Supporting Information

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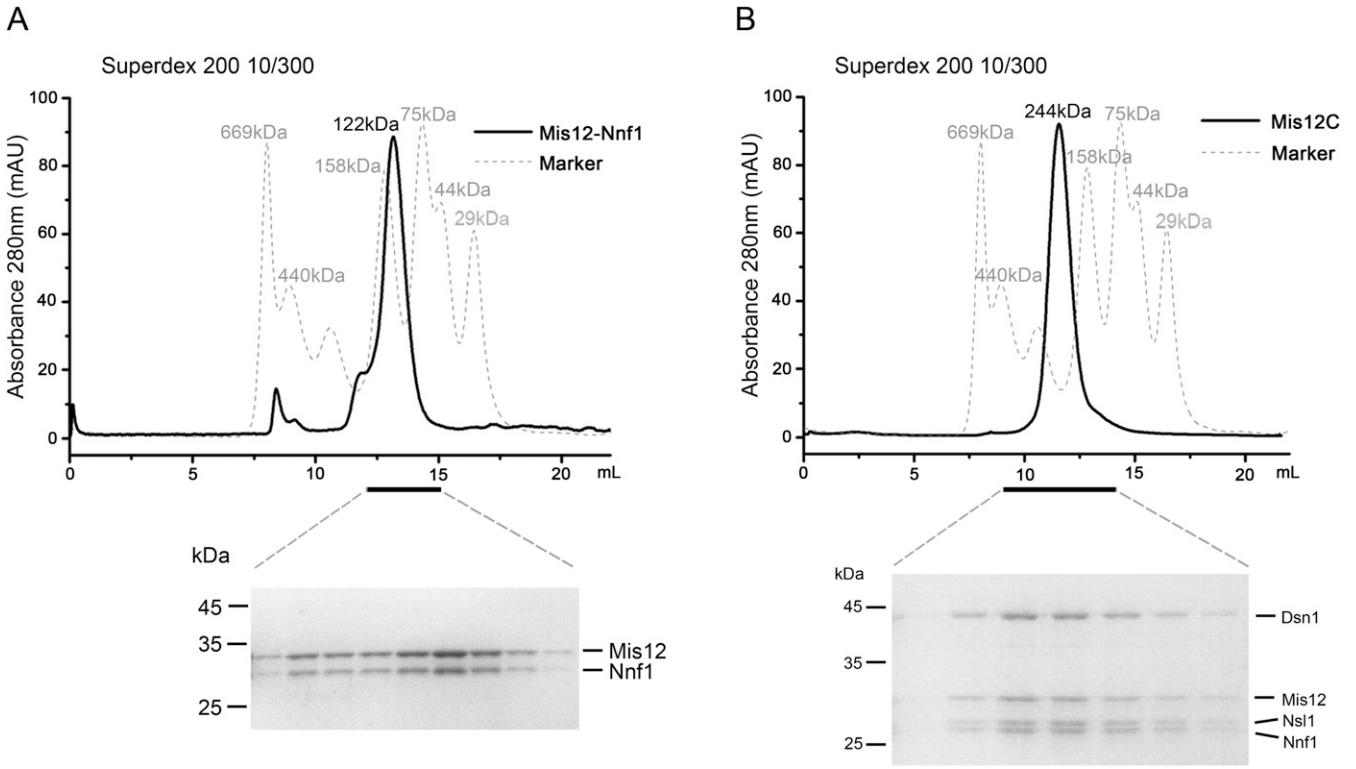

Fig. S1. SEC of Mis12–Nnf1 and Mis12C. SEC of the Mis12–Nnf1 subcomplex (*A*) and Mis12C (*B*) with corresponding SDS/PAGE analysis stained with Coomassie brilliant blue. The dotted gray lines indicate elution markers of SEC, and their molecular masses are shown.

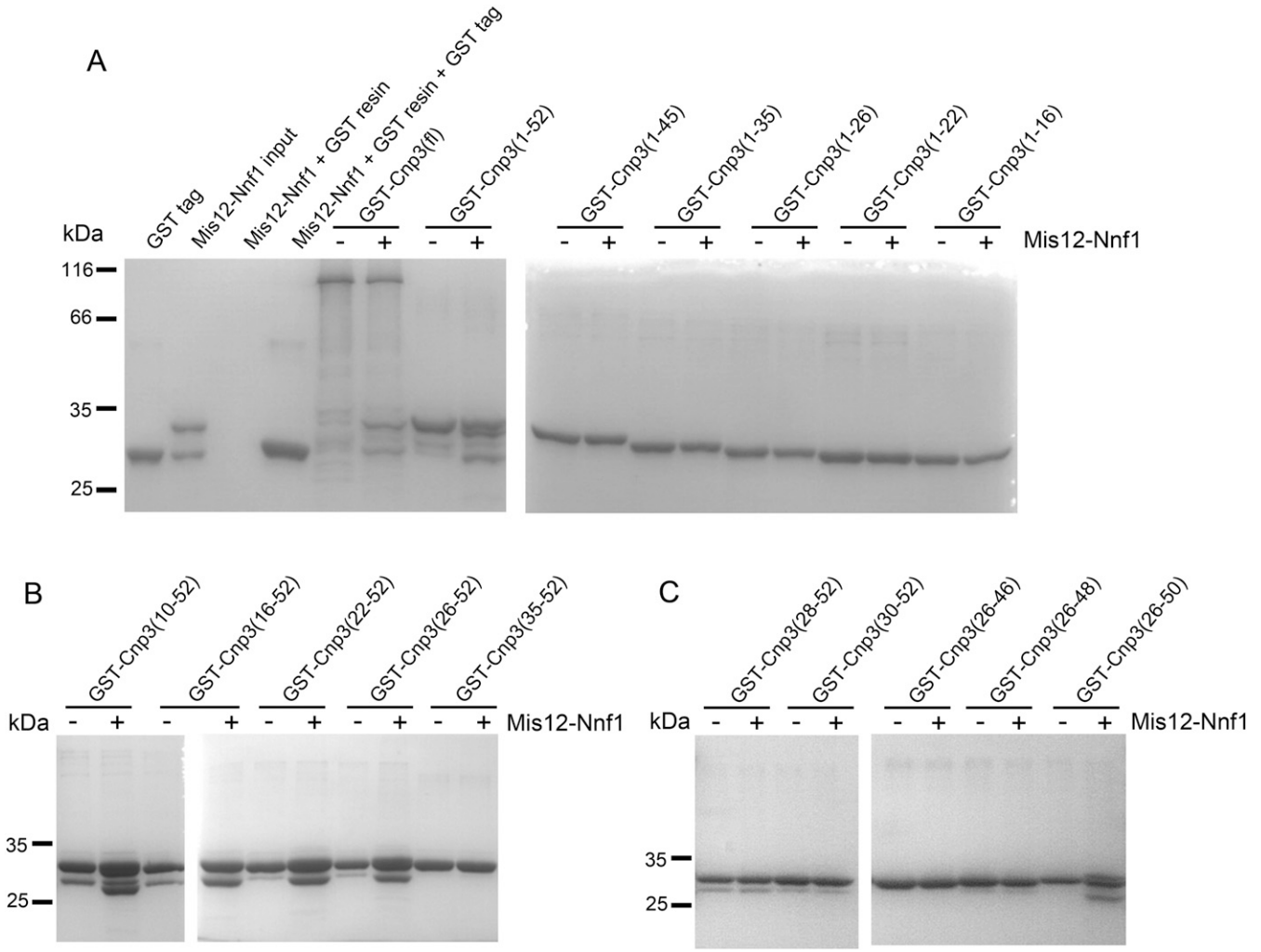

Fig. S2. N-terminal region of Cnp3 interacts with Mis12–Nnf1. (*A*–*C*) GST pull-down assay of Mis12–Nnf1 with several Cnp3 N-terminal truncations fused to the GST tag. The GST-Cnp3(fl) served as positive controls. The grouping blots in each panel were cropped from different gels and separated with white spaces.

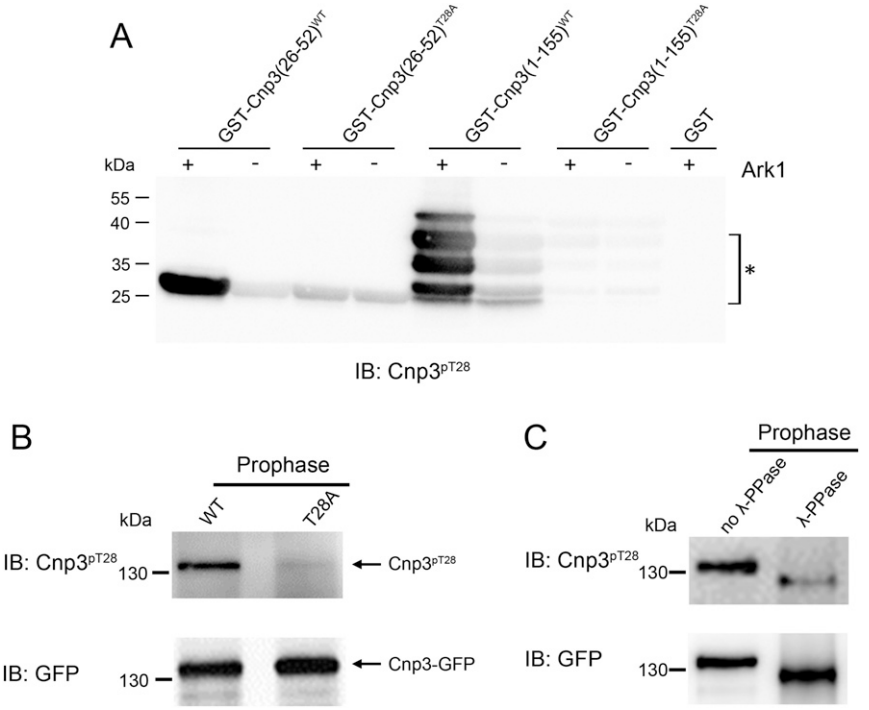

Fig. S3. Phosphorylation of Cnp3 at Thr28 is specifically recognized by the generated antibody. (*A*) Western blot analysis of the site-specific anti-pT28 of Cnp3 antibody in vitro kinase assay. GST-Cnp3(26–52)$^{WT/T28A}$ and GST-Cnp3(1–155) $^{WT/T28A}$ with or without Ark1 are analyzed. GST tag is used as the negative control. Asterisk indicates the degradation of GST-Cnp3(1–155). (*B*) In vivo phosphorylation at Thr28 of Cnp3. The indicated *S. pombe* cells carrying *nda3-KM311* and GFP-tagged Cnp3^{WT} or Cnp3^{T28A} were inoculated at 30 °C and arrested at the mitosis by shifting to the restrictive temperature (16 °C). The cell extracts were analyzed by Western blotting using the GFP antibody and anti-phosphoThr28 antibody. (*C*) Immunoprecipitated proteins of mitotic-arrested cells were treated with or without λ-PPase and analyzed with the indicated antibodies.

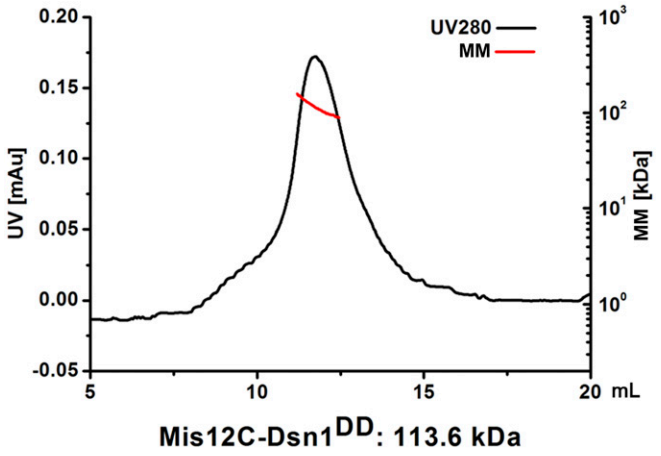

Fig. S4. Molecular masses of Mis12C–Dsn1^{DD} measured by SEC-MALS. Chromatograms show elution profiles measured in UV280 and calculated molecular mass (MM). The *x* axis shows elution volume in milliliters.

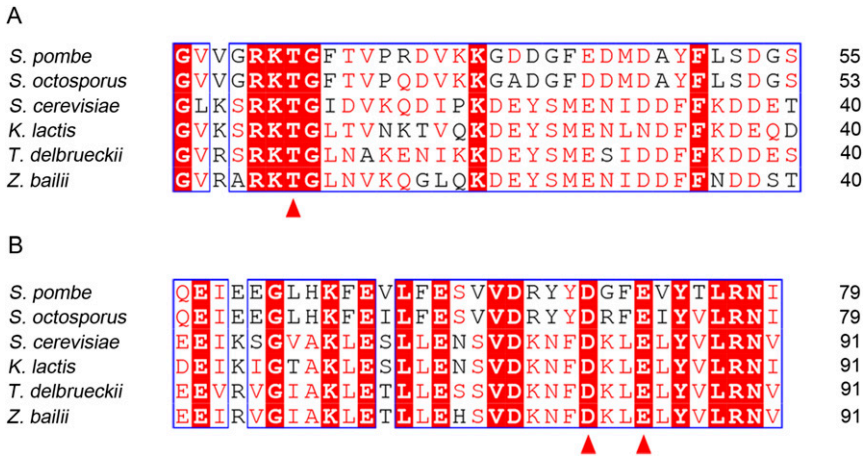

Fig. S5. Sequence alignment of the N terminus of CENP-C and Mis12. (*A*) Sequence alignment of the N terminus in CENP-C from different species. Red triangle indicates the conserved threonine that was phosphorylated by Aurora B. (*B*) Sequence alignment of the N terminus in Mis12 from different species. Red triangle indicates the conserved negative residues that interact with the N terminus of CENP-C.

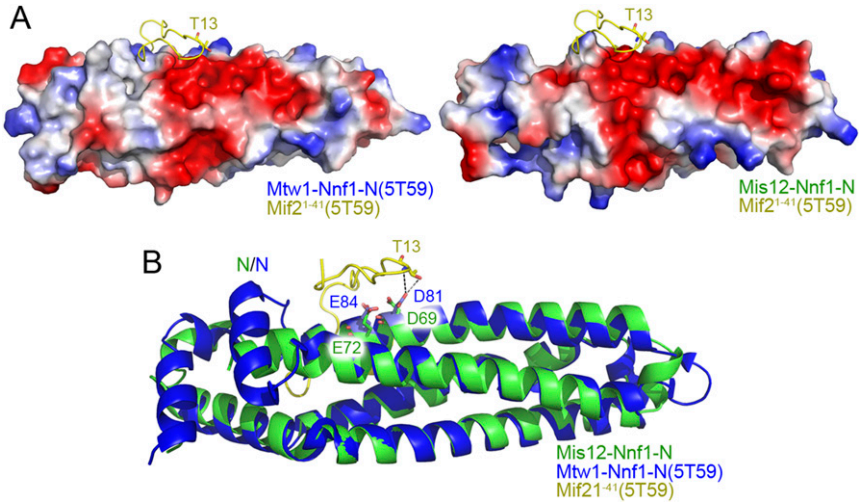

Fig. S6. Comparison of the Mis12–Nnf1 subcomplex structures. (*A*) Surface representation of the N-terminal part of the Mis12–Nnf1 and Mif2$^{1-41}$ (PDB ID code 5T59) structure (PDB ID code 5T59, *Left*; our Mis12–Nnf1 subcomplex structure superimposed, *Right*). Surfaces with positive electrostatic potential are blue, while surfaces with negative electrostatic potential are red. (*B*) Comparison of the N-terminal part of our Mis12–Nnf1 subcomplex structure with the *K. lactis* MN–C2–Mif2$^{1-41}$ structure (PDB ID code 5T59); Mis12–Nnf1, Mtw1–Nnf1 (PDB ID code 5T59), and Mif2$^{1-41}$ (PDB ID code 5T59) are colored green, blue, and yellow. N indicates the amino terminal, while C indicates the carboxyl terminal.

Table S1. Kinetic studies of Ark1 with Dsn1 and Cnp3 peptides as the substrates

Peptides	V_{max}, μM·min^{-1}	K_m, μM	K_{cat}, s^{-1}	K_{cat}/K_m, s^{-1}·μM^{-1}
Dsn1(66–92)	0.80 ± 0.06	76.31 ± 20.54	0.0053	7.0×10^{-5}
Cnp3(26–52)	0.58 ± 0.05	277.49 ± 63.38	0.0038	1.4×10^{-5}

Table S2. Yeast strains used in this study

Strain	Genotype	Figure	Source
CF.3968	*nda3-KM311 ark1-as3*:HygR cnp3Δ:NatR ade6-m210? Leu1-32:ase1P*-cnp3-GFP ura4-D18? h?	Figs. 3*C* and 5*G* and Fig. S3 *B* and *C*	This study
CF.3969	*nda3-KM311 ark1-as3*:HygR cnp3Δ:NatR ade6-m210? Leu1-32:ase1P*-cnp3 (28T→A)-GFP ura4-D18? h?	Fig. 3*C* and Fig. S3*B*	This study
CF.3768	cnp3Δ:NatR mis12-FLAG:URA+ Leu1-32:ase1P*-cnp3-GFP lys1-131? h-	Fig. 4*C*	This study
CF.3770	cnp3Δ:NatR mis12-FLAG:URA+ Leu1-32:ase1P*-cnp3(28T→E)-GFP lys1-131? h-	Fig. 4*C*	This study
CF.3696	cnp3Δ:NatR mis12-GFP-HA:KanR mCherry-atb2:HygR ade6-m210? Leu1-32:ase1P*-cnp3-VC ura4-D18? lys1-131? h90	Fig. 4 *D*, *F*, and *I*	This study
CF.3698	cnp3Δ:NatR mis12-GFP-HA:KanR mCherry-atb2:HygR ade6-m210? Leu1-32:ase1P*-cnp3 (28T→E)-VC ura4-D18? lys1-131? h90	Fig. 4 *E*, *F*, and *I*	This study
PT.286	WT ade6-m210 Leu1-32 ura4-D18 h-	Fig. 4 *H* and *I*	Phong Tran (Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia)
CF.3574	cnp3Δ:NatR cnp1-GFP:HygR sid4-mTomato: HygR ade6-m210? Leu1-32:ase1P*-cnp3-VC ura4-D18 h-	Figs. 4*G* and 5*A*	This study
CF.3576	cnp3Δ:NatR cnp1-GFP:HygR sid4-mTomato: HygR ade6-m210? Leu1-32:ase1P*-cnp3(28T→E)-VC ura4-D18 h-	Figs. 4*G* and 5*B*	This study
CF.3144	cnp3Δ:KanR ade6-m210 Leu1-32:ase1P*-cnp3-GFP h-	Fig. 4*H*	This study
CF.3238	cnp3Δ:KanR ade6-m210 Leu1-32:ase1P*-cnp3(28T→E)-GFP h-	Fig. 4*H*	This study
CF.3529	cnp3Δ:NatR sid4-GFP:HygR mCherry-atb2: HygR ade6-m210? Leu1-32:ase1P*-cnp3-VC ura4-D18 h-	Fig. 5*E*	This study
CF.3531	cnp3Δ:NatR sid4-GFP:HygR mCherry-atb2: HygR ade6-m210? Leu1-32:ase1P*-cnp3(28T→E)-VC ura4-D18 h-	Fig. 5*E*	This study
CF.3926	cnp3Δ:KanR bub1-GFP:URA4+ sid4-mTomato: NatR ade6-m210? Leu1-32:ase1P*-cnp3-VC ura4-D18? h?	Fig. 5*F*	This study
CF.3928	cnp3Δ:KanR bub1-GFP:URA4+ sid4-mTomato: NatR ade6-m210? Leu1-32:ase1P*-cnp3(28T→E)-VC ura4-D18? h?	Fig. 5*F*	This study

Table S3. Plasmids used in this study

Plasmid	Genotype	Source
pCF.2090	pJK148-ase1P*-cnp3-GFP	This study
pCF.2134	pJK148-ase1P*-cnp3(28T→A)-GFP	This study
pCF.2135	pJK148-ase1P*-cnp3(28T→E)-GFP	This study
pCF.2143	pJK148-ase1P*- cnp3-VC	This study
pCF.2145	pJK148-ase1P*- cnp3(28T→E)-VC	This study

Table S4. Imaging conditions for the strains in this study

Figure	Parameters
Fig. 4*D*	Maximum projection live-cell *z* stack images: 11 optical planes, 0.5 μm step size, 2 min intervals, 5% laser power, 500 ms mCherry exposure, 5% laser power, 500 ms GFP exposure
Fig. 4*E*	Maximum projection live-cell *z* stack images: 11 optical planes, 0.5 μm step size, 2 min intervals, 5% laser power, 500 ms mCherry exposure, 5% laser power, 500 ms GFP exposure
Fig. 5*A*	Maximum projection *z* stack images: 21 optical planes, 0.25 μm step size, 1 min intervals, 500 ms mTomato exposure, 500 ms GFP exposure, 3× EM gain
Fig. 5*B*	Maximum projection *z* stack images: 21 optical planes, 0.25 μm step size, 1 min intervals, 500 ms mTomato exposure, 500 ms GFP exposure, 3× EM gain
Fig. 5*E*	Maximum projection live-cell *z* stack images: 11 optical planes, 0.5 μm step size, 30 s intervals, 5% laser power, 500 ms mCherry exposure, 5% laser power, 500 ms GFP exposure
Fig. 5*F*	Maximum projection live-cell *z* stack images: 11 optical planes, 0.5 μm step size, 1 min intervals, 5% laser power, 500 ms mCherry exposure, 5% laser power, 500 ms GFP exposure