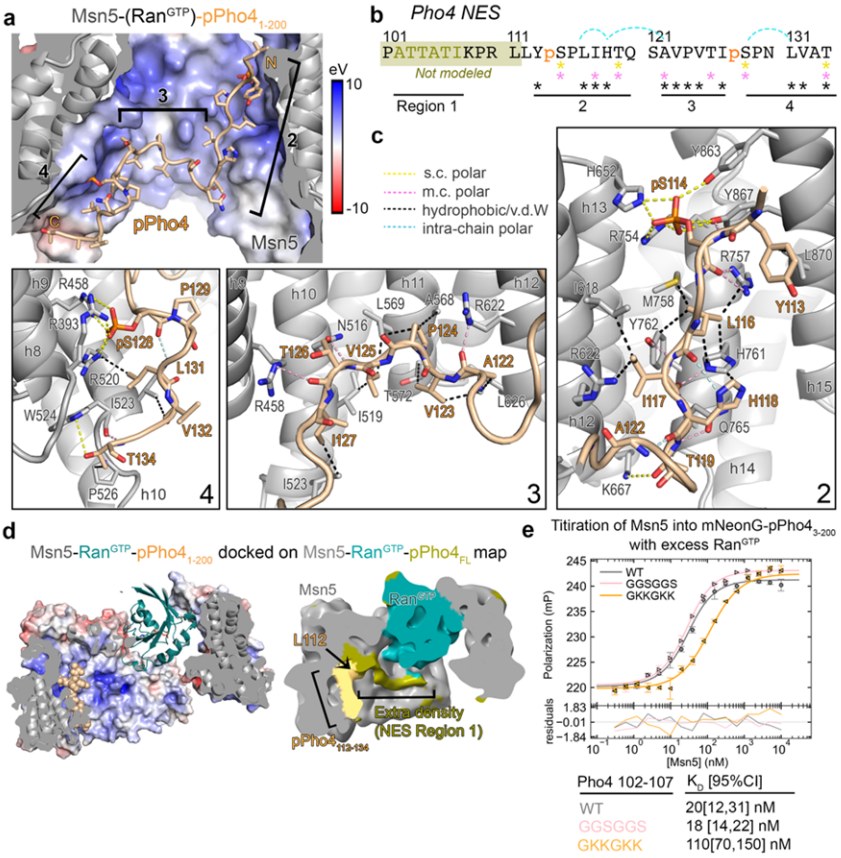
REVIEWER #2's COMMENT

13). Repeat point for Figure 2. This author is very enthusiastic about Figure 2 However the colors are not bright enough to see readily and could be improved. Also it is hard to visualize the Msn5 NES binding to the central concave portion of Msn5 in Figure 1, it could be improved to stand out more and also have another view

AUTHORS' RESPONSE

Thank you for noting the difficulty in viewing the figures. Again, instead of changing the colors, we added white or black outlines for the labels in Fig. 2 and all other relevant main and supplementary figures to improve visualization. We have also added additional views (Supplementary Fig. 8) to improve visualization of the NES binding to the central concave portion of Msn5.

Updated Fig. 2 is shown below:



REVIEWER #2's COMMENT

14). Lines 272-280. This reviewer is enthusiastic about this part of the manuscript and extended table 2. However, the authors ought to include Pho4 in the table referencing themselves as "this study". In addition, the authors only show the proteins that conform to their proposed consensus. I think they need to at a minimum list the remainder of the 16 they examined in the table legend with the references to those papers and let us know whether those papers had done a detailed enough analysis to narrow down potential interaction site.

AUTHORS' RESPONSE

We have added information to the new Supplementary Table 4 for all eighteen proteins that were reported to be Msn5 cargoes. We also referenced "this study" for the Pho4 entry in the table.

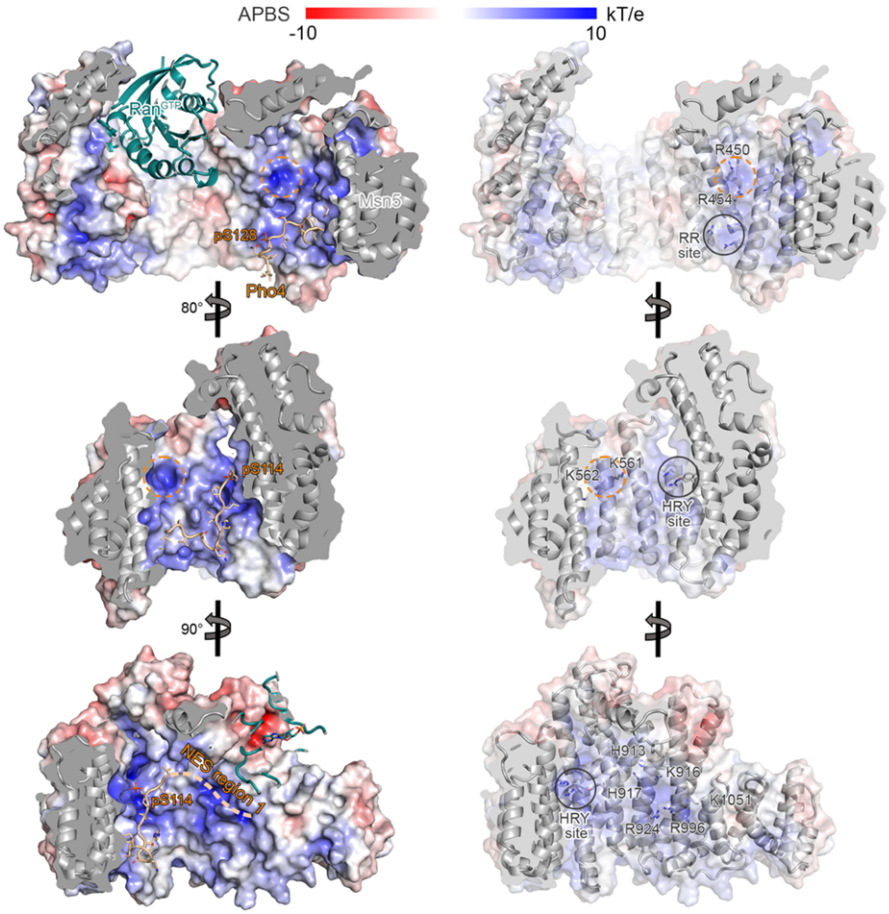
REVIEWER #2's COMMENT

15). Lines 281-289: It would be clearer if the authors stated the stretch of Pho4 that is binding to the basic patches and also explain whether different cargoes would be predicted to bind to the same basic patches or to different ones on Msn5. It seems either possibility is feasible.

AUTHORS' RESPONSE

We have expanded that paragraph (now lines 318-328) to include more analysis and speculation: "We also note that the prediction of Msn5-binding NESs is likely further complicated by the likelihood that Msn5-NES binding modes and the sequences/structures of the NESs might be quite diverse. The Pho4 NES binding site that spans HEAT repeats h8-h15 of Msn5 is large and open, and Pho4 pS114 and pS128 bind only two (Msn5 HRY and RR sites) of the four basic patches on this Msn5 surface. Additional Msn5 basic patches at nearby Msn5 residues K561/K562 and R450/R454 may bind phospho-serine/threonine residues of other cargoes (Supplementary Fig. 13). The combinatorial use of four basic potential phosphate-binding sites could result in very diverse modes of Msn5 binding to phosphorylated NESs. The extent of structural and sequence diversity of NESs that bind at repeats h8-h18 of Msn5 will only be evident with additional cargo-bound structures."

New Supplementary Fig. 13 is shown below:



Supplementary Fig. 13. Additional basic patches in Msn5 concave surface. Left: APBS electrostatic potential of Msn5, shown as surface in three views. Msn5 is the gray cartoon

underneath the surface, and Ran^{GTP} and Pho4 are shown as cyan and wheat cartoon respectively. Pho4 phosphoserines are labelled, and NES region 1 is shown as dotted line in the bottom. Right: Transparent version of the surface on the left, and Pho4 and Ran^{GTP} are hidden. Residues that contribute to the strong basic patches are shown as sticks and labelled. Two additional basic patches, circled in orange dotted line, can be seen near the RR and HRY sites, formed by residues R450/R454 (top) and K561/K562 (middle). Additional patches along the C-terminal of Msn5 interacts with NES region 1 (Fig. 1d).

REVIEWER #2's COMMENT

16). Lines 162-175 would benefit from having a table that has the various affinities of the Pho4 and Msn5 mutants and is combined with the discussion in Extended results "Comparison of pPho4 S114A and S128A mutants and the Msn5 mutants at their respective binding sites "As described in the main text, we mutated the Pho4 serines that are phosphorylated (Pho4 S114A, Pho41-200 S128A and Pho41-200 S114/128AA) and the phosphate binding sites on Msn5 (Msn5 HRYAAA and Msn5 RRAA). The 5-fold affinity decrease of the Msn5 HRYAAA mutant (KD 230[160,350 nM) compared to Msn5 WT matches the 6-fold decrease seen for pPho4 S114A vs. pPho4 WT. However, the 50-fold affinity decrease of Msn5 RRAA (KD 2.2[1.0,6.1] µM) vs the 3-fold decrease of pPho4 S128A is surprising as Msn5 R393 and R458 contact only the phosphate group of pS128 in the structure (Fig. 2c, left panel). It is possible that the loss of the phosphate group in pPho4 S128A is less detrimental because Msn5 R393 and R458 may still interact with the alanine at position 128 or its neighboring residues. Alternatively, in the absence of pS128, pS114 may bind at the Msn5 RR site (see above). " are important and a shortened version ought to be in the main text with a table of affinities".

AUTHORS' RESPONSE

Thank you for your interest in these additional findings. We have moved this paragraph back into the main text. All affinity data generated in this study is now summarized in the new Supplementary Table 2.

REVIEWER #2's COMMENT

17). Line 144: the sentence …three 90° turns of the zig-zagging chain (Fig. 2a and b) is an apt description that helps one visualize the Msn5-Pho4 interaction and might be repeated earlier in the text when the structure is first described.

AUTHORS' RESPONSE

We took the reviewer's suggestion and now repeat the description earlier in the text, when the structure is first described in the new lines 135-138:
"Strong and continuous map features adjacent to the concave surface of Msn5 B helices of h8-h15 allowed confident modeling of 23 Pho4 residues that form three 90° related segments of a zig-zagging chain (Fig. 2a–c and Supplementary Fig. 8)."

REVIEWER #2's COMMENT

18). The authors discuss the different conformers (states) of the Msn5/Pho4/Ran complexes. Can they speculate on the relationship of those conformers to the life-cycle of the complex viz association in cytosol and nucleus and release after export. Do they have any favored models?

AUTHORS' RESPONSE

The maps give us six snapshots of the Msn5 solenoid flexing to different extents. The Msn5/Pho4/Ran complex likely samples all six states and additional ones in a dynamic manner. It is unclear which states are more favored while the complex is the nucleus, translocating across the nuclear pore complex (NPC) or as it exits the NPC on the cytoplasmic side before it encounters RanBP1 and RanGAP. There is a lack of information in the field for how Kap-cargo complex conformations and dynamics may change or not change across the nuclear-cytoplasmic transport cycles. There is no basis for us to even speculate here.

REVIEWER #2's COMMENT

19). The authors cite as " interesting" that : "Interestingly, phosphomimic mutations do not mimic Pho4 phosphorylation: Pho41-200 140. 158 S114/128DD and Pho41-200 S114/128EE bind Msn5 weakly, like unphosphorylated Pho41-141. 159 200, with KDs ~2-3 μM (Extended Data Fig. 6a)."

This reviewer thinks the word "Surprisingly" rather than Interestingly is better suited for the sentence because it is not expected at all that the phosphomimetic mutations would reduce binding, the expectation is that they should mimic phosphorylation. What is the effect of these amino acid substitutions on the structure of Pho4 and does this differ from the phosphorylated Pho4 structure and explain the reduced binding? Extended data figure 6 supports what has been written but some summary statement about why is important to the results section description. one might consider the impact of the phosphomimetic EE mutations on this structure described by the14 non-phospho mutations later in the results section and how this region would be impacted by the EE mutations.

AUTHORS' RESPONSE

If either $Pho4_{1\text{-}200}$ $S114/128_{DD}$ or $Pho4_{1\text{-}200}$ $S114/128_{EE}$ had successfully mimicked the phosphorylated $pPho4_{1\text{-}200}$, the DD or EE mutants would bind Msn5 very tightly, with K_Ds in the low nanomolar range, just like how the pPho4 (WT) NES binds Msn5 with a K_D of 20-50 nM. However, both $Pho4_{1\text{-}200}$ $S114/128_{DD}$ and $Pho4_{1\text{-}200}$ $S114/128_{EE}$ bound Msn5 like unphosphorylated $Pho4_{1\text{-}200}$, all of them binding Msn5 weakly with K_Ds ~2-3 μM. Please note than the phosphomimetic Pho4 mutants do not have reduced Msn5 binding compared to the unphosphorylated Pho4 WT construct; they bind with about the same low affinity, that is all three hardly bind to Msn5. This was very inconvenient for our biochemical analysis because this meant we had to phosphorylate every Pho4 construct to study Msn5 binding; we could not just use a S to E/D mutant construct.

The key point here is that the Pho4 S114E/D and S128E/D phosphomimic mutants do not mimic the Msn5-binding effects of pPho4. It is inconvenient but not rare or surprising when E/D phosphomimic mutations do not mimic the effects of phosphorylating serine/threonine residues. Other examples include the activating loops of PAK1 kinase (Ng et al, Structure, 2010) and AGC kinases (Somale et al, Sci Rep, 2020). Therefore, we feel that "interesting" describes these results better than "surprising".

Addition of a phosphate group to a side chain results in 1) addition of negative charges that could potentially participate in new charged-charged or electrostatic interactions and/or 2) addition of hydrogen-bonding potential from the electronegative oxygen atoms of the phosphate group. The S/T to E/D phosphomimetic mutations attempt to mimic phosphorylation by adding a negatively

charge side chain that mimics the negatively charged phosphate group. These mutations could substitute successfully for phospho-S/T only if it uses electrostatic forces in binding. However, not all interactions with phospho-S/T involve electrostatic interactions; many participate in hydrogen-bonding instead. S/T to E/D phosphomimetic mutations may not provide these hydrogen bonding capabilities and thus could not substitute for phosphorylation of the S/T side chain.

We included this explanation to the Results section (lines 163-173):
"Although the structures clearly show phosphorylated Pho4 100-134 bound to Msn5, a Pho4 construct of these residues is insufficient for Msn5 binding because it is missing the kinase binding sites and therefore cannot be phosphorylated efficiently. Synthetic phosphopeptides were unsuitable for binding studies because of aggregation. Furthermore, phosphomimic mutations unfortunately do not substitute for Pho4 phosphorylation: $Pho4_{1-200}$ $S114/128_{DD}$ and $Pho4_{1-200}$ $S114/128_{EE}$ bind Msn5 weakly, like unphosphorylated $Pho4_{1-200}$, with K_Ds ~2-3 μM (Supplementary Fig. 9a). The ineffectiveness of these phosphomimic mutations suggests that the phosphate groups of pS114 and pS128 bind Msn5 via hydrogen-bonding rather than through charged-charged or electrostatic interactions. Though inconvenient, this finding is not entirely surprising as the efficacy of phosphomimic mutations is often case-dependent[46-49]."

REVIEWER #2's COMMENT

19). The sentence Lines 161-162 Phospho-serines pS114 and pS128 were previously reported to be critical for Msn5-mediated nuclear export Ref5. is inaccurate, because reference 5, Komeili, A., and O'Shea, E.K. (1999). Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. Science 284, 977-980. 10.1126/science.284.5416.977 IS THE WRONG REFERENCE FOR THEIR STATEMENT AND THEIR STATEMENT IS INCORRECT. The correct reference is REF7 Kaffman, A., Rank, N.M., O'Neill, E.M., Huang, L.S., and O'Shea, E.K. (1998). The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. Nature 396, 482-486. 10.1038/24898 . However, in Ref 7, the Kaffman and O'Shea paper did not delineate which of 5 phosphorylated sites were critical for binding to Msn5. I suggest the authors revisit the excellent Kaffman and O'Shea paper and properly describe what they showed. In my opinion it is the work in this Fung et al manuscript that is the first delineation of the individual phosphorylated residues required for binding to Msn5 and a strong description should be made.

AUTHORS' RESPONSE

Both the 1998 (Kaffman et al, Nature) and the 1999 (Komeili and O'Shea, Science) papers from the O'Shea lab are excellent and important papers on Pho4 phosphorylation and Msn5-mediated nuclear export. We stand by our referencing of the Komeili 1999 paper for the sentence "Phospho-serines pS114 and pS128 were previously reported to be critical for Msn5-mediated nuclear export". We wish we could claim that we are the first to figure out that S114 and S128 must be phosphorylated for nuclear export by Msn5, but Erin O'Shea's group did this 25 years ago and therefore we simply cannot claim the discovery.

Komeili & O'Shea, in their 1999 Science paper (old REF 5/new REF 26), tested various serine to alanine mutants of Pho4's 5 phosphorylation sites (their S1 site is S100, S2 – S114, S3 – S128, S4 – S152 and S6 – S223). We quote here an excerpt from the 3rd paragraph of the Science report: "To determine which of the five phosphorylation sites are required for the export of Pho4, we tested the ability of Pho4 mutants to be exported from the nucleus. $Pho4^{SA1}$-GFP, $Pho4^{SA4}$-GFP, and $Pho4^{SA6}$-GFP, containing an individual Ser→Ala substitution at phosphorylation site 1,4, or 6, had no defect in nuclear export (8). However, $Pho4^{SA2}$-GFP and $Pho4^{SA3}$-GFP, containing a

Ser→Ala substitution at sites 2 and 3, respectively, could not be exported (Fig. 1A).” Komeili & O’Shea, 1999, had indeed determined that S114 and S128 must be phosphorylated for nuclear export (by Msn5) to occur. The earlier old REF 7/new REF 7 (Kaffman, 1998) figured out that phosphorylated Pho4 is exported by Msn5, but they did not map which phosphorylation site is important for export.

REVIEWER #2’s COMMENT

20). Figure 4 RNA-seq analysis is an excellent addition to support their findings. However, it is standard to include tables of the raw data with full lists of genes and fold effects in supplemen this information should be added to the manuscript.

AUTHORS’ RESPONSE

We fully agree with this comment and a file with the normalized counts of all genes and fold effects is now included in Supplementary Table 3. We apologize for this oversight.

REVIEWER #2’s COMMENT

21). Extended Figure 7 growth data. It is quite amazing that the growth curves of overexpressed WT and HRY RR AAA AA point mutant Msn5 are nearly superimposable. How can this be if the HRYRR AAAAA point mutant is not binding cargo as efficiently? Does this mean that the growth impairment is independent of binding to cargo? This is a confusing finding. Is the mutant a dominant negative? Was it tested in a wild type background?

AUTHORS’ RESPONSE

We thank the reviewer for this comment, as we ourselves were surprised by this result. It was previously noted in Douglas et al., G3 Genes|Genomes|Genetics, 2012 that overexpression of wild type Msn5 resulted in reduced competitive fitness. Our initial hypothesis/rationale for this growth curve experiment was that overexpressing the $HRYRR_{AAAAA}$ mutant would rescue this phenotype. However, the results suggest that the growth impairment is independent of the specific Msn5-export cargo binding studied in our current manuscript. The growth results may instead suggest that the growth impairment may derive from one or multiple export cargoes not recognized at the either or both HRY and RR sites of Msn5, or may be driven by other functions of Msn5 outside of protein export. Our experiment has not been tested in a wild type background, as we feel that following up on this phenotype would expand the scope of the investigation beyond the purview of this current manuscript.

REVIEWER #2’s COMMENT

22). The authors analyze 14 other non-phospho-serine residues within the Pho4 core residues for their role in binding to Msn5. Lines 233-234 “Mutating all the non-phospho-serine Msn5-interacting residues in region 2 (113YpSPLIHT119 to GpSPGGSG; R2mut) decreased Msn5-binding by 40-fold…” AND the other mutants discussed therein. It is not clear how these mutations affect the overall structure and stability of Pho4 and whether this might impact binding to Msn5. A discussion of this caveat is needed in addition to their conclusion sentence “In summary, many hydrophobic and small polar side chains in each of Pho4 NES regions 2, 3 and 4 contribute substantially to total binding energy. They are as important as phosphoserines pS114 and pS128 for high-affinity Msn5-binding. Importantly, though, many Pho4-Msn5 contacts also involve the Pho4 main chain. “

AUTHORS' RESPONSE

The Pho4 NES is located within a large ~250 residues long IDR, which lacks tertiary structure. Therefore, we do not have to worry about further loss of overall structure. In fact, we designed the mutations, substituting the amino acids with glycines and serines which are known to favor intrinsic disorder, to prevent any inadvertent gain of secondary/tertiary structure upon mutating the Pho4 NES.

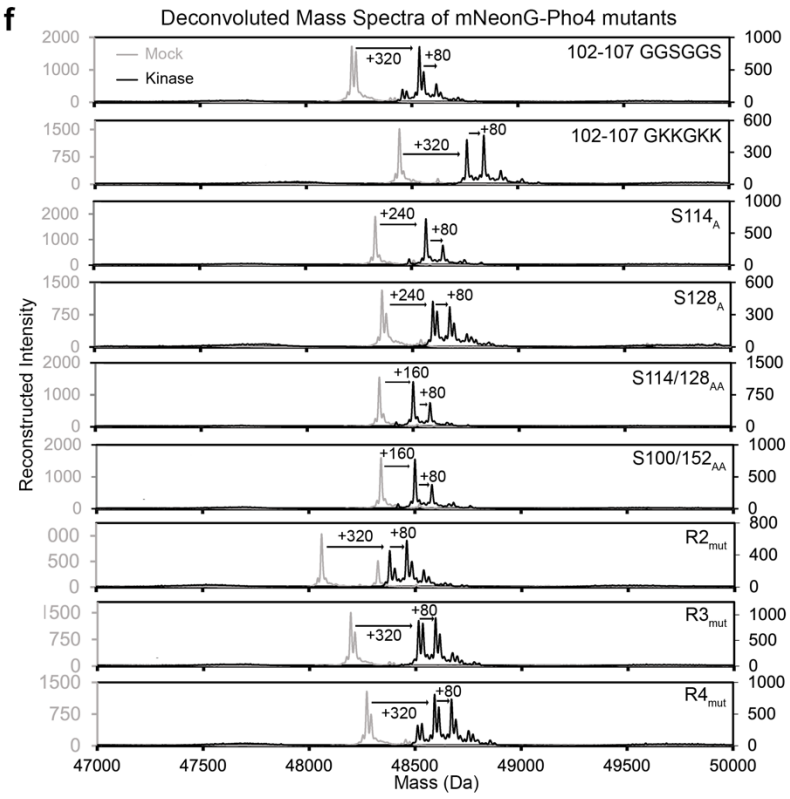
We observed no aggregation of these mutant constructs during purification with size exclusion chromatography. Furthermore, the mutants were also efficiently phosphorylated, another indication that none of them were aggregated. The choice of residues to mutate Pho4 side chains to and the monitoring of aggregation and other complicating new biochemical behavior upon mutation are all common strategies when performing structure-guided mutagenesis. We also used intact mass analysis to confirm phosphorylation of all Pho4 proteins used in this study, and no degradation of the proteins can be observed, suggesting the variants are all stable like the wild-type protein. We have now included this information to our revised manuscript.

Lines 258-262: "Mutating all the non-phospho-serine Msn5-interacting residues in region 2 to generate the $R2_{mut}$ (^{113}YpSPLIHT^{119} mutated to GpSPGGSG; glycine and serine residues were chosen to preserve intrinsic disorder of the Pho4 segment) decreased Msn5-binding by 40-fold (K_D 800[500,1200] nM; Fig. 3c), underscoring the substantial contributions of these non-phospho-serine residues to Msn5 binding (Fig. 3b)."

Lines 619-622 in methods: "All Pho4 mutants expressed and behaved similarly during all steps of purification as the wild-type protein, suggesting that the mutations have preserved the physical property of Pho4 and did not induce aberrant aggregation or instability."

Lines 637-640 in methods: "Samples before and after phosphorylation was sent to intact mass analysis to ensure that phosphorylation was complete for all Pho4 variants that were used in this study (Supplementary Fig. 1e and 9f)"

New Supplementary Fig. 9f is shown below:



REVIEWER #2's COMMENT

23.) Fig6 and Extended Fig10 of structure of unliganded Msn5 provides additional important information. In Fig6 it would be better to have the pink Msn5 also be pink with Pho4 and RanGTP in the trimeric complex so one can more easily pick it out and compare. A superimposed picture of unliganded and liganded Msn5 (minus Ran and pho4) would be very helpful even if certain parts of liganded Msn5 may not be so well defined because of occlusion by binding partners.

AUTHORS' RESPONSE

For clarity and consistency, we have changed the color of all Msn5 molecules in the main figures to the gray, irrespective of whether it is liganded or unliganded. Superposition of unliganded Msn5 and liganded Msn5 (with Ran^{GTP} and Pho4 not displayed) would indeed be helpful, but due to the already crowded figure space limitation and the consistently gray Msn5 in Fig. 6, we decided to show the superposition in a supplementary figure panel, now Supplementary Fig.17.

Updated Fig. 6:

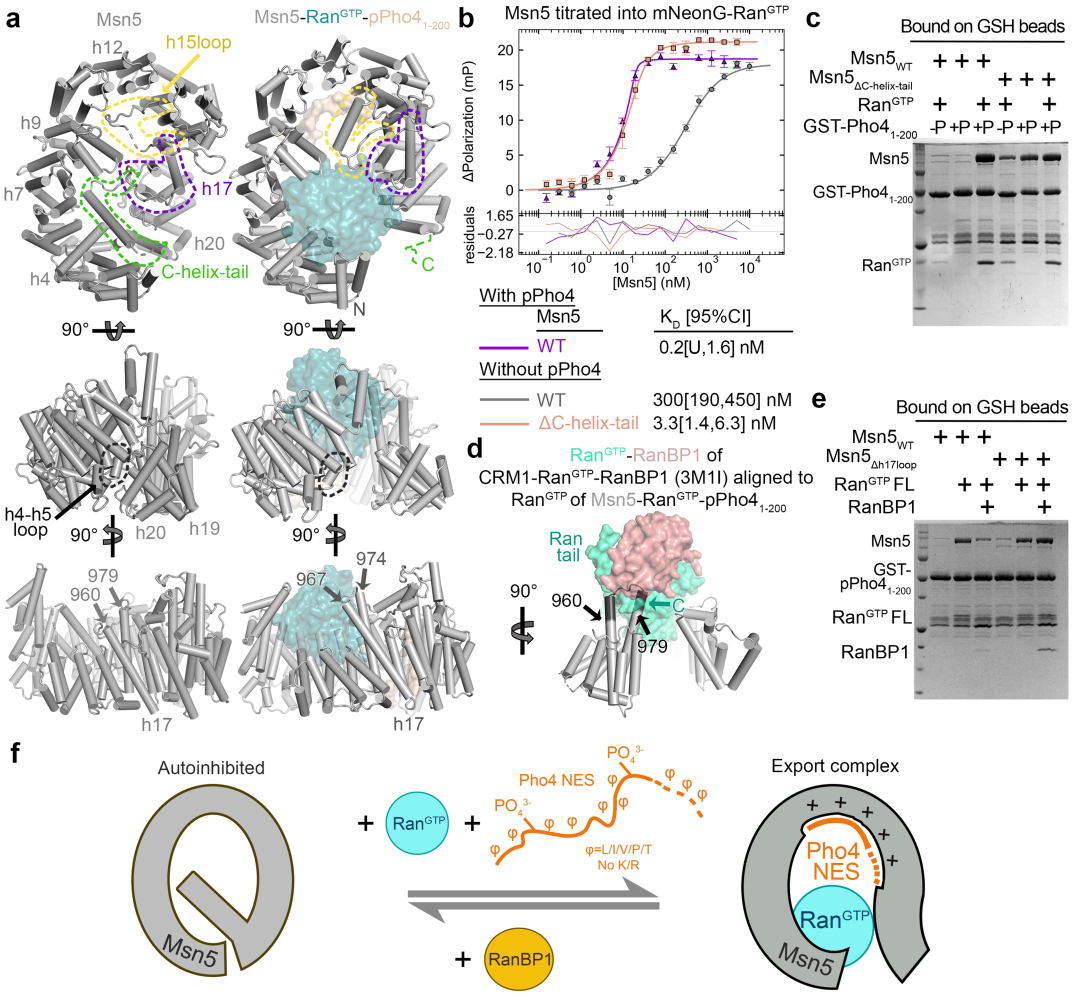
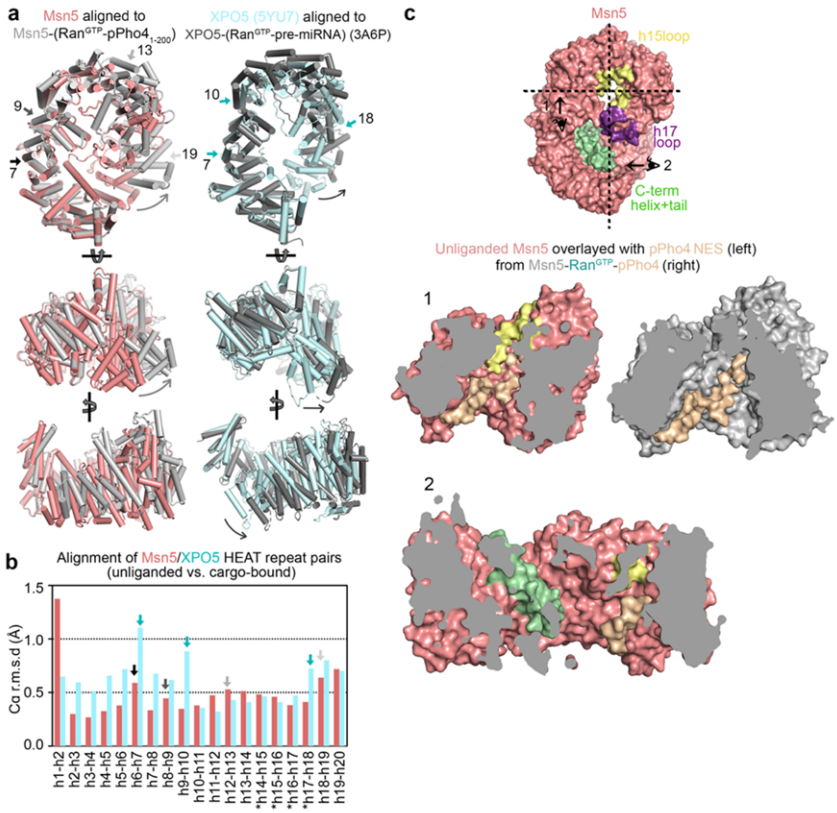


Fig. 6. Unliganded Msn5 and the mechanisms for loading/unloading Pho4. (a) Unliganded Msn5 (gray, left) and Msn5-Ran^{GTP}-pPho4$_{1-200}$ (gray-teal-wheat, right). Structural differences at h15loop, h17 and C-helix-tail are highlighted by yellow, purple and green dashed lines, respectively. The h4-h5loop that contacts h19-h20 in unliganded Msn5 but not in ligand-bound Msn5, is highlighted with black dashed lines. (**b)** FP titration of Msn5 WT or mutant ΔC-helix-tail to mNeonG-Ran^{GTP} ± excess pPho4$_{1-200}$. Data points are mean and s.d. of triplicate measurements, fitted with 1-site fitting (solid line) and fitting residuals are plotted below. **(c)** Bound proteins (after extensive washing) in an *in vitro* pull-down assay of immobilized unphosphorylated (-P) or phosphorylated GST-Pho4$_{1-200}$ (+P) incubated with Msn5 (WT or ΔC-helix-tail) ± Ran^{GTP}, visualized by Coomassie stained SDS-PAGE. **(d)** Msn5 (gray; h17 residues 960-979 in dark gray)-Ran^{GTP}-pPho4$_{1-200}$ and CRM1-Ran^{GTP}-RanBP1 (3M1I) structures aligned at Ran^{GTP}. CRM1 and pPho4 are not displayed for clearer visualization of the docked Ran^{GTP}-RanBP1 (aquamarine and pink, respectively). **(e)** As in **c**, pull-down assay of GST-pPho4$_{1-200}$ incubated with Msn5 WT or Δh17loop (residues 962-980 replaced with GGSGGS) ± Ran^{GTP} FL ± RanBP1 as labeled. **(f)** Cartoon model of cargo loading and unloading.

Supplementary Fig. 17:



REVIEWER #2's COMMENT

24). The final data in Fig 6E regarding the h17loop and RanBP and allosteric switch is interesting. Figure 6D legend states: "CRM1 and pPho4 are not shown and the docked Ran-RanBP1 is aquamarine and purple, respectively." Whereas the results text states lines 367-371 Alignment of RanGTP- RanBP1 from the XPO1-bound structure (3M1I51) with the RanGTP of Msn5-RanGTP-pPho41-200 reveals a steric clash between the Msn5 h17loop-extended h17B helix with the superimposed Ran tail and RanBP1 (Fig. 6d)." This reviewer was unable to visualize the steric clash being described and suggest improvements in the figure.

AUTHORS' RESPONSE

We changed the color of the RanBP1 surface to pink and the color of Msn5 h17 helix portions (residues 960-979) to dark grey to better show these Msn5 helix portions will clash sterically with the RanBP1 and the Ran^{GTP} tail (see the page above).

REVIEWER #2's COMMENT

25). LINES 377-381 on a model for Msn5 function are important and would benefit from a cartoon model summary at the end of the figures that describes the overall model described in these lines and also points out the Pho4 binding pocket and pho4 binding site with key residues cartooned in.

AUTHORS' RESPONSE

Thank you for this suggestion. We have included a cartoon model in a similar style as the one for comparison to the XPO1 system (Fig 5d) as the new Fig 6f. This new cartoon model in Fig 6f summarizes the proposed mechanisms of autoinhibition and positive cooperativity upon Ran^{GTP} binding (see the page above).

REVIEWER #2's COMMENT

26). The figure legends are overall quite explanatory but what is needed is knowledge of the ations of proteins used in all of the experiments. This information needs to b added either in legends or some kind of paragraph describing each figure in extended methods.

AUTHORS' RESPONSE

Protein concentrations used in the assays are listed in detail in the methods section to reduce clutter from the already long figure legends.

Methods, lines 642-654:
"For Fig. 1, 1 µM Msn5, 5 µM Ran^{GTP} and ~1-2 µM GST proteins were mixed in buffer B in 200 µL reactions with ~10-20 µL glutathione Sepharose beads and rotated at 4 °C for 30 mins. Samples were then spun at 6000 xg for 1 min and flowthrough was removed. Buffer B was used to wash the beads for 3 times, 500 µL each. Lastly, 20 µL 2X SDS sample buffer was added to beads and boiled for 3 mins and bound proteins were visualized on SDS-PAGE gel for Coomassie staining. ~2.5 µM GST-$Pho4_{1-200}$ or GST-$pPho4_{1-200}$ + 1 µM Msn5 (WT or mutants) ± 5 µM Ran^{GTP} for assay in Fig. 6c, or ~2.5 µM GST-$pPho4_{1-200}$ + 1 µM Msn5 ± 5 µM Ran^{GTP} FL ± 5 µM RanBP1 for assay in Fig. 6e, were assembled and treated the same way as described above. For binding assay in Supplementary Fig.1c, GST-Pho4 proteins were expressed and purified in small scale (35 mL). Lysate was sonicated over nice before they were clarified and bound to GSH beads and washed. Beads were used directly for binding assay with 5 µM Msn5 and 20 µM Ran^{GTP} following procedures described above."

Methods, lines 726-730:
"In brief, triplicates of sixteen 20 µL samples were assembled with final concentration of 20 nM mNeonG-$pPho4_{1-200}$, 30 µM Ran^{GTP} and a 1:1 serially diluted wild type or mutant Msn5 from 10 µM, in a 384 well black bottom plate (Corning). For Ran^{GTP} binding affinities, samples contain 20 nM mNeonG-Ran^{GTP} with serial dilution of wild type or mutant Msn5 from 10 µM."

Reviewer #3 (Remarks to the Author):

REVIEWER #3's COMMENTs

The manuscript studied the phosphate-dependent nuclear export mechanism of the transcription factor Pho4, facilitated by the yeast exportin Msn5. The authors resolved multiple Msn5 structures, including the Msn5 apo state and Msn5-Ran-Pho4 complex states, revealing a novel 35-residue NES motif that differs from the canonical NES motif recognized by XPO1. The study highlighted the critical role of phosphorylation at S114 and S128 in regulating the interaction between Pho4 and Msn5, enhancing our understanding of the mechanisms governing nuclear transport and cellular signaling pathways. These structural findings were also corroborated by

biochemical and cellular experiments on mutants. Overall, I believe the innovation of the paper is strong, and the new scientific discovery serves as a valuable addition to the existing nuclear localization mechanism. It can be considered for publication with several modifications.

AUTHORS' RESPONSE

We thank the reviewer for their encouragement and constructive comments.

REVIEWER #3's COMMENT

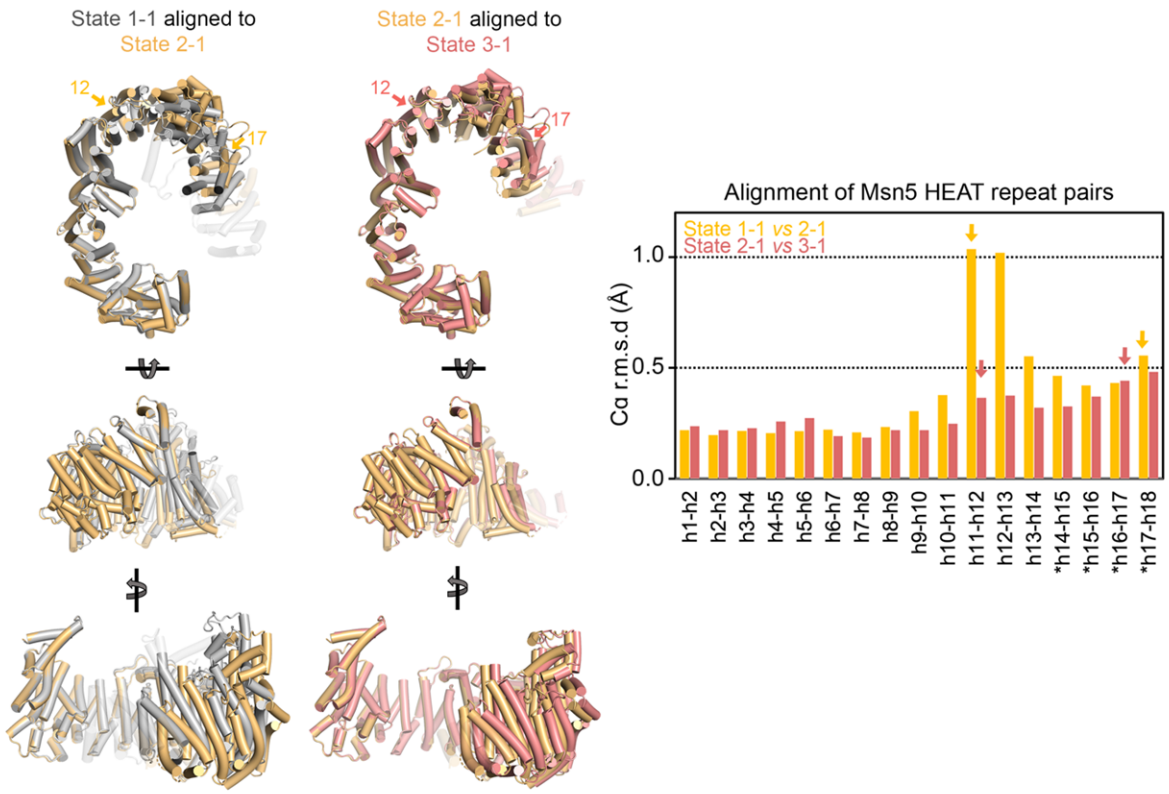
1. Since I cannot find Extended Data Movie 1, the structural variability details of Msn5 remain unclear to me. I find this to be an intriguing point. Given that the structural resolution of Msn5 proteins in most structural states is high enough for modeling, it is recommended to construct their atomic structure models and subsequently compare the different structural states based on these models. This would aid in understanding the complete molecular mechanism of Msn5.

AUTHORS' RESPONSE

We apologize for missing the upload of Extended Data Movie 1. This has now been uploaded with the resubmission as intended.

We have modeled the Msn5 and Ran^{GTP} molecules in the State 2-1 and State 3-1 structures, as suggested by the reviewer. Comparative structural analysis of States 1-1, 2-1 and 3-1 informs on the origins of structural variability that results in the opening/closing of the Msn5 solenoid.

The result of the analysis is presented in the new Supplementary Fig. 5, and the figure legend describes the findings. See below:



Supplementary Fig. 5. Comparative structural analysis of the States 1-1, 2-1 and 3-1 pPho4$_{1-200}$-bound Msn5 solenoids. The left panel shows State 1-1 (gray) Msn5 aligned at repeats h1 and h2 to State 2-1 Msn5 (yellow). The middle panel shows State 2-1 aligned to State 3-1 (coral) in a similar manner. Ran^{GTP} and pPho4 are omitted to focus on conformational changes of the Msn5 solenoid. The right panel shows a histogram of the Cα r.m.s.d values of the indicated HEAT repeat pairs. In the panels showing the aligned Msn5 structures, Msh5 loop regions that were not used for alignment (h15loop 822-847, h17loop 961-978 and h17-h18loop 1002-1019) and residues beyond h18 that are not well-defined in the map due to poor local resolution (affected alignments are indicated by * on the histograms) are drawn transparent. Structural alignments of both State 1-1 with 2-1 and State 2-1 with 3-1 show that the largest conformational differences are concentrated at repeats h11-h13, which is the binding site for NES region 2, and at repeats h17-h18, which produce the most flexible C-terminal region of Msn5 (map features here are the least defined). Conformational differences comparing States 2-1 and 3-1 and those comparing States 1-1 with 2-1 follow the same trend, with the former showing smaller differences than the latter.

We also refer to the results in Supplementary Fig. 5 in the Results section (lines 111-117):
"3D variability analysis revealed conformational variability of the Msn5 solenoid (Supplementary Movie 1), prompting us to split the particles into six classes. The six reconstructions show three states of the Msn5 solenoid that vary in the extent of compactness (Supplementary Fig. 2). We modeled the dominant species of each state, revealing differences in HEAT repeat orientations at repeats h11-13 and h17-20 (Supplementary Fig. 5)."

REVIEWER #3's COMMENT

2. I did not find the data processing flowchart, which is essential. This figure should encompass all classifications for the various structural states.

AUTHORS' RESPONSE

We have added new data processing flowcharts (Supplementary Fig. 2, 4 and 14), which summarize the data processing and include classifications of structural states. More detailed information is described in the methods section as quoted earlier in the response to Reviewer #2 point #9 in pages 10-12.

REVIEWER #3's COMMENT

3. The model-map matching qualities should be provided. In addition to Fig. 2c, for the significant domains or motifs in different structures, particularly in the local regions where side chain conformation or residue distances are discussed, it would be beneficial to display the fitting qualities of the structural model to the density map.

AUTHORS' RESPONSE

We thank the reviewer for this suggestion. Two different views for assessing the map quality of the Msn5 side chains are now in the new Supplementary Fig. 8b, which can be seen in page 13 in this document.

REVIEWER #3's COMMENT

4.I believe many readers will be curious about how similar the structures of the Msn5 monomer and the Msn5-Ran-Pho4 complex are compared to the corresponding structures predicted by AlphaFold2 or AlphaFold3.

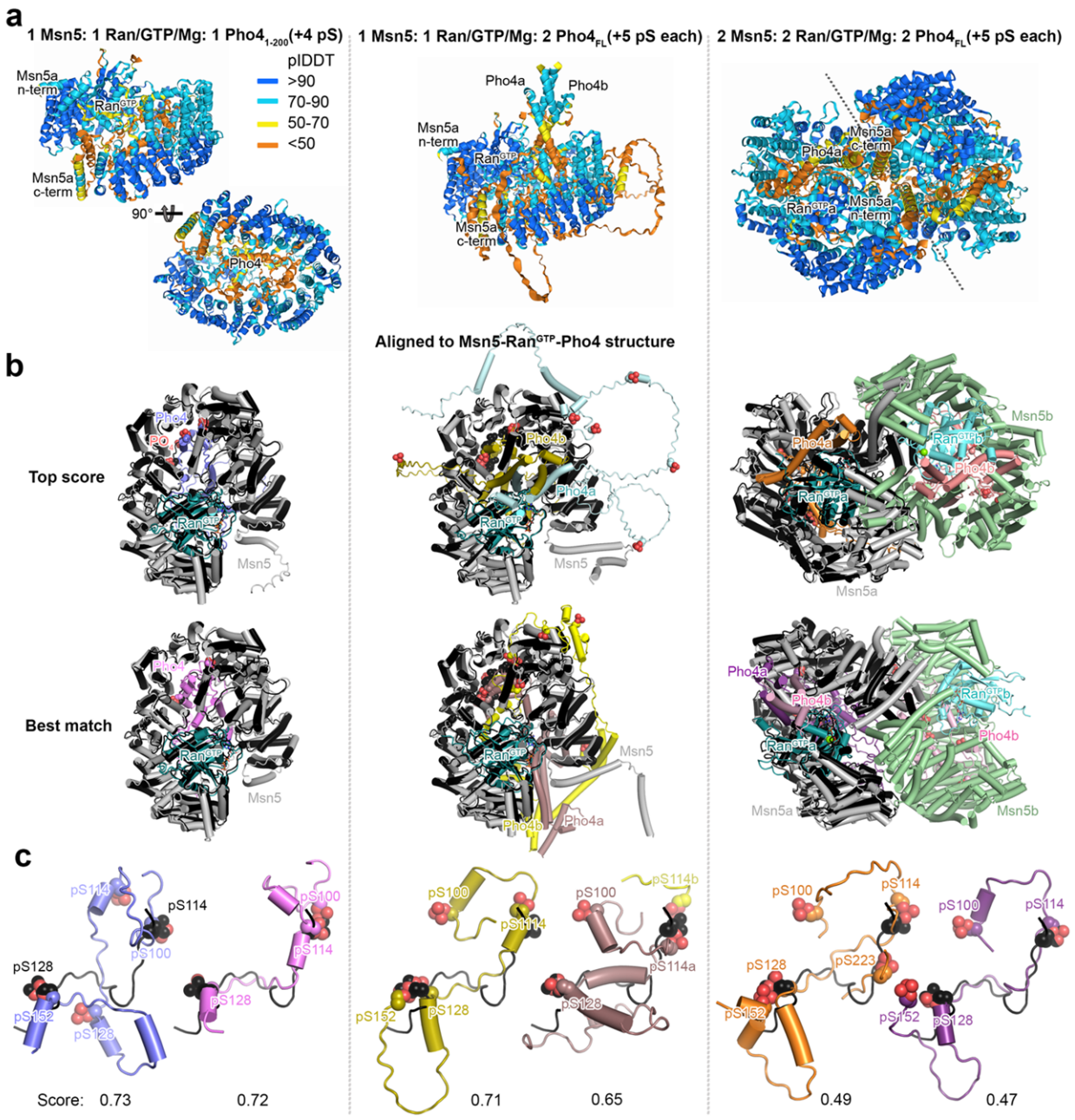
AUTHORS' RESPONSE

We have used the AlphaFold 3 (AF3) web server to predict the structures of all relevant Msn5/Pho4/Ran complexes that we can think of, and of the unliganded Msn5 structure. The results are now briefly mentioned in the main text, and extensively discribed in Supplemental text (pages 3-6 of Supplementary Information), accompanied with new Supplementary Fig. 12 (Msn5/Pho4/Ran complexes) and Fig. 16 (unliganded Msn5). The latter also includes the AF2 database model for comparison with the AF3 model and our cryo-EM structure.

Lines 289-299: "We compared our cryo-EM structure to AlphaFold3 (AF3)[53] models predicted with Msn5, Ran^{GTP} and either $Pho4_{1-200}$ or $Pho4_{FL}$ (detailed analysis in Supplementary text and Fig. 12). AF3 predicted the structure of Ran-bound Msn5 but not the Msn5-Pho4 binding mode observed in our structure. When using $Pho4_{1-200}$, the predictor placed Pho4 pS100 and pS152 rather than pS114 and pS128 into the Msn5 HRY and RR sites, respectively. Some of the predictions using $Pho4_{FL}$ placed the phosphates of pS114 and pS128 correctly, but others placed the wrong phosphoserines at the HRY or RR sites. Interestingly, the top scoring AF3 models fared worse than lower scoring models in placing the correct Pho4 phosphoserines at the Msn5 HRY and RR sites. None of the AF3 models accurately predicted the Pho4 conformation/path spanning NES regions 2 - 4 nor the placement of NES region 1 at the acidic patch of Msn5."

Lines 393-395: "AlphaFold successfully predicted the closed ring structure of Msn5 but failed to predict the conformations of h15loop and h17loop (more on AF analysis in Supplementary text)."

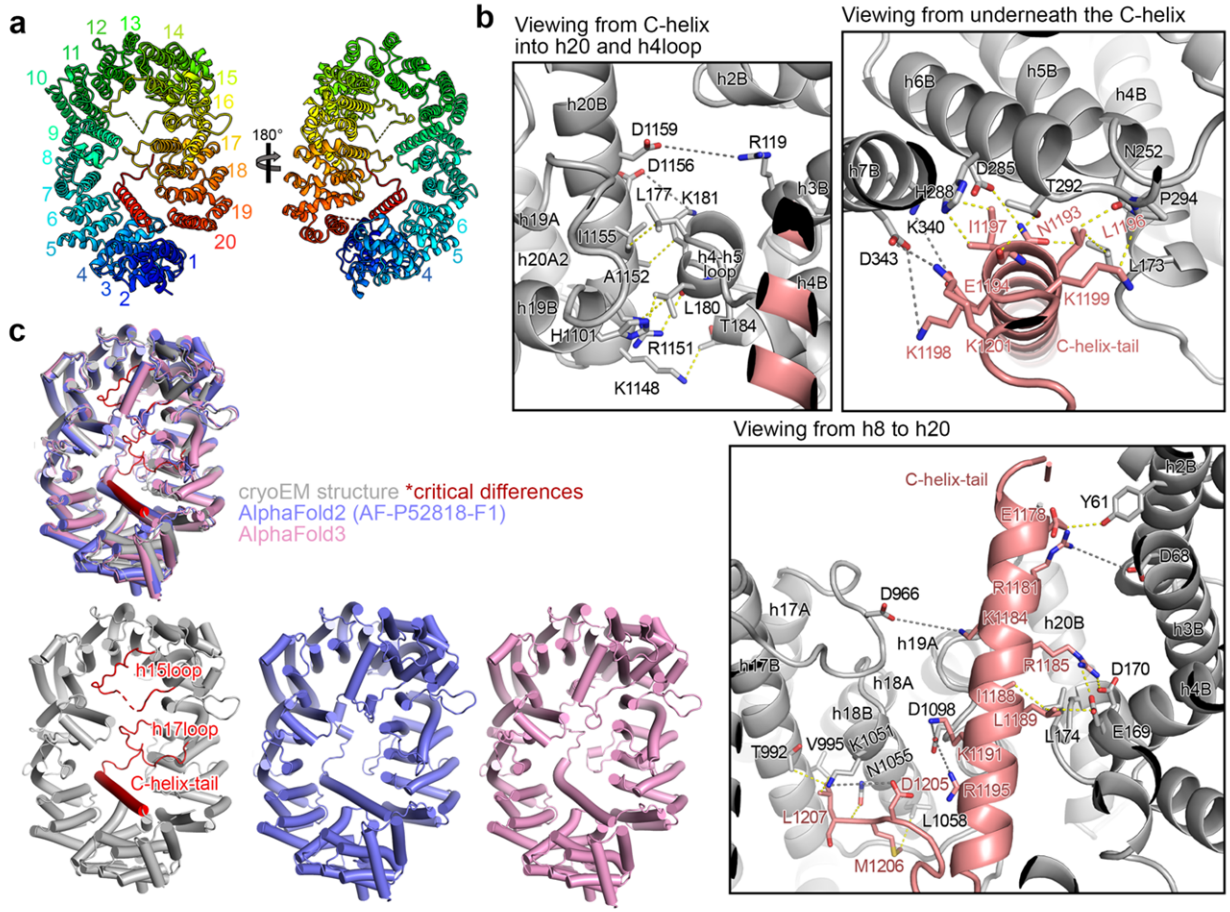
New Supplementary Fig. 12:



Supplementary Fig. 12. AlphaFold3 (AF3) prediction of Pho4-Msn5 complexes. (a) AF3 output of two models, colored by the pIDDT confidence score (higher indicates more confidence), for three inputs of different Pho4-Msn5 complexes. The left panel shows the results of an input of 1 Msn5: 1 Ran (along with one each GTP and Mg): 1 $Pho4_{1\text{-}200}$ (has 4 phospho-serines). The middle panel shows the result for 1 Msn5: 1 Ran/GTP/Mg: 2 $Pho4_{FL}$ (each with 5 phospho-serines). The right panel shows the result for 2 Msn5: 2 Ran/GTP/Mg: 2 $Pho4_{FL}$. All AF3 models predicted Msn5 and Ran^{GTP} with relatively high confidence but Pho4 predictions were of low confidence. **(b)** The Msn5 of AF3 models were aligned to the Msn5 of our Msn5-Ran^{GTP}-$Pho4_{1\text{-}200}$ cryo-EM structure (all three proteins colored black). Phosphoserines of the models (various colors) and cryo-EM structure (black) are drawn as spheres. The top row shows the top scoring

AF3 models while the bottom row shows the models that best match our cryo-EM structure. **(c)** Zoomed-in views of the Pho4 NES of our structure (black) overlayed with the AF3 Pho4 models.

Updated Supplementary Fig. 16:



Supplementary Fig. 16. Unliganded Msn5: autoinhibitory contacts and AlphaFold prediction. (a) The structure of unliganded Msn5 is shown as cartoon, colored and labeled by HEAT repeats in rainbow (N, blue to red, C). **(b)** The autoinhibited conformation of Msn5 appears to be mediated by intramolecular contacts between its N- (h3-h4) and C-terminal HEAT repeats h19-h20 (left panel) and interactions involving the C-helix-tail (salmon), shown in the 2 right panels. Contacts (<4 Å) and long-range electrostatic interactions of <8 Å are shown with yellow and grey dashed lines, respectively. Additional intramolecular interactions not displayed here involve two loops (h15loop and h17loop) that reach across the Msn5 ring to contact repeats h9-h11. **(c)** Top panel: AlphaFold2 (blue) and AlphaFold3 (pink) models of unliganded Msn5 aligned to our cryoEM structure (gray) show overall good agreement. Differences are observed only at the C-helix-tail, h15loop and h17loop (drawn in red in the cryoEM structure). The individual models are shown below.

REVIEWER #3's COMMENT

5.The authors did not mention the structure of the Msn5-Ran complex. Has this been included in the Msn5-Ran-Pho4 dataset? Has there been any effort to resolve this structure? By comparing it to the Msn5 monomer and the Msn5-Ran-Pho4 ternary complex structure, it seems that a more complete understanding of the Msn5-Pho4 interaction process could be elucidated.

AUTHORS' RESPONSE

Msn5 binds Ran^{GTP} alone with substantially lower affinity than in the presence of Pho4. The Msn5-Ran^{GTP} K_D is 300 nM in the absence of Pho4 vs. single digit to sub nanomolar in the presence of Pho4 (Fig. 6b). We are not confident that this complex would survive the grid preparation, and therefore we did not attempt to solve its structure.

Determining the XPO5-Ran^{GTP} structure did not seem straightforward. Yamazawa et al. (Structure, 2018) solved the XPO5-Ran^{GTP} crystal structure by keeping the complex in low to no salt condition throughout purification and crystallization. The complex was stored in buffer containing 20 mM Tris-HCl (pH7.4), 2 mM $MgCl_2$, 2 mM DTT and the crystallization reservoir solution contained 50 mM MES buffer, pH 6.5, 50 mM DTT, 100 mM $MgCl_2$, 2 mM spermine tetrahydrochloride, 6.5-8% PEG3350. We do not believe that our proteins would survive the solution conditions that were favorable for the XPO5-Ran^{GTP} purification and structure determination.

Like all exportins, Msn5 binds Ran^{GTP} and cargo synergistically, with positive cooperativity. Msn5 binds either ligand with low affinity but binding of one stabilizes the Msn5 conformation that binds the other with high affinity. The synergy is of course reciprocal: Msn5 binds Ran with lower affinity when Pho4 is absent, and Msn5 binds Pho4 with lower affinity when Ran^{GTP}. When both are present, they bind with high affinity. We don't know the kinetic pathway of the ternary complex assembly - if Ran^{GTP} binds before Pho4 or *vice versa*, or if they bind simultaneously – but this topic is for a future rigorous biophysical study. The focus of our manuscript is first to understand how Msn5 recognizes Pho4 and what the Pho4 NES is. Secondary to that is comparing the Ran^{GTP} and Pho4 bound nuclear export complex that we expect will form in the nucleus and translocate through the NPC, with the unliganded Msn5 that is expected after dissociation of both ligands upon GTP hydrolysis in the cytoplasm, to get at the basis of positive cooperativity of the two ligands binding to Msn5.

Future structural studies to understand how Msn5-Ran^{GTP} binds RanBP1 will be needed to examine the mechanism for cargo release by RanBP1 that we proposed in this study, and to complete our understanding of the entire allosteric cycle of Msn5 function. These studies are beyond the scope of our current work which is prioritizes cargo recognition.

REVIEWER #3's COMMENT

6.The authors note that the Msn5 protein itself can form an autoinhibited state and cannot bind to Pho4. It is only upon binding to Ran that the Msn5 protein can interact with Pho4. I think the structural basis for this process is crucial. However, the authors primarily focus on analyzing the Ran binding site, rather than the Pho4 binding sites. I believe that a more in-depth investigation of the differences at the Pho4 binding site in these structures is required to clarify the regulatory mechanism by which Msn5 interacts with Pho4.

AUTHORS' RESPONSE

The reviewer's suggestion for a more in-depth comparative analysis of the Pho4 binding site in the structures is an excellent one. We have expanded on our analysis and elaborated more on the ligand binding sites in lines 396-412:

"The Ran^{GTP}-binding site at the N-terminal h1-h4 repeats of the Msn5-Ran^{GTP}-$pPho4_{1-200}$ structure is occupied by HEAT repeats h17-h20 and the C-helix-tail in the unliganded Msn5 structure (Fig. 6a). Both h15loop and h17loop also contact Ran^{GTP} in the former (Fig. 1a and Supplementary Fig.

7b), and they undergo conformational changes when Msn5 is unliganded. These structural differences suggest that large conformational changes accompany Ran^{GTP} binding.
The NES binding site (h8-h18) of unliganded Msn5 is substantially more compact, occluding the groove at h12-h15 that binds region 2 and 3 of the Pho4 NES (Supplementary Fig. 17c). The same Msn5 region is also the hinge for conformational changes that result in the multiple states of Pho4-bound Msn5, some of which do not interact persistently with the Pho4 NES region 2 (Supplementary Fig. 5 and 6). Interestingly, the dominant phospho-serine binding Msn5 RR site appears fully accessible in unliganded Msn5, potentially allowing initial partial binding of the Pho4 NES (Supplementary Fig. 17c). These structural differences suggest that the unliganded conformation is incompatible with high affinity Pho4 NES binding which requires engagement of both phosphoserines in NES regions 2 and 4. Unliganded Msn5 is thus autoinhibited."

REVIEWER #3's COMMENT

7.The authors mention that Msn5 recognizes a specific NES motif on the Pho4 protein. This unique NES sequence and recognition mechanism significantly differ from those of common NESs. This is very interesting; however, there is a lack of in-depth discussion. What important biological implications might arise from this unique NES sequence and recognition mechanism during the evolutionary process? A thorough discussion on this would enhance the depth of the research presented in this study.

AUTHORS' RESPONSE

This is also an excellent suggestion. We added three paragraphs of "Discussion" to that Results and Discussion section. They are lines 357-383:

"The long Pho4 NES, with its complex and vague sequence features that seem required for binding Msn5 and distribution of energetic interactions across several segments, bears similarities to the proline-tyrosine (PY) NLSs, which are recognized by the importin TNPO1/Kapβ2[71,72]. Like the PY-NLS, which uses 3-4 NLS epitopes to interact with a large, open and expansive concave surface of its karyopherin, the Pho4 NES exhibits a more diffuse mode of karyopherin-signal recognition. This contrasts with the more compact and energy-concentrated interaction seen with monopartite classical NLSs[28,73] and cNESs.

In addition to these structural features, the mechanism by which phosphorylated cargoes are recognized by their karyopherins remains poorly understood. The only other nuclear targeting signal known to be phosphorylated for karyopherin engagement is the arginine-serine (RS) NLS, which is recognized by the importin TNPO3/Transportin-SR[74-76]. Interestingly, some RS-NLSs do not require phosphorylation, as they contain phosphomimic sequences, such as the RS-like domain in CPSF6 protein[77]. RS-NLSs are also complex, typically binding TNPO3 through both an IDR or linear element containing RpS repeats and a folded, globular RNA-recognition motif (RRM) domain[75,77]. It remains unclear whether other Msn5 cargoes rely solely on their Pho4-like NESs for Msn5 binding or if the NES can function in conjunction with protein or RNA domains.

The different complexity of karyopherin recognition may also reflect the distinct cellular roles for different nuclear export pathways. For instance, the Pho4 NES and the classical NES may have evolved to export cargoes that are regulated in distinct ways in the cell. The narrowly defined affinity range for active nuclear export of Msn5 cargoes suggests that phosphorylation of either of the two critical sites within the NES tightly controls Pho4 export by Msn5. In contrast to the broad, three orders of magnitude for XPO1-cNES active nuclear export, the Msn5 system may be better suited for more precise and rapid regulation of nuclear-cytoplasmic localization in response to environmental signals."

REVIEWER #3's COMMENT

8.This study presents a substantial amount of experimental results but lacks a systematic summary. After a more comprehensive and in-depth analysis of the various structural models in this work, it is recommended that the author include a flowchart illustrating all the structural changes involved in Msn5's recognition of the Pho4 protein. This should clearly convey information in a well-organized and logical manner, encompassing self-inhibition, phosphorylation, conformational changes, Ran regulation, and more.

AUTHORS' RESPONSE

We added a schematic cartoon to illustrate the conformational changes involved in the nuclear export of Pho4 by Msn5 in Fig. 6f, as shown in page 24 in this document.

Reviewer #2 (Remarks to the Author):

Reviewer 2 is happy with the authors response to the review. They have improved the paper which is a significant advance to the field and also carefully documented. The paper should be accepted for publication.

Two minor points:

Minor point #1

Comment on: [[We have revised the paragraph to make it clear that NESs refer to linear sequence elements that are most often found in IDRs. The new lines 63-65 now reads: "Exportins are thought to recognize their protein cargoes by binding to linear sequence elements known as nuclear export signals (NESs) that occur within intrinsically disordered regions (IDRs) of cargoes or to the folded domains of cargoes4,28,29."]].

I believe it is more prudent to write that NESs ["often"- single word addition to sentence] occur within IDRs… The authors discuss that there are also folded NESs ["or to the folded domains of cargoes "] which presumably are unlikely to be within IDRs.

Authors' Response:

The word "often" has been added to that sentence in the now line 69.

Reviewer's minor point #2:

In supplemental the Sup Table 2 goes to Sup Table 4 and skips Sup Table 3. Confusion here. Sup Table 3 is on a separate file. Perhaps authors should stick in Sup Table 3 here in addition to having separate file or insert a sentence saying Sup Table 3 is a separate file.

Authors' Response:

Supplementary Table 3 is now in Source Data to comply with the journal's formatting requirements, which resolves the problem.

Reviewer's minor point #3:

Supplementary Table 4 has typos. In the third column next to Ste5 the sequence of ref 9 appears to be jumbled up with incorrect numbering. Mig1 which appears alphabetically later in the table has ref 5 which suggests the reference order has to be corrected.

Authors' Response:

We have reformatted Supplementary Table 4 as an excel spreadsheet where the references for all the putative cargoes are in a separate column. We also caught an error in the Mig1 footnote, which we have corrected in the new Supplementary Data file.