

Supplementary Note 1. Further analysis of Pho4 density/map features in additional cryoEM maps in this study.

The Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ complex. We divided Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ particles into 3 groups named State 1, State 2 and State 3 according to the different extent of compact to open curvatures of their Msn5 solenoids. The Msn5 in the State 1 is most compact and State 3 most open. Each state is further divided into two populations (e.g., State 1-1 and State 1-2) that have small differences in Msn5 solenoid conformations (Supplementary Fig. 2). Cryo-EM map features for Pho4 NES can be observed to varying degrees in all six cryo-EM maps generated from the six group of particles (Supplementary Fig. 6b-d). In the main text, we focused on describing the most compact State 1-1 map of Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ and its resulting structural model. Here, we analyze the differences in the additional maps that show the multiple conformations and elaborate on the structural flexibility of Msn5.

In the State 1-1 map of Msn5-Ran^{GTP}-pPho4₁₋₂₀₀, which has the most compact Msn5 conformation, Pho4 NES density is continuous and well-defined cross NES regions 2 to 4. In the map of the slightly less compact State 1-2, the density for the pPho4 NES region 2 is also continuous but not as well-defined as in the State 1-1 map (Supplementary Fig. 6b). In the States 2-1, 2-2, 3-1 and 3-2 maps of Msn5-Ran^{GTP}-pPho4₁₋₂₀₀, Pho4 densities are no longer continuous. The best-defined features of Pho4 in the States 1-1 and 1-2 maps are at Pho4 NES region 4 (¹²⁸pSPNL¹³¹), which is adjacent to Msn5 repeats h8-h10 containing the Msn5 RR site (Supplementary Fig. 6c and d). Interestingly, the features at this location in the State 2-1, 2-2, 3-1 and 3-2 maps seem to match the sequence ¹¹³ApSPLA¹¹⁷ better than ¹²⁸pSPNL¹³¹ (Supplementary Fig. 19a). The features of these maps are too ambiguous to confidently model Pho4, but they raise the possibility that the Msn5 RR site could possibly also engage pS114. Regardless, the maps suggest that a phosphoserine containing segment of the Pho4 NES likely always occupies the Msn5 RR site, consistent with mutagenesis data suggesting that Msn5 RR is the more important phosphoserine binding site (Fig. 3b).

Several maps also contain extra density sandwiched between Ran^{GTP} and the h6-h8 repeats of Msn5 (Supplementary Fig. 6). Alanine mutations of Msn5 residues facing the extra density in h6-h8, D285, H288 and D343, did not affect binding to Ran^{GTP} or to pPho4₃₋₂₀₀ (Supplementary Fig. 19b). It is possible that this density is the NP-40 detergent.

The Msn5-Ran^{GTP}-pPho4_{FL} complex. Like the Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ particles, the Msn5- Ran^{GTP}-pPho4_{FL} particles also displayed flexibility. Two Msn5- Ran^{GTP}-pPho4_{FL} reconstructions were obtained, which matched the Msn5 solenoid shapes of the States 1 and 3 maps obtained from the pPho4₁₋₂₀₀ dataset (Supplementary Fig. 4). The resulting map features for Pho4 NES are continuous in the State 1 map (most compact Msn5) of pPho4_{FL}-bound Msn5 but not continuous in the State 3 map (most open Msn5 conformation; Supplementary Fig. 6e). The State 3 map has features for Pho4 NES regions 1 and 4 but not for regions 2 and 3. Interestingly, NES region 1 of pPho4_{FL} appears persistently bound to Msn5 even though the same region of pPho4₁₋₂₀₀ is not observed bound to Msn5 (Supplementary Fig. 6). It is possible that additional pPho4_{FL} residues that are not present in the shorter pPho4₁₋₂₀₀ construct may engage Msn5 to produce more persistent interaction between the NES region 1 of pPho4_{FL} with Msn5. Alternatively, additional residues in pPho4_{FL} residues also include a putative oligomerization domain at residues 201-300, which may facilitate Pho4 dimerization/oligomerization, influencing Pho4 NES accessibility and Msn5 binding. The 2-site binding curve of mNeonG-pPho4_{FL} for Msn5 suggests that when pPho4 dimerizes, one pPho4 subunit is less accessible to Msn5 when the other subunit is already bound to Msn5 (Supplementary Fig. 1d). These results are consistent with AlphaFold prediction suggesting that the model that best match our Pho4 NES cryoEM structure is an asymmetric dimer of Pho4 (see more in last section below; Supplementary Fig. 13).

Supplementary Note 2. Interactions of pPho4 S114A and S128A with Msn5 HRY_{AAA} and RR_{AA} mutants

We combined Pho4 and Msn5 mutations to validate the pairing of Pho4 pS114 binding at Msn5 HRY and pS128 at Msn5 RR. Consistent with the former pair, the affinity loss of pPho4 binding to Msn5 HRY_{AAA} (vs WT) is not exacerbated by additionally mutating S114 (K_D to Msn5 HRY_{AAA} = 300[170,520] nM for S114_A vs. 230[160,350] nM for WT; Supplementary Fig. 9d). The same is observed for the latter pair (K_D to Msn5 RR_{AA} = 2.8[2.4,3.1] μ M for S128_A vs. 2.2[1.0,6.1] μ M for WT; Supplementary Fig. 9e). In contrast, mutations involving mis paired sites, pPho4 S114_A binding Msn5 RR_{AA} or pPho4 S128_A binding Msn5 HRY_{AAA}, showed additional affinity decreases (Supplementary Fig. 9d and e). In summary, mutagenic analysis is consistent with the cryo-EM structure which shows Pho4 pS114 binding at the Msn5 HRY site, and pS128 binding at the Msn5 RR site.

Supplementary Note 3. Comparison of Msn5-Ran^{GTP}-pPho4 cryo-EM structure with AlphaFold 3 models

The new AlphaFold 3 (AF3) structure predictor allows for ligands and post-translational modifications¹, and we used the web server to predict the structure of Msn5 bound to Ran^{GTP} and pPho4 for comparison with our cryo-EM structure. We first predicted the monomeric complex that we used for cryo-EM structure determination – a complex containing 1 Msn5: 1 Ran (along with one each GTP and Mg): 1 Pho4₁₋₂₀₀ (has 4 phospho-serines) (left column, Supplementary Fig. 13). We also predicted the structure of a complex containing 1 Msn5: 1 Ran/GTP/Mg: 2 Pho4_{FL} (each with 5 phospho-serines) as Pho4_{FL} is expected to form a dimer; we refer to this assembly as the sub-stoichiometric dimer complex (middle column, Supplementary Fig. 13). To allow both Pho4 NESs of the Pho4_{FL} dimer to bind Msn5, we also predicted the stoichiometric 2 Msn5: 2 Ran/GTP/Mg: 2 Pho4_{FL} dimeric complex (right column, Supplementary Fig. 13). We examined all five AF3 output models from each of the three predictions. The scores of the five models are very similar: 0.72-0.73 for the monomeric complex, 0.63-0.71 for the sub-stoichiometric dimer and 0.47-0.49 for the stoichiometric dimer complex.

Prediction of the individual proteins. The structure of Ran^{GTP} is accurately predicted with high confidence. The Msn5 solenoids of all the AF3 predictions align somewhat well with that of the cryo-EM structure, but they all differ in compactness and none of the prediction is spot-on. The difficulty in accurately predicting HEAT repeat orientations of the Msn5 solenoid is not surprising as cryoEM data suggests that it is highly flexible (Supplementary Fig. 13b). AF3 predicted the Msn5-bound Pho4₁₋₂₀₀ to have multiple helices of various lengths, quite different from the AF and PSIPRED predictions of Pho4 AF alone, which found Pho4 to be largely intrinsically disordered. AF3 prediction of Msn5-bound Pho4_{FL} found Pho4 dimerization via its bHLH domain (residues 259-312; moderately high confidence) in all sub-stoichiometric dimer complex models (Fig. 1a, Supplementary Fig. 13a, middle). None of the AF3 models show the

oligomerization region of Pho4 participating in dimerization, bringing into doubt its previously assigned role in oligomerization.

Prediction of Msn5-Ran^{GTP} interactions. AF3 correctly predicted interactions between Ran^{GTP} with the N-terminal HEAT repeats, h9loop, h15loop and h17B helix of Msn5. (Supplementary Fig. 7b and 13a). The critical h17loop coil to helix transition, at Msn5 residues 960-979 upon Ran^{GTP} binding (Fig. 6d), was correctly predicted in all AF3 models. This transition produced a longer straight h17A helix in the cryoEM structure (Fig. 6a and d), which is predicted in only two of the five models for the monomeric and sub-stoichiometric dimer complexes, and not at all for the stoichiometric dimer complex. The other three models predicted a kinked h17A helix rather than the straight unkinked one in our structure. The kinked h17A helix in these AF3 models places the h17loop to make extra contacts with the Switch 1 region of Ran^{GTP} switch I loop that we did not see in the cryoEM structure.

Prediction of Msn5-Pho4 interactions. Most if not all the Pho4 residues in the IDR (residues 1-259) in the AF3 models are placed snugly onto the concave side of the Msn5 solenoids (Supplementary Fig. 13b). 1) Monomeric complex with truncated Pho4₁₋₂₀₀: Only residues 33-65 extend beyond of the Msn5 solenoid as a coil in two of five models (Supplementary Fig. 13b, left). 2) Sub-stoichiometric Pho4 dimer complex: In general, one subunit of the Pho4 dimer is placed at the concave Msn5 surface while the other Pho4 subunit is placed against both the concave and convex surfaces of Msn5 (Supplementary Fig. 13b, middle). The only exception is the best scoring model, which predicted one subunit of Pho4 mostly not contacting Msn5 (light cyan in Supplementary Fig. 13b, middle top). 3) Stoichiometric dimer complex: Most AF3 models show Msn5, Ran^{GTP} and Pho4 arranged symmetrically through intermolecular Msn5-Msn5 and Msn5-Pho4 contacts. Interestingly, the two Pho4 subunits do not interact to form dimers (Supplementary Fig. 13b, right top), except for the lowest scoring model where a Pho4 dimerization domain is positioned asymmetrically relative to the Msn5-Ran dimer (pink and purple in “Best match” model,

Supplementary Fig. 13b, right bottom). Except for the dimerized bHLH domain and the preceding linker regions, the rest of Pho4 (residues 1-230) make the same Pho4-Msn5 contacts in both subunits of the dimeric complex.

Prediction of phospho-serine binding sites. The AF3 predictions generally failed to place Pho4 pS114 and pS128 into the Msn5 HRY and RR sites. 1) Monomeric complex: Different pairs of phosphates, such as pS100/pS152 or pS128/pS152, are placed into the Msn5 HRY and RR sites instead. The correct pairings of Pho4 pS114/Msn5 HRY and Pho4 pS128/Msn5 RR are predicted in only one of the lower scoring models (“Best match” in Supplementary Fig. 13b and c, left). 2) Sub-stoichiometric dimer complex: Both the top scoring/best match model correctly placed pS114 with HRY and pS128 with RR. However, the remaining phosphoserines pS100 and pS152 as well as pS114 and pS128 from the other Pho4 subunit are also placed nearby. For example, pS152 was placed near the RR site in the top score model; pS114 from another Pho4 subunit placed near the HRY site in the best match model (Supplementary Fig. 13c, middle). The other AF3 models failed to place pS114 and pS128 at HRY and RR, respectively, or they placed did not place any phosphoserine into the Msn5 two sites. 3) Stoichiometric dimer complex: Predictions using this complex performed better than the sub-stoichiometric dimer complex, consistently placing at least two phosphoserines into the HRY and RR pockets. Two of the five models (top score and best match models) placed the correct phosphoserines in the respective Msn5 sites but additional phosphoserines are also placed nearby (Supplementary Fig. 13c, right). The remaining models incorrectly paired phosphoserines with Msn5 HRY/RR, such as pS114/pS152-HRY/RR and pS100/pS128-HRY/RR.

Prediction of the bound NES conformation. In the best-matching models of all three complexes, the 90° turns between NES region 2 and 3 and NES region 3 and 4 are somewhat predicted, but the peptide paths predicted differed from the Pho4 path in the cryoEM structure. This is also not entirely surprising as our cryoEM structure is likely of the NES conformation that

is most stably bound to the flexible Msn5 solenoid. Furthermore, the conformation of NES region 1 seen in the Msn5-Ran^{GTP}-pPho4_{FL} map is not predicted in any of the Pho4 FL models.

Supplementary Note 4. Comparison of the unliganded Msn5 structure with AF2 and AF3 models

We compared the structure of unliganded Msn5 with the AF2 model (AF-P52828-F1) and the AF3 model (Supplementary Fig. 16c). Overall, the predictions align with the solved structure. The autoinhibitory C-terminal helix was consistently predicted to interact similarly with the N-terminal HEAT-repeats like in the cryo-EM structure. The C-terminal tail extension takes on a different path, possibly reflecting the flexibility of the tail. Interestingly, the AF models are unable to predict the correct conformations of h15loop and h17loop. The AF h15loops adopt the conformation of the Pho4- and Ran^{GTP}-bound Msn5. Similarly, the unliganded Msn5 AF3 h17loops take on the extended h17 helix conformation, like in the cargo-bound structure,

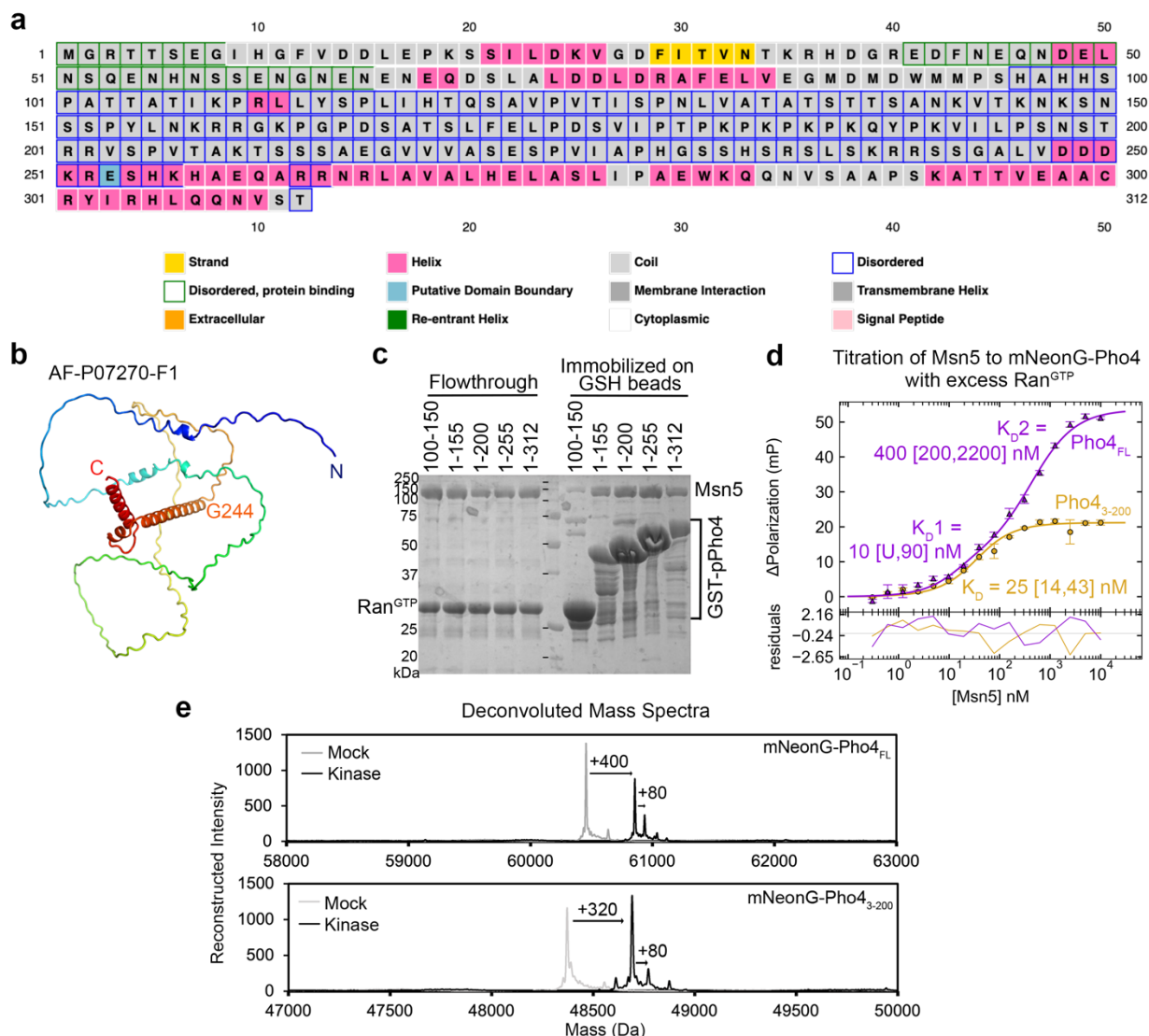
Supplementary Table 1. Cryo-EM data and map statistics of additional maps of pPho4 complexes.

Complex	Msn5-Ran ^{GTP} -pPho4 ₁₋₂₀₀		
State	1-2 (EMD-46557)	2-2 (EMD-46560)	3-2 (EMD-46556)
Magnification	105,000		
Voltage	300 kV		
Electron exposure	50 e ⁻ /Å ²		
Defocus range	-0.8 to -2.5 μm		
Pixel size	0.83 Å		
Symmetry imposed	C1		
Initial particle images (after initial clean-up)	1,574,329		
Final particle images	93,291	85,048	121,812
Map resolution (Å) ²	3.37	3.33	3.23
FSC Threshold	0.143	0.143	0.143
Map resolution range (Å) ²	2.88-6.48	2.87-6.49	2.78-6.64
B factor (Å ²)	92.2	81.8	100.2
Complex	Msn5-Ran ^{GTP} -pPho4 _{FL}		
State	1 (EMD-46562)	3 (EMD-46561)	
Magnification	81,000		
Voltage	300 kV		
Electron exposure	50 e ⁻ /Å ²		
Defocus range	-0.8 to -2.5 μm		
Pixel size	1.03 Å		
Symmetry imposed	C1		
Initial particle images (after initial clean-up)	1,104,150		
Final particle images	108,248	111,752	
Map resolution (Å) ²	4.91	5.12	
FSC Threshold	0.143	0.143	
Map resolution range (Å) ²	4.43-11.07	4.48-11.77	
B factor (Å ²)	325.9	387.4	

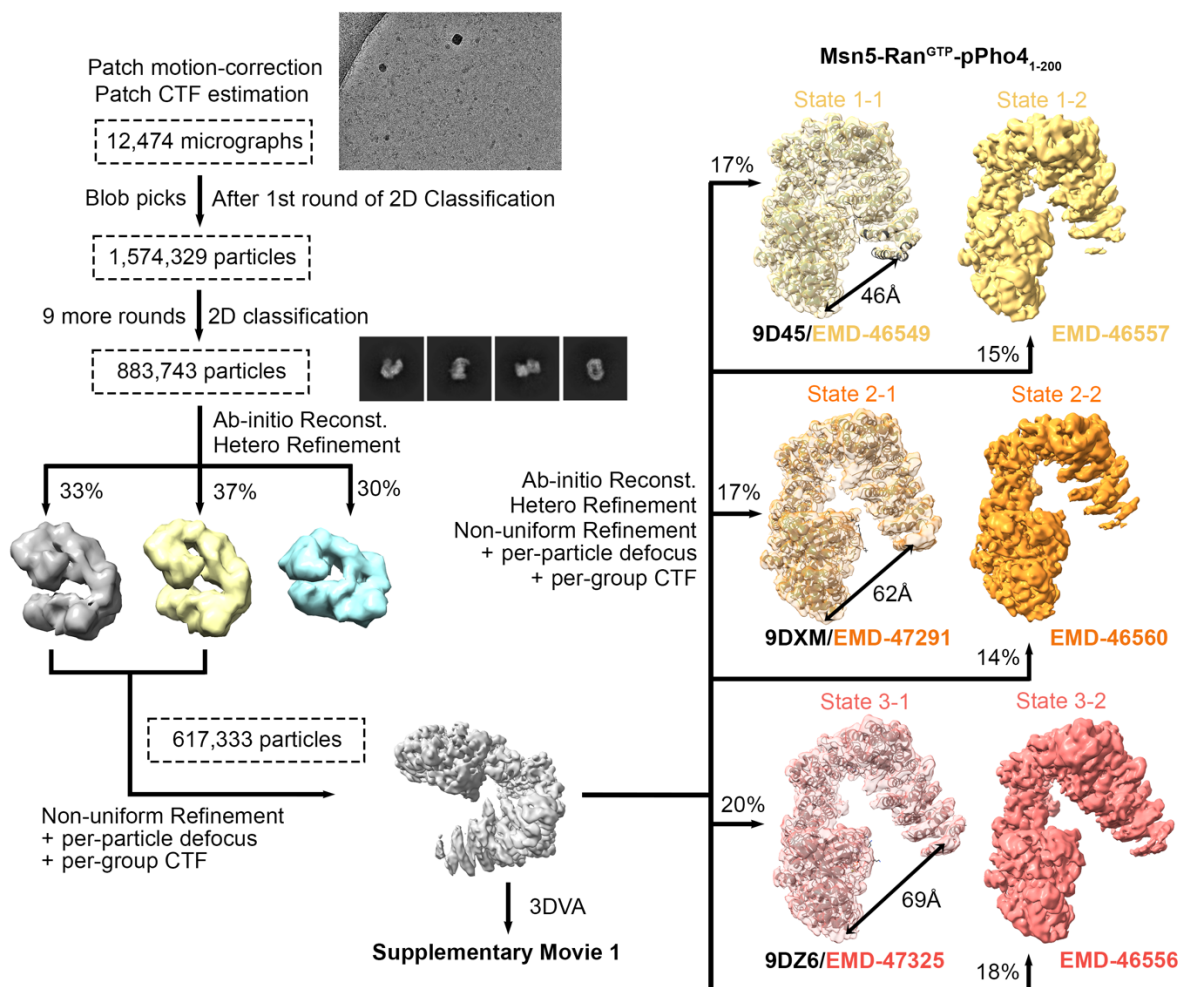
Supplementary Table 2. Binding affinities measured by fluorescence polarization.

Titrant	mNeonG-fusion	Excess	K_D [95% CI] nM; U=undetermined
<i>Msn5</i>	<i>pPho4_{FL}</i>		
WT*	WT	Ran ^{GTP}	1: 10 [U, 90]; 2: 400 [200,2200]
<i>Msn5</i>	<i>pPho4₃₋₂₀₀</i>		
WT*	WT	Ran ^{GTP}	25 [14,43]
WT	WT	Ran ^{GTP}	20 [12,31]; 44[26,71]; 23 [6,52]
HR _Y _{AAA}	WT	Ran ^{GTP}	230 [160,350]
RR _{AA}	WT	Ran ^{GTP}	2200 [1000, 6100]
DHD _{AAA}	WT	Ran ^{GTP}	38 [25,62]
WT	102-107 GGSGGS	Ran ^{GTP}	18 [14,22]
WT	102-107 GKKGKK	Ran ^{GTP}	110[70,150]
WT	R2 _{mut}	Ran ^{GTP}	800 [500,1200]
WT	R3 _{mut}	Ran ^{GTP}	280 [190,400]
WT	R4 _{mut}	Ran ^{GTP}	150 [90,260]
WT	S114A	Ran ^{GTP}	250 [170,370]
HR _Y _{AAA}	S114A	Ran ^{GTP}	330 [170,520]
RR _{AA}	S114A	Ran ^{GTP}	17000 [15000, 20000]
WT	S128A	Ran ^{GTP}	120 [90,160]
HR _Y _{AAA}	S128A	Ran ^{GTP}	510 [410,630]
RR _{AA}	S128A	Ran ^{GTP}	2800 [2400, 3100]
WT	S114A S128A	Ran ^{GTP}	1000 [700, 1400]
HR _Y _{AAA}	S114A S128A	Ran ^{GTP}	1900 [1200,2800]
RR _{AA}	S114A S128A	Ran ^{GTP}	8100 [6500,10200]
WT	S100A S152A	Ran ^{GTP}	46 [38,56]
<i>Msn5</i>	<i>Pho4₃₋₂₀₀</i>		
WT	WT	Ran ^{GTP}	2400 [1900, 3200]
WT	S114D S128D	Ran ^{GTP}	2900 [2600, 3200]
WT	S114E S128E	Ran ^{GTP}	3300 [2800, 4000]
<i>Msn5</i>	<i>Ran^{GTP}</i>		
WT*	WT	-	300 [190,450]
WT*	WT	pPho4 ₁₋₂₀₀	0.2 [U, 1.6]
ΔC-helix-tail*	WT	-	3.3 [1.4, 6.3]
WT	WT	-	140 [110, 170]
HR _Y _{AAA}	WT	-	100 [60,150]
RR _{AA}	WT	-	150 [110,210]
DHD _{AAA}	WT	-	180 [150, 210]

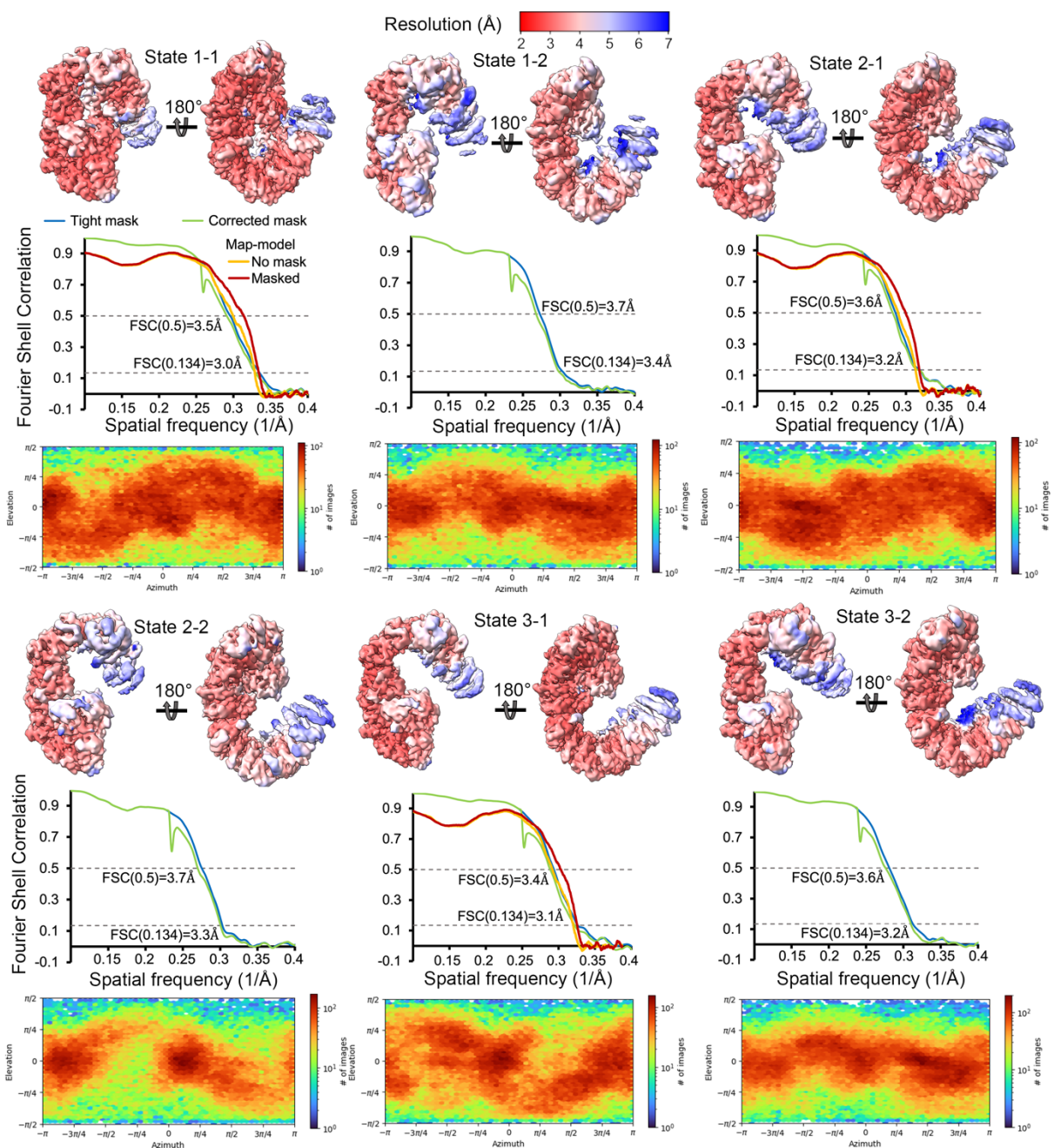
*N-term His tag removed, the rest of the Msn5 are from C-term His tag construct with tag removed.



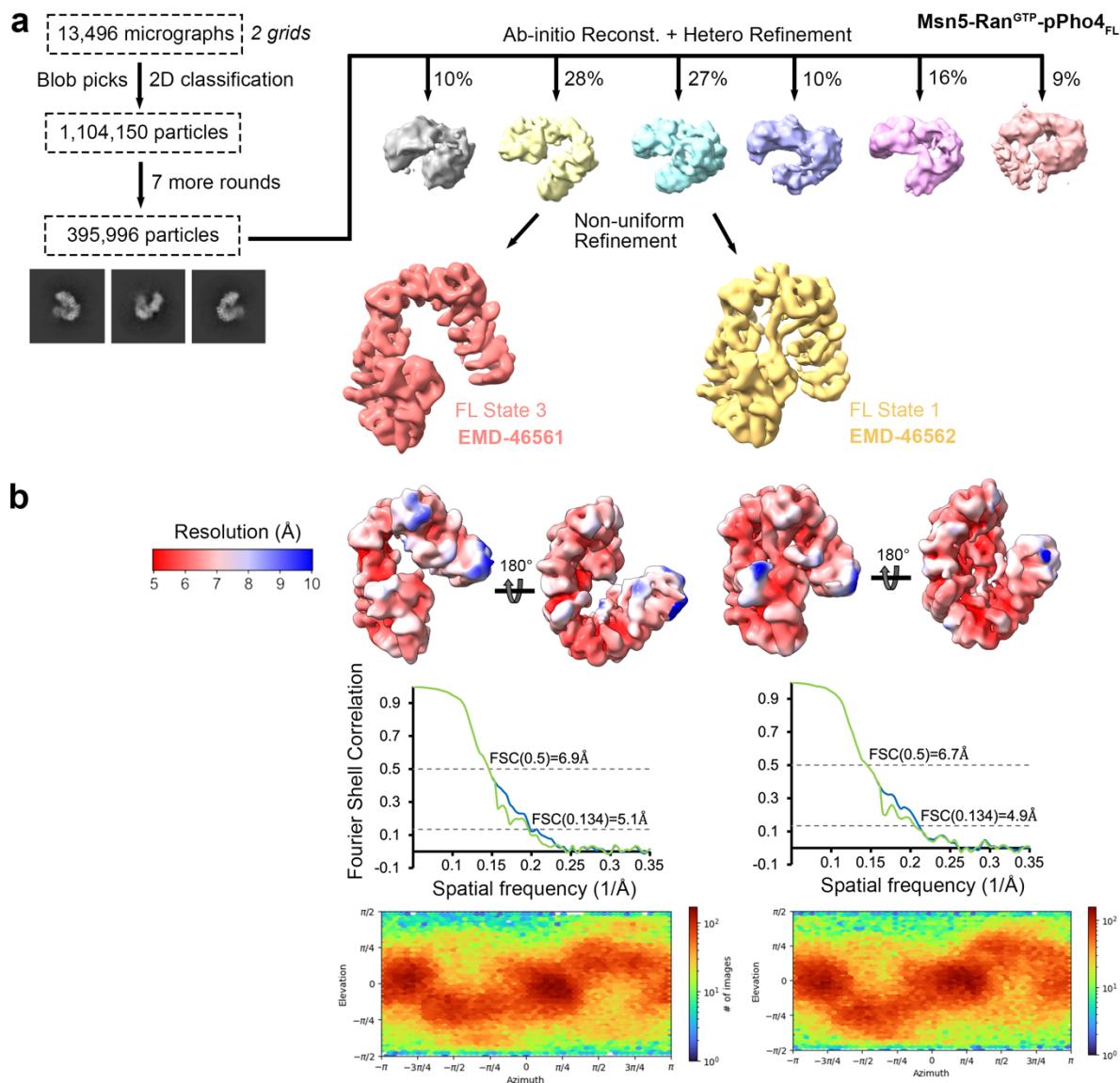
Supplementary Figure 1. Biochemical analysis of Pho4 binding to Msn5. **a** Secondary structure prediction of Pho4 by PSIPRED. **b** The AlphaFold model of Pho4 (rainbow, blue to red from N- to C-terminus). Both **a** and **b** predict that residues 1-243 to be intrinsically disordered. **c** GST-Pho4 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 bound proteins were visualized by SDS-PAGE and Coomassie staining. Image from the only experiment is shown. **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 n=3 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 Pho4₃₋₂₀₀ 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-Pho4₃₋₂₀₀ has 4 phosphates. Source data for **c-d** are provided as a Source Data file.



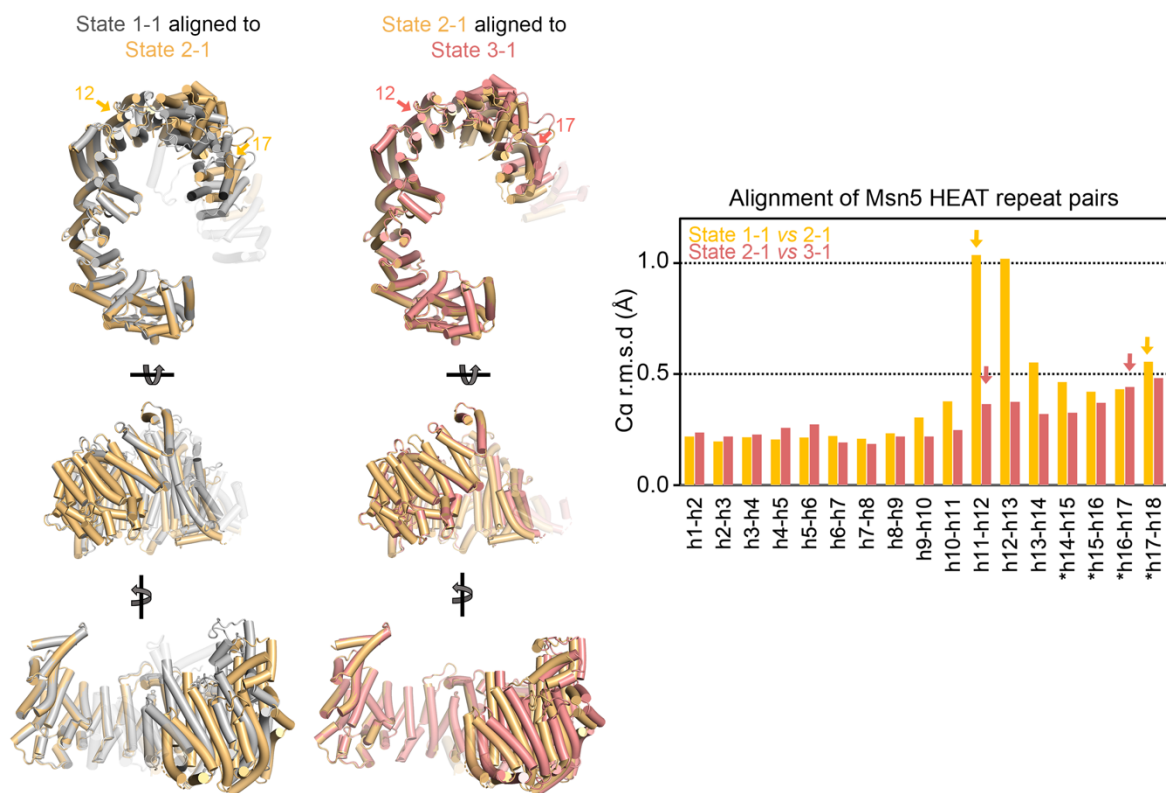
Supplementary Figure 2. Summary of data processing for Msn5-Ran^{GTP}-pPho4₁₋₂₀₀. Details of data processing are described in methods. In brief, particles from the Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ cryo-EM dataset (representative micrograph shown) were picked by blob picker and 2D classified; representative 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 1 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.



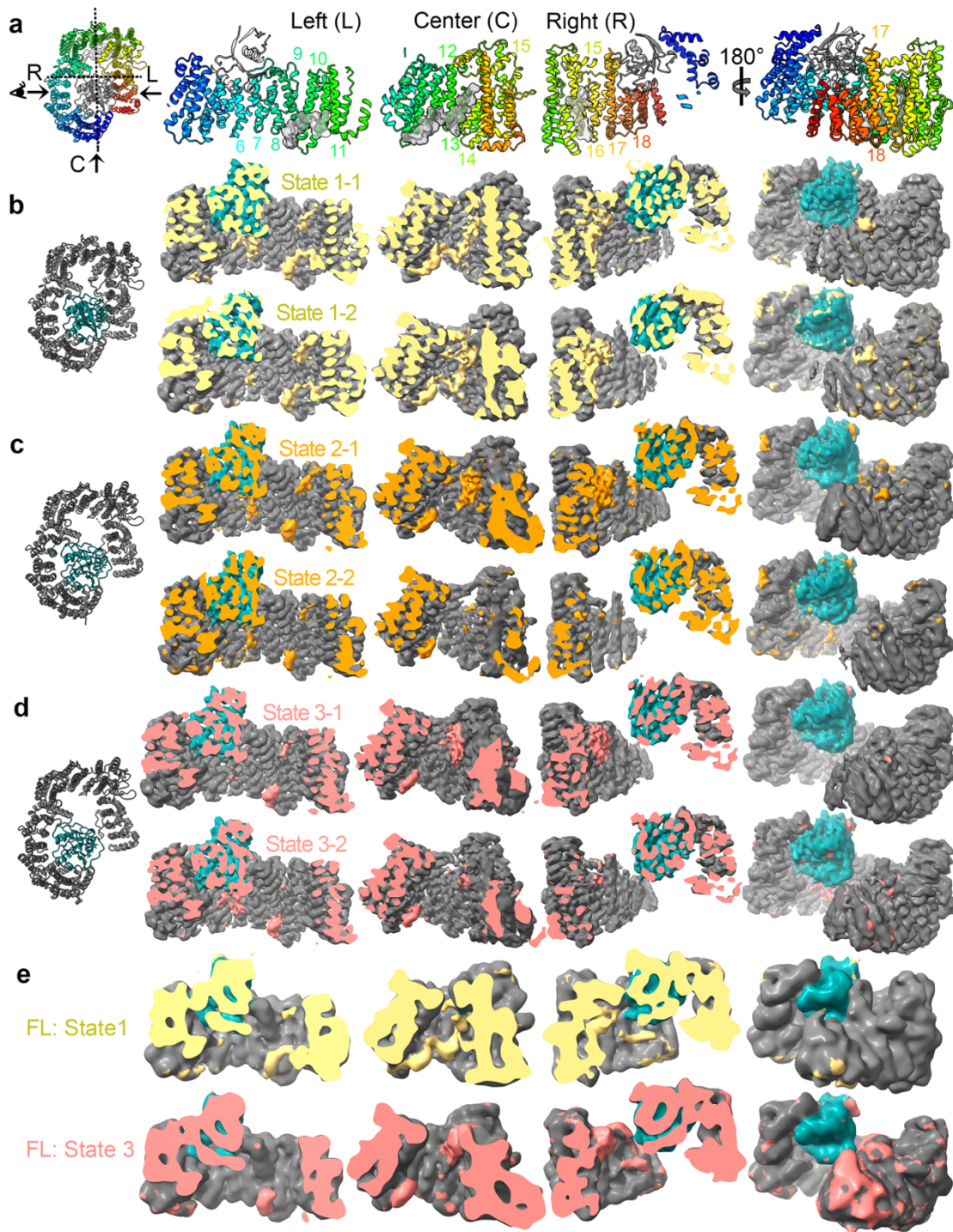
Supplementary Figure 3. Local resolution and FSC curves for Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ maps. CryoSPARC FSC output is shown with tight mask in blue and corrected mask in green. Map-model FSC output from PHENIX is plotted for the maps with associated PDBs, yellow without a mask and red when masked. Viewing direction distribution is shown below.



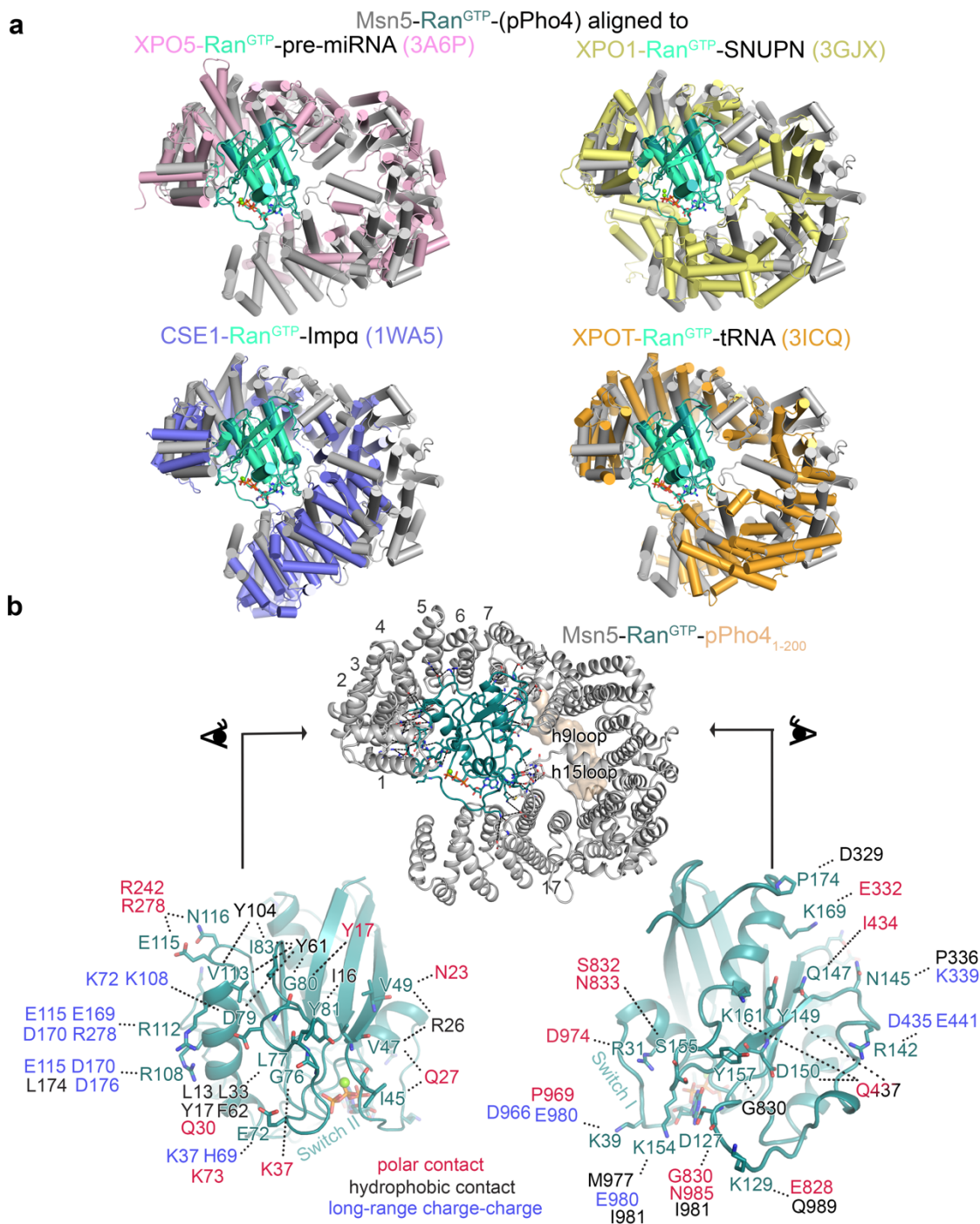
Supplementary Figure 4. Simplified data processing workflow and statistics for Msn5-Ran^{GTP}-pPho4_{FL} maps. **a** Data processing summary (details in methods) with particle distribution indicated in percentages. Representative 2D classes and the 6 classes from heterogeneous refinement are shown. Maps were assigned their states designation by comparison with the Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ maps. Final maps are shown as surfaces with their EMD codes, where State 1 is in yellow and State 3 in coral. **b** Map statistics of the two maps in **a**, shown as in **Supplementary Fig. 3**.



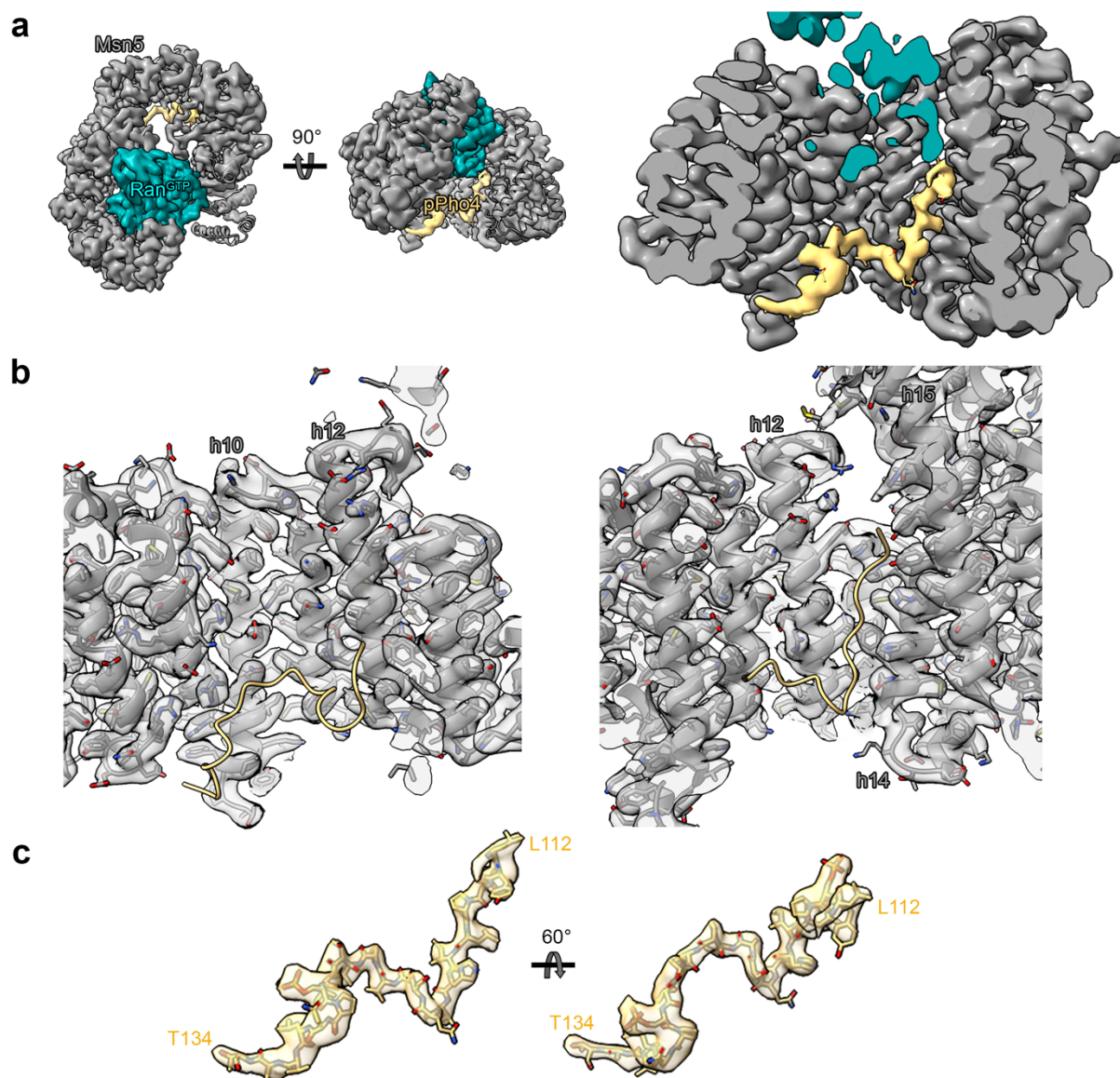
Supplementary Figure 5. Comparative structural analysis of the States 1-1, 2-1 and 3-1 pPho4₁₋₂₀₀-bound Msn5 solenoids. The left panel shows State 1-1 (gray) Msn5 aligned at repeats h1 and h2 to State 2-1 Msn5 (yellow). The middle panel shows State 2-1 aligned to State 3-1 (coral) in a similar manner. 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.



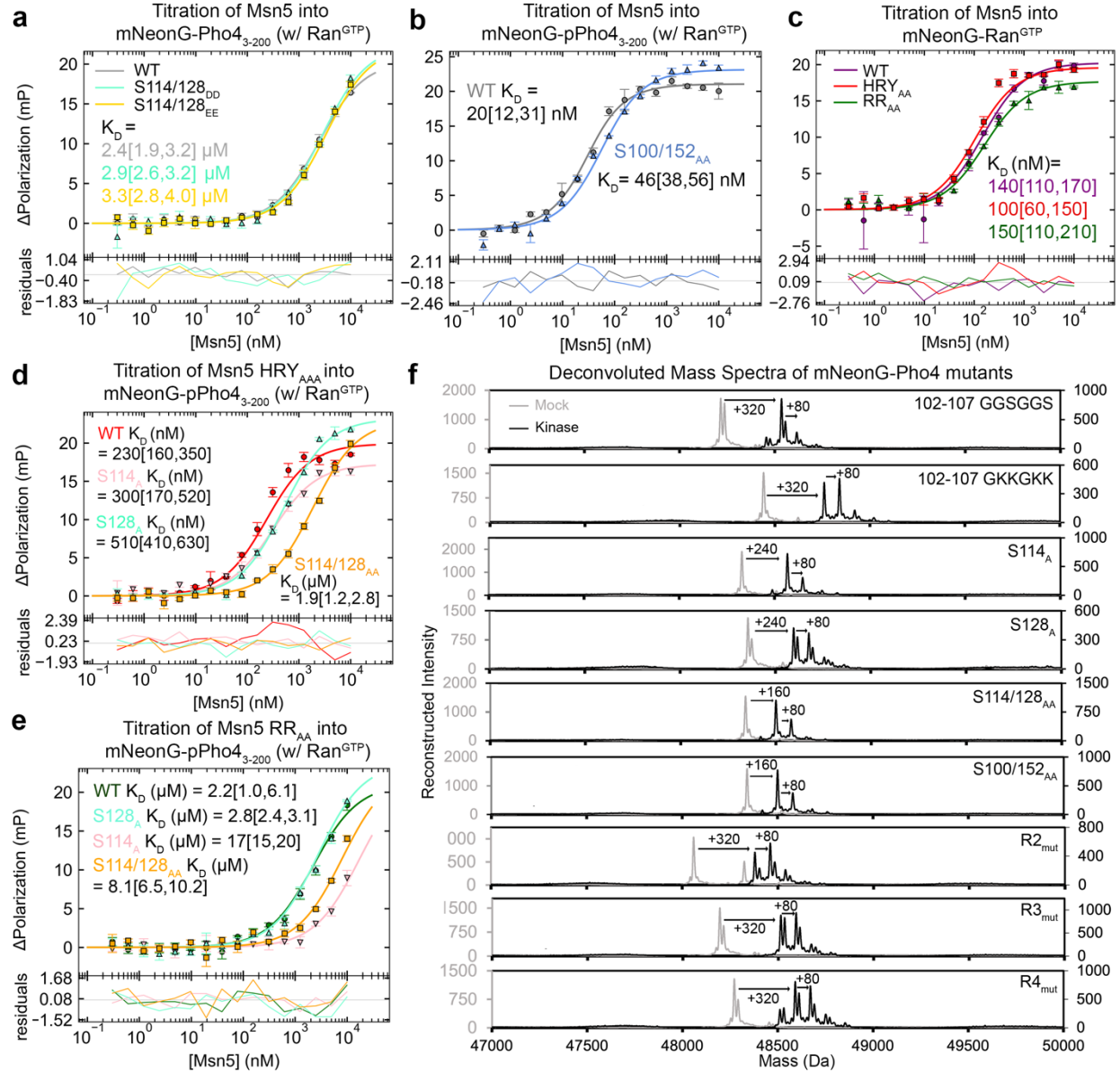
Supplementary Figure 6. Cryo-EM maps of Msn5-Ran^{GTP}-pPho4 complexes. **a** Cryo-EM structure of Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ in cartoon (Msn5 colored in rainbow: blue to red = N to C; the rest in grey). View angles for the left (L), right (R) and center (C) are indicated on the top-down view on the left. **b-e** Cryo-EM maps of indicated states are colored by the reference model on the left containing only Msn5 (dark gray) and Ran^{GTP} (cyan) so that any extra densities are apparent (yellow, orange and coral for states 1-3 respectively).



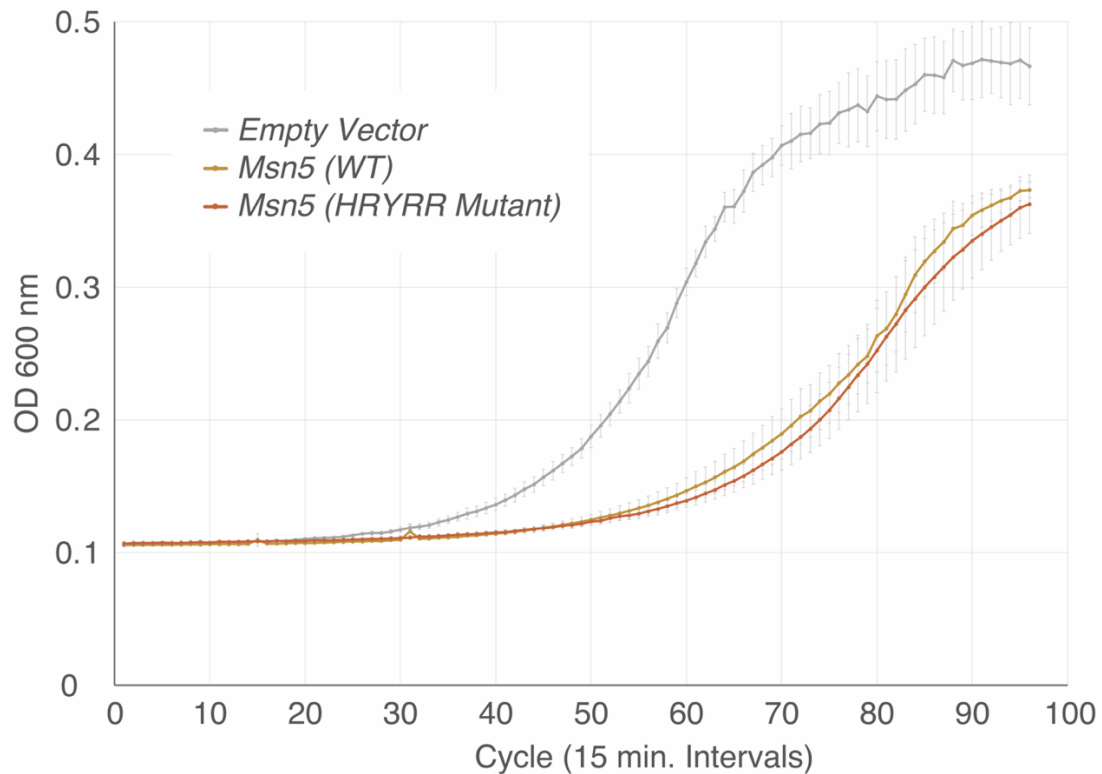
Supplementary Figure 7. Interactions between Msn5 and Ran^{GTP}. **a** The cryo-EM structures of Msn5 (gray)-Ran^{GTP} (dark cyan)-pPho4₁₋₂₀₀ (not shown) and other Exportin-Ran^{GTP}-cargo complexes are aligned by their GTPases (for clarity, cargoes are not displayed)²⁻⁵. **b** Contacts between Msn5 and Ran^{GTP} in Msn5-Ran^{GTP}-pPho4₁₋₂₀₀ (wheat surface) shown in dotted lines. As there are many contacts that cannot be viewed easily, the front and back views of Ran^{GTP} are displayed below with their interacting residues on Msn5 indicated and colored by the nature of the contact.



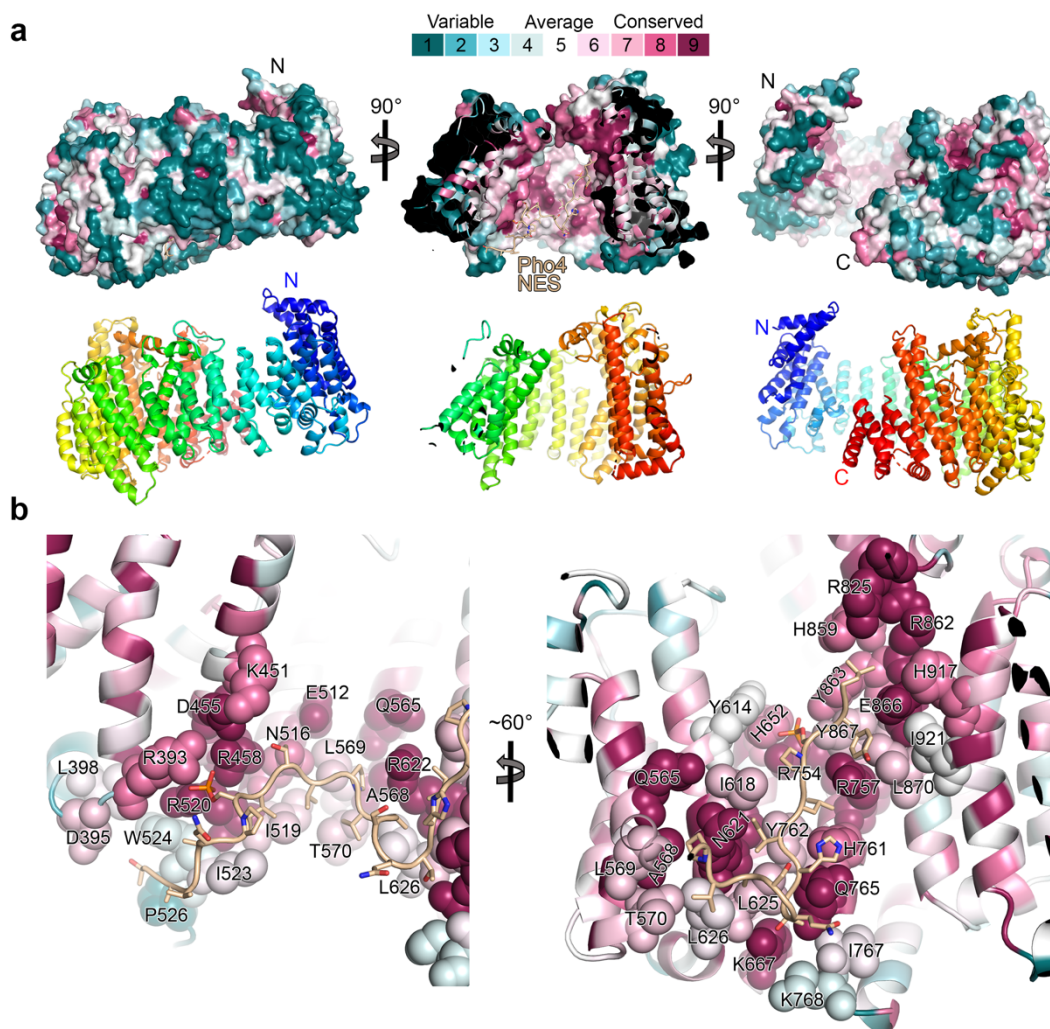
Supplementary Figure 8. Map quality of the Pho4-binding interface of Msn5. **a** Top (left) and front (middle) views of the Msn5 (gray)-Ran^{GTP} (dark cyan)-pPho4₁₋₂₀₀ (yellow) State 1-1 map, colored as the overlaid final model. The rightmost panel is an enlarged front view (same as in the middle panel), 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, overlaid with the peptide shown as sticks, in two views. The last and first residues modelled are labeled.



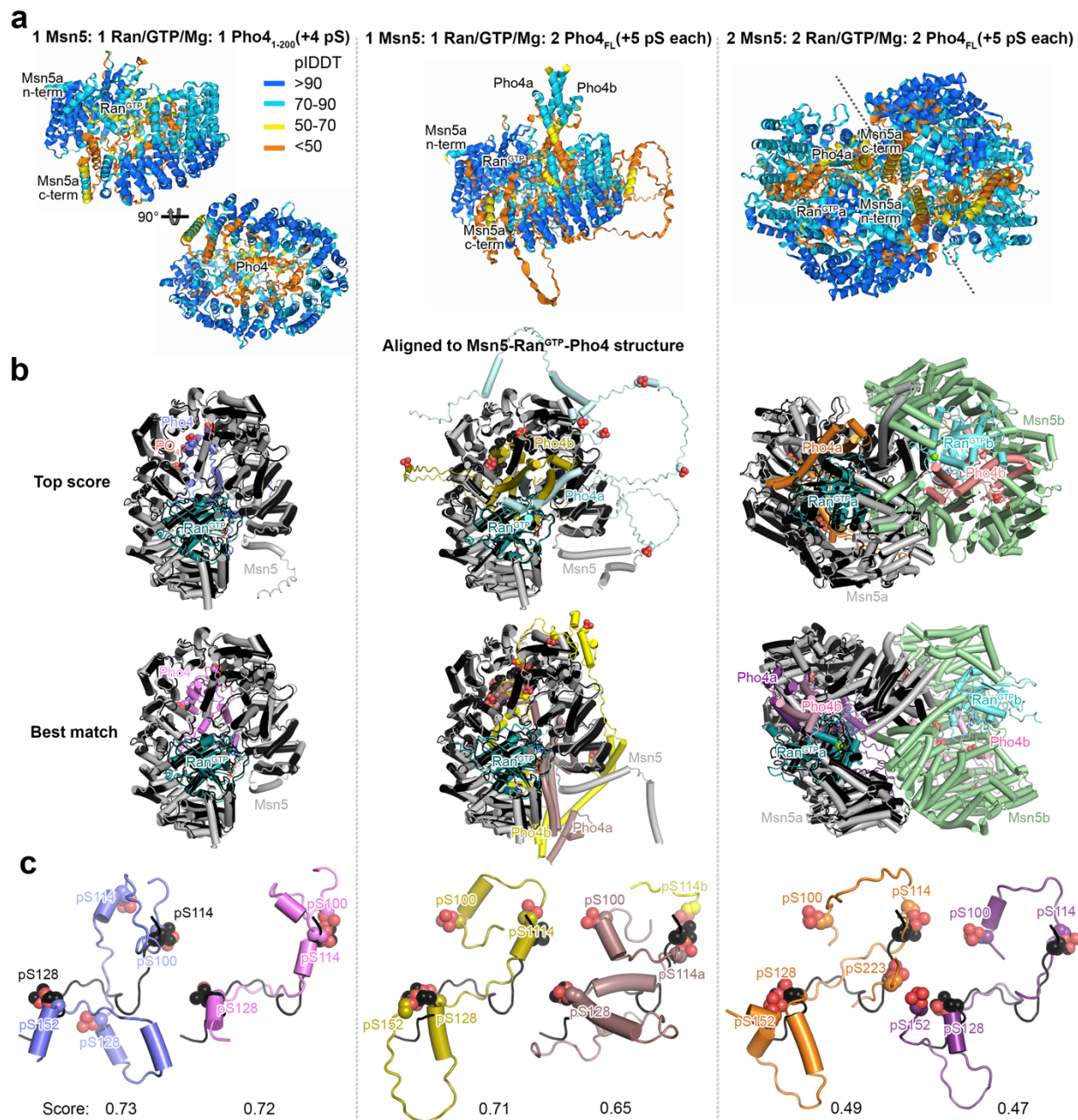
Supplementary Figure 9. FP assays of wild-type (WT) and mutant Msn5 binding to phosphorylation site mutants of Pho4. **a** WT Msn5 binding to unphosphorylated WT (gray), phosphomimetic mutant S114/128_{DD} (light cyan; S114 and S128 mutated to aspartic acid) or S114/128_{EE} (light yellow; S114 and S128 mutated to glutamic acid) mNeonG-Pho4₃₋₂₀₀, in presence of excess Ran^{GTP}. All three mNeonG-Pho4₃₋₂₀₀ constructs bind Msn5 with similar affinities. **b** WT Msn5 binding to Pho4 WT (gray) and S100/152_{AA} mutant (blue; S100 and S152 were mutated to alanine). WT titration is duplicated from **Fig. 2e**. **c** Control for WT (purple), HRY_{AA} (red) and RR_{AA} (green) Msn5 from **Fig. 3** binding to mNeonG-Ran^{GTP}. **d-e** As in **Fig. 3b**, HRY_{AA} (D) or RR_{AA} (E) Msn5 binding to mNeonG-pPho4₃₋₂₀₀ S114_A (pink), S128_A (light cyan) and S114/128_{AA} (orange) mutants. The WT pPho4 traces (red for Msn5 HRY_{AA} and green for Msn5 RR_{AA}) are duplicated from **Fig. 3b**. All data points represent mean $\pm$ s.d. of $n=3$ measurements. Lines represent 1-site binding fit and residuals of the fits are plotted below. Dissociation constants (K_D) with 95% confidence intervals obtained using error-surface projection are displayed. **f** Deconvoluted mass spectra from intact mass analysis of mNeonG-Pho4 mutants used in this study. Source data are provided as a Source Data file.



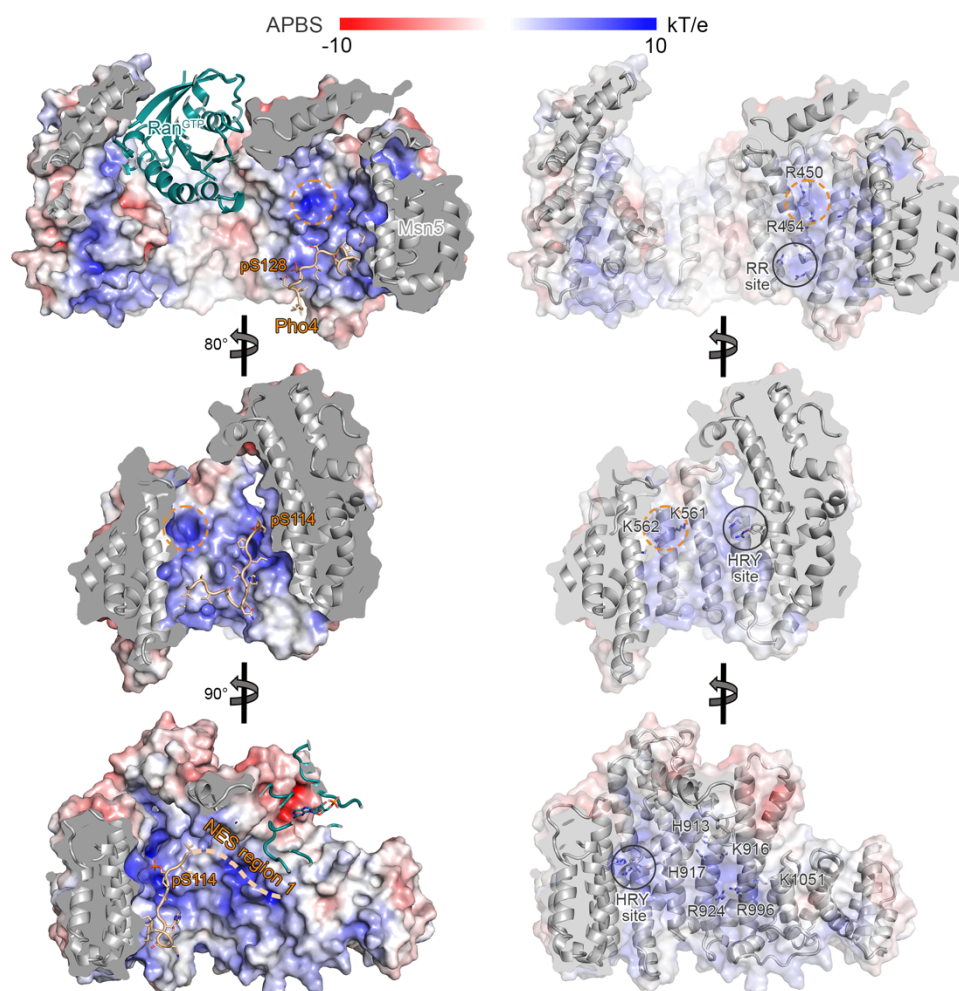
Supplementary Figure 10. Growth curve of strains carrying an empty vector control, a WT Msn5 or the HRYRR_{AAAAA} double mutant. There has been demonstrated evidence that overexpression of Msn5 can cause deleterious effects to cell growth⁶⁻⁸. We used the base ASY788 (Msn5 KO) strain and transformed in an empty vector, a WT Msn5, or an HRYRR_{AAAAA} double mutant in a pRS414 vector, with the powerful ADH1 promoter to induce substantial overexpression. Data points are mean and standard deviation of n=4. Source data are provided as a Source Data file.



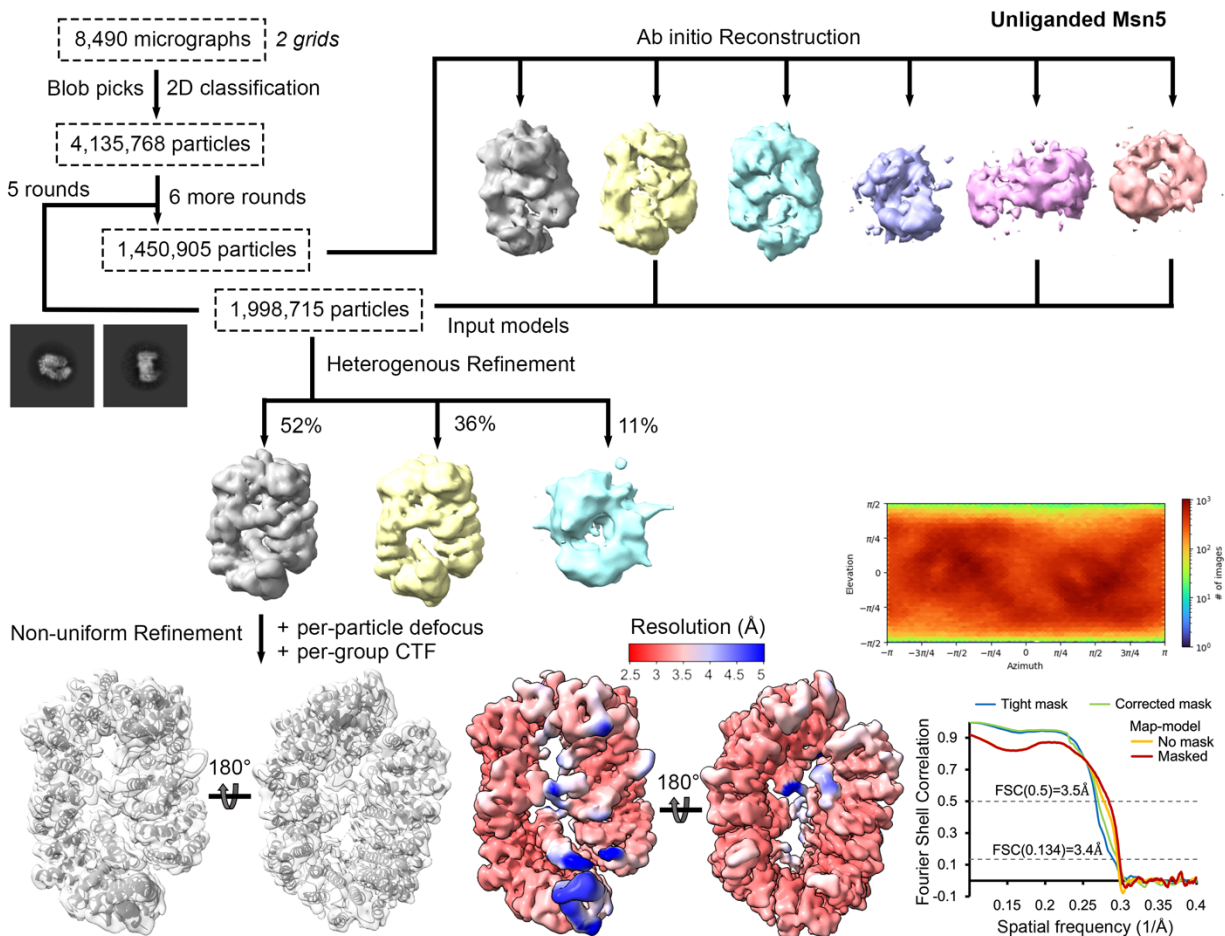
Supplementary Figure 11. ConSURF analysis of 101 fungal homologs of Msn5. a Conservation analysis of Msn5 is performed on the ConSURF server⁹ and displayed in PyMOL, colored according to the conservation score in the key. Msn5 is shown as surface, and pPho4 NES peptide in wheat cartoon, Ran^{GTP} is not shown. Cartoon of the Ran^{GTP} and pPho4₁₋₂₀₀ bound Msn5 in the three views are shown below, colored in rainbow (N to C: blue to red). **b** Zoom-in view of the pPho4 NES interface on Msn5. Msn5 residues at the Pho4 interface (by PISA analysis¹⁰) are drawn as spheres and labelled.



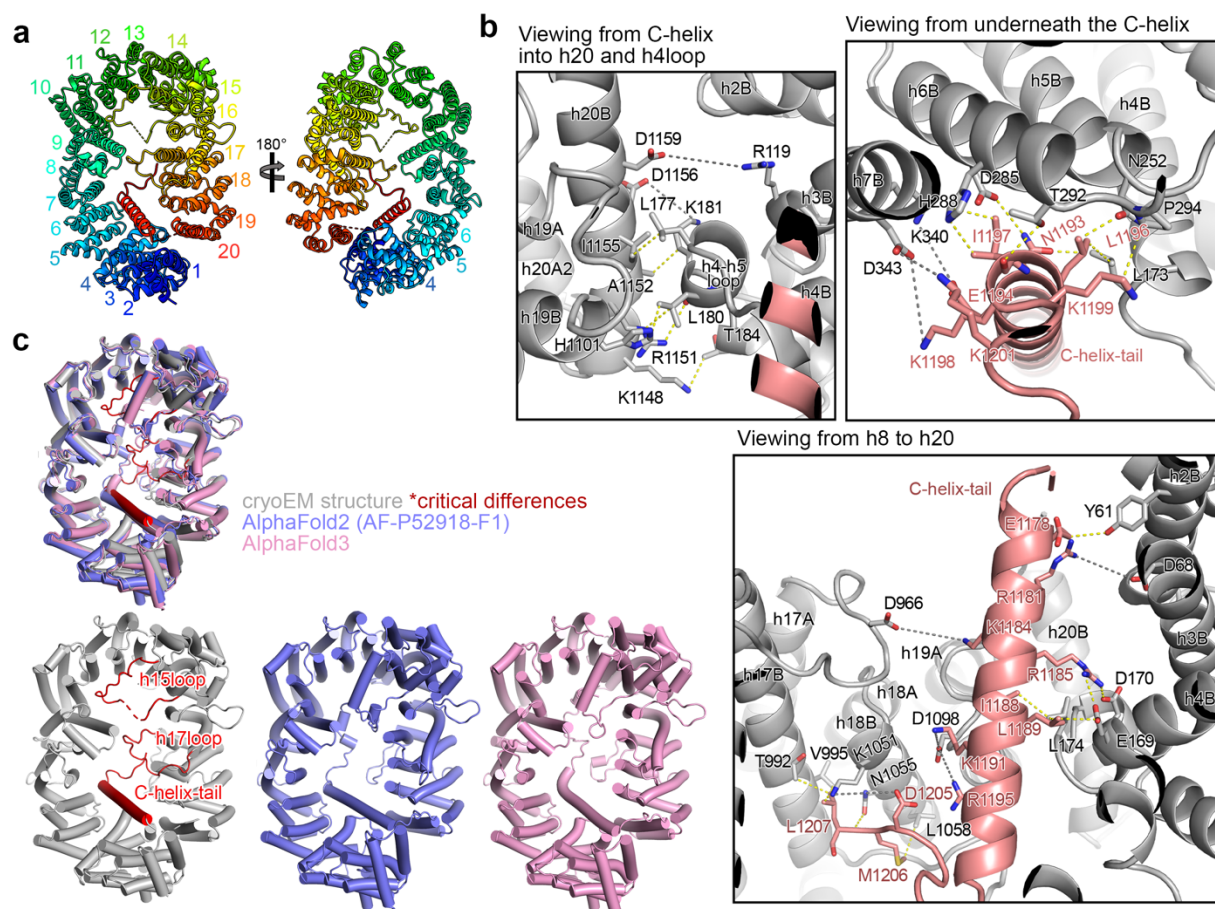
Supplementary Figure 12. AlphaFold3 (AF3) prediction of Pho4-Msn5 complexes. **a** AF3 output of two models, colored by the pLDDT confidence score (higher indicates more confidence), for three inputs of different Pho4-Msn5 complexes. The left panel shows the results of an input of 1 Msn5: 1 Ran (along with one each GTP and Mg): 1 Pho4₁₋₂₀₀ (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}-Pho4₁₋₂₀₀ 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) overlaid with the AF3 Pho4 models.



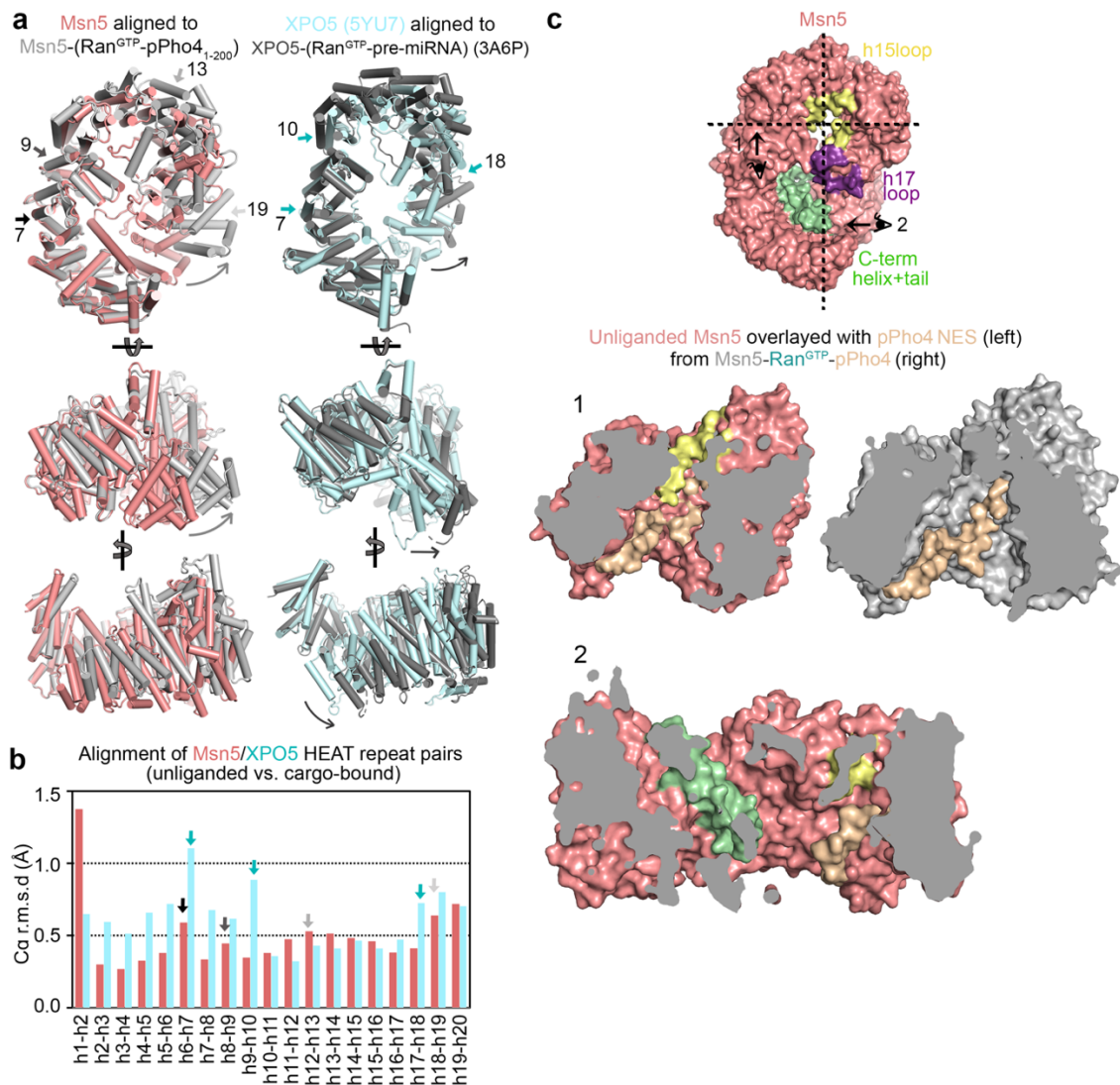
Supplementary Figure 13. Additional basic patches in Msn5 concave surface. Left: APBS electrostatic potential of Msn5, shown as surface in three views. Msn5 is the gray cartoon underneath the surface, and Ran^{GTP} and Pho4 are shown as cyan and wheat cartoon respectively. Pho4 phosphoserines are labelled, 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).



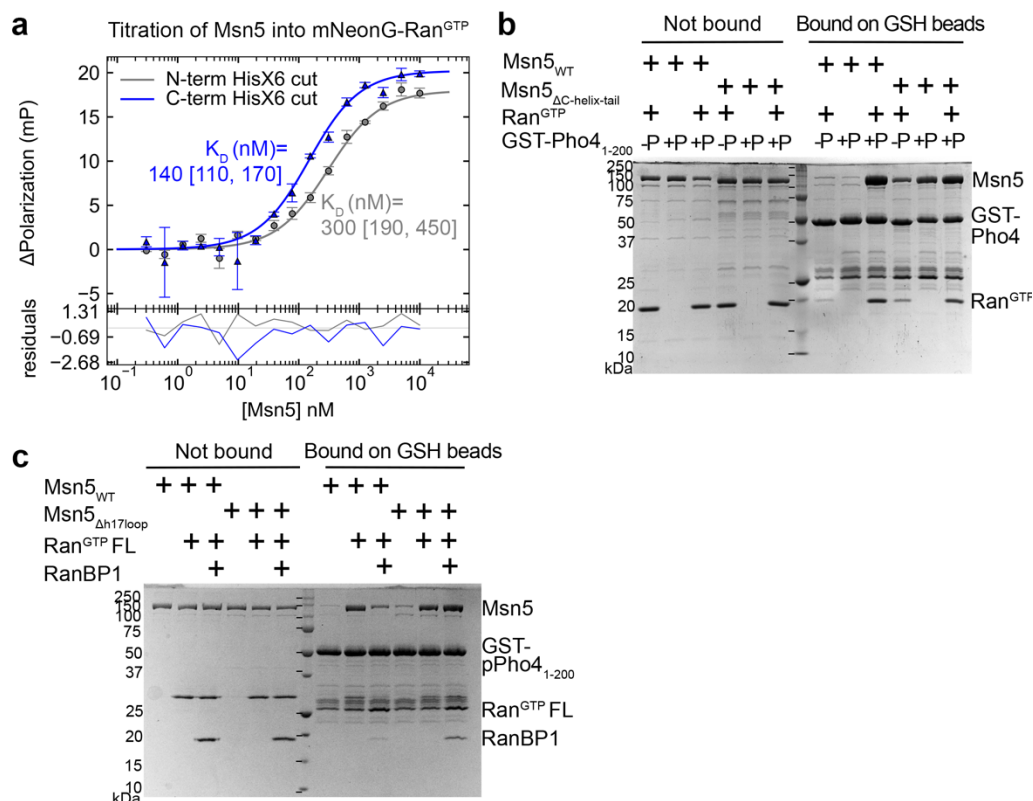
Supplementary Figure 15. Unliganded Msn5 cryo-EM data processing workflow and map and model statistics. Summary of data processing of the unliganded Msn5 cryo-EM data, with representative 2D classes shown. Six *ab initio* reconstructions are shown, and the three reconstructions selected for heterogeneous refinement are noted. Two of the heterogeneous refined maps are highly similar but inclusion of the class 1 particles (yellow) did not improve the class 0 map (gray), suggesting small conformational variability/flexibility of unliganded Msn5. The final map, obtained using non-uniform refinement, is shown as transparent surface at the bottom, overlaid with the final structure of unliganded Msn5 (left), and colored by local resolution (middle). Viewing direction distribution and FSC curves from cryoSPARC (tight and corrected mask; blue and green) and PHENIX map-model FSC (with and without mask; yellow and red) are plotted on the right.



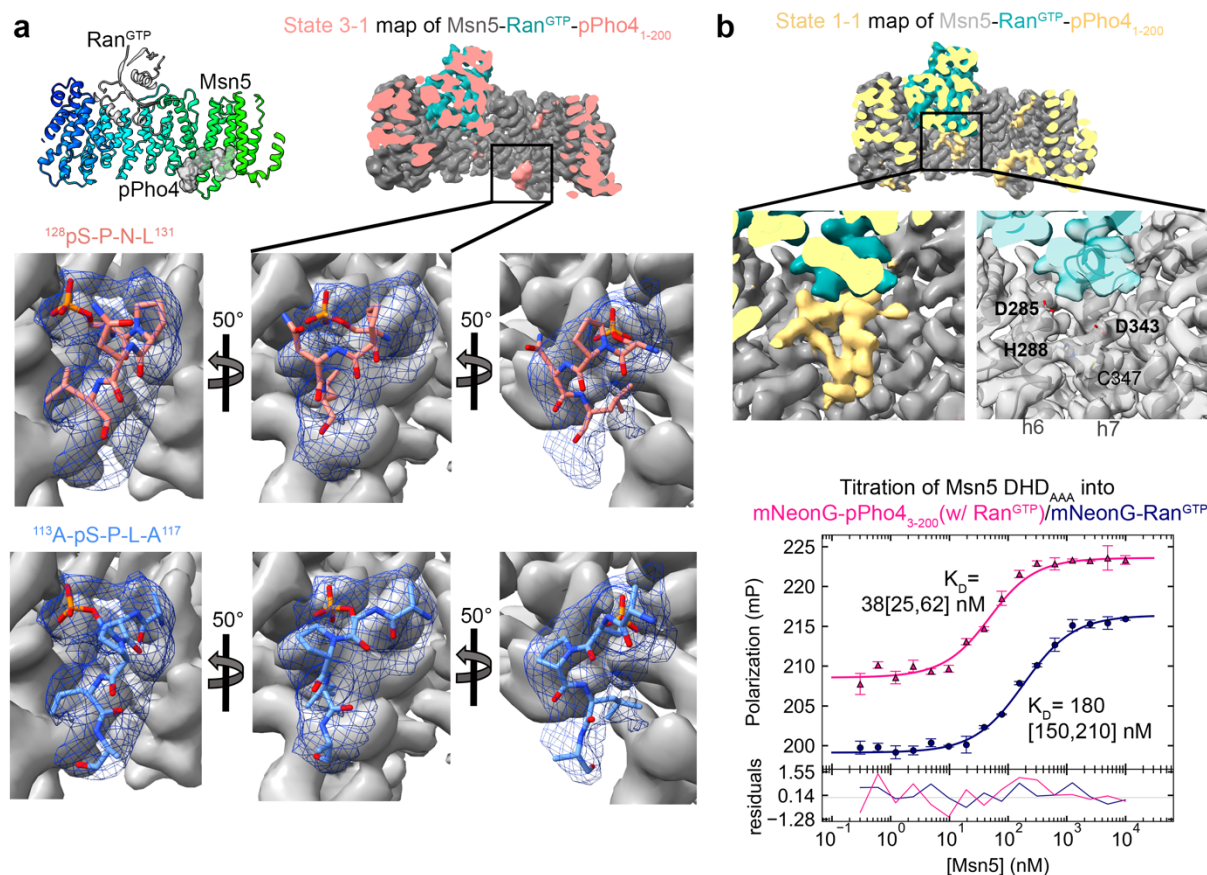
Supplementary Figure 16. Unliganded Msn5: autoinhibitory contacts and AlphaFold prediction. **a** The structure of unliganded Msn5 is shown as cartoon, colored and labeled by HEAT repeats in rainbow (N, blue to red, C). **b** The autoinhibited conformation of Msn5 appears to be mediated by intramolecular 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 ($<4 \text{ \AA}$) and long-range electrostatic interactions of $<8 \text{ \AA}$ 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.



Supplementary Figure 17. Comparison of unliganded and cargo-bound Msn5/XPO5 structures. **a** Left: Unliganded Msn5 unliganded (pink) aligned with Ran^{GTP}-pPho4₁₋₂₀₀-bound Msn5 (light grey; Ran and Pho4 not shown). Right: Unliganded XPO5 unliganded (cyan; 5YU7) aligned with Ran^{GTP}-pre-miRNA-bound XPO5 (dark grey; Ran and pre-miRNA not shown; 3A6P).^{2,12} Both Msn5 and XPO5 solenoids are more open when bound to cargo. **b** C α root-mean-square-deviation (r.m.s.d) of aligned HEAT repeat pairs of unliganded and cargo-bound Msn5 (pink) and XPO5 (cyan) structures shown in **a**. No large changes are observed at any HEAT repeats. Structural differences are distributed across the whole solenoid with small increases in r.m.s.d at a few HEAT repeats that are marked with arrows (also marked in **a**) that may act as hinges. **c** Unliganded Msn5 structure shown as colored surface in two cut-out views, view 1 (middle) and view 2 (bottom), as indicated on the top-down view on the top. In left panel for view 1, pPho4 NES overlayed from the Msn5 (gray)-Ran^{GTP} (not shown)-pPho4₁₋₂₀₀ (wheat) structure (right panel) clash with the unliganded Msn5 – compare visibility of the pPho4 NES in right and left panels. In view 2, the entire pPho4 NES region 4 can be seen with no obstruction from unliganded Msn5.



Supplementary Fig 18. Binding assays of Msn5 with Ran and RanBP1. **a** Cryo-EM structure determination was performed using Msn5-HisX6 where the HisX6 tag was removed by Tev protease. Here, FP analysis was performed with HisX6-Msn5 and Msn5-HisX6 where the N- or C-terminal HisX6 tags, respectively, were removed with TEV protease. Msn5 was titrated into 20 nM mNeonG-Ran^{GTP} and polarization signal was measured. Data points represent mean $\pm$ s.d. of $n=3$ measurements. Line represents 1-site binding fit and residuals of the fits are plotted below. Dissociation constants (K_D) with 95% confidence intervals is displayed. Msn5 binds Ran^{GTP} with K_D 140 nM or 300 nM. Such moderate affinity of Msn5 for Ran^{GTP} in the absence of cargo is unusual exportins, which usually bind with much lower affinities to Ran^{GTP} alone.¹³ This finding suggests that although a large population of unliganded Msn5 adopts a compact ring conformation, the autoinhibited ring has some flexibility and small populations of other states with open rings and exposed Ran^{GTP} binding site also exist. The presence of additional residues from the TEV cleavage site on the C-terminus of Msn5 increases Ran^{GTP} affinity 2-fold, supporting the importance of the Msn5 C-terminus in stabilizing the closed-ring autoinhibited Msn5 conformation. **b-c** Full gels for the binding assays in **Fig. 4c** and **e**, which include the unbound proteins of the assays. Source data are provided as a Source Data file.



Supplementary Figure 19. CryoEM map densities that cannot be confidently modeled. **a**

The Msn5 (rainbow cartoon)-Ran^{GTP} (gray cartoon)-pPho4 (gray cartoon and surface) structure is shown on top left as in **Supplementary Fig. 6a**. The Pho4 density in State 3-1 map of Msn5 (gray)-Ran^{GTP} (cyan)-pPho4₁₋₂₀₀ (salmon) is shown on top right, marked by a box and in the same orientation as the structure on the left. Six zoom-in panels of the boxed area are shown below with Msn5 density in grey and the Pho4 density near the Msn5 RR site as blue mesh. The center 2 panels are in approximately the same orientation as the boxed area. Two possible pPho4 models, each with a phosphoserine (¹²⁸pSPNL¹³¹ in salmon or ¹¹³ApSPLA¹¹⁷ in blue), can be fitted into the Pho4 density. The ¹¹³ApSPLA¹¹⁷ model (blue) seems to fit better in all orientations. However, it is unclear which one is correct, and it is also possible that the density represents a mix of both models. The same can be seen in the State 2-1 maps. In both final deposited models for State 2-1 and State 3-1, Pho4 is not included. **b** The state 1-1 map of the Msn5 (gray)-Ran^{GTP} (cyan)-pPho4₁₋₂₀₀ (yellow) complex with unmodeled and unexplained density close to Ran^{GTP} marked with a box (top). Middle left panel: Zoom-in view of unexplained density. Middle right panel: The same map is shown transparent, to see the Msn5 cartoon and sticks below the transparent extra density. Bottom panel: FP titrations of Msn5 mutant where D285/H288/D343 are mutated to alanines (DHD_{AAA}) binding to mNeonG-pPho4₃₋₂₀₀ in the presence of excess Ran^{GTP} (pink) or to mNeonG-Ran^{GTP} (navy). Data points represent mean ± s.d. of n=3 measurements. Line represents 1-site binding fit and residuals of the fits are plotted below. Dissociation constants (K_D) with 95% confidence intervals obtained using error-surface projection are displayed. Source data are provided as a Source Data file.

References

1. Abramson, J. *et al.* Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**, 493-500 (2024). <https://doi.org/10.1038/s41586-024-07487-w>
2. Okada, C. *et al.* A high-resolution structure of the pre-microRNA nuclear export machinery. *Science* **326**, 1275-1279 (2009). <https://doi.org/10.1126/science.1178705>
3. Monecke, T. *et al.* Crystal structure of the nuclear export receptor CRM1 in complex with Snurportin1 and RanGTP. *Science* **324**, 1087-1091 (2009). <https://doi.org/10.1126/science.1173388>
4. Matsuura, Y. & Stewart, M. Structural basis for the assembly of a nuclear export complex. *Nature* **432**, 872-877 (2004). <https://doi.org/10.1038/nature03144>
5. Cook, A. G., Fukuhara, N., Jinek, M. & Conti, E. Structures of the tRNA export factor in the nuclear and cytosolic states. *Nature* **461**, 60-65 (2009). <https://doi.org/10.1038/nature08394>
6. Niu, W., Li, Z., Zhan, W., Iyer, V. R. & Marcotte, E. M. Mechanisms of cell cycle control revealed by a systematic and quantitative overexpression screen in *S. cerevisiae*. *PLoS Genet* **4**, e1000120 (2008). <https://doi.org/10.1371/journal.pgen.1000120>
7. Douglas, A. C. *et al.* Functional analysis with a barcoder yeast gene overexpression system. *G3 (Bethesda)* **2**, 1279-1289 (2012). <https://doi.org/10.1534/g3.112.003400>
8. Duffy, S. *et al.* Overexpression screens identify conserved dosage chromosome instability genes in yeast and human cancer. *Proc Natl Acad Sci U S A* **113**, 9967-9976 (2016). <https://doi.org/10.1073/pnas.1611839113>
9. Ashkenazy, H. *et al.* ConSurf 2016: an improved methodology to estimate and visualize evolutionary conservation in macromolecules. *Nucleic Acids Res* **44**, W344-350 (2016). <https://doi.org/10.1093/nar/gkw408>
10. Krissinel, E. & Henrick, K. Inference of macromolecular assemblies from crystalline state. *J Mol Biol* **372**, 774-797 (2007). <https://doi.org/10.1016/j.jmb.2007.05.022>
11. Holm, L. & Laakso, L. M. Dali server update. *Nucleic Acids Res* **44**, W351-355 (2016). <https://doi.org/10.1093/nar/gkw357>
12. Yamazawa, R. *et al.* Structural Basis for Selective Binding of Export Cargoes by Exportin-5. *Structure* **26**, 1393-1398 e1392 (2018). <https://doi.org/10.1016/j.str.2018.06.014>
13. Hahn, S. & Schlenstedt, G. Importin beta-type nuclear transport receptors have distinct binding affinities for Ran-GTP. *Biochem Biophys Res Commun* **406**, 383-388 (2011). <https://doi.org/10.1016/j.bbrc.2011.02.051>