Supplementary information
Synthetic-evolution reveals narrow paths to regulation of the *Saccharomyces cerevisiae* mitotic kinesin-5 Cin8

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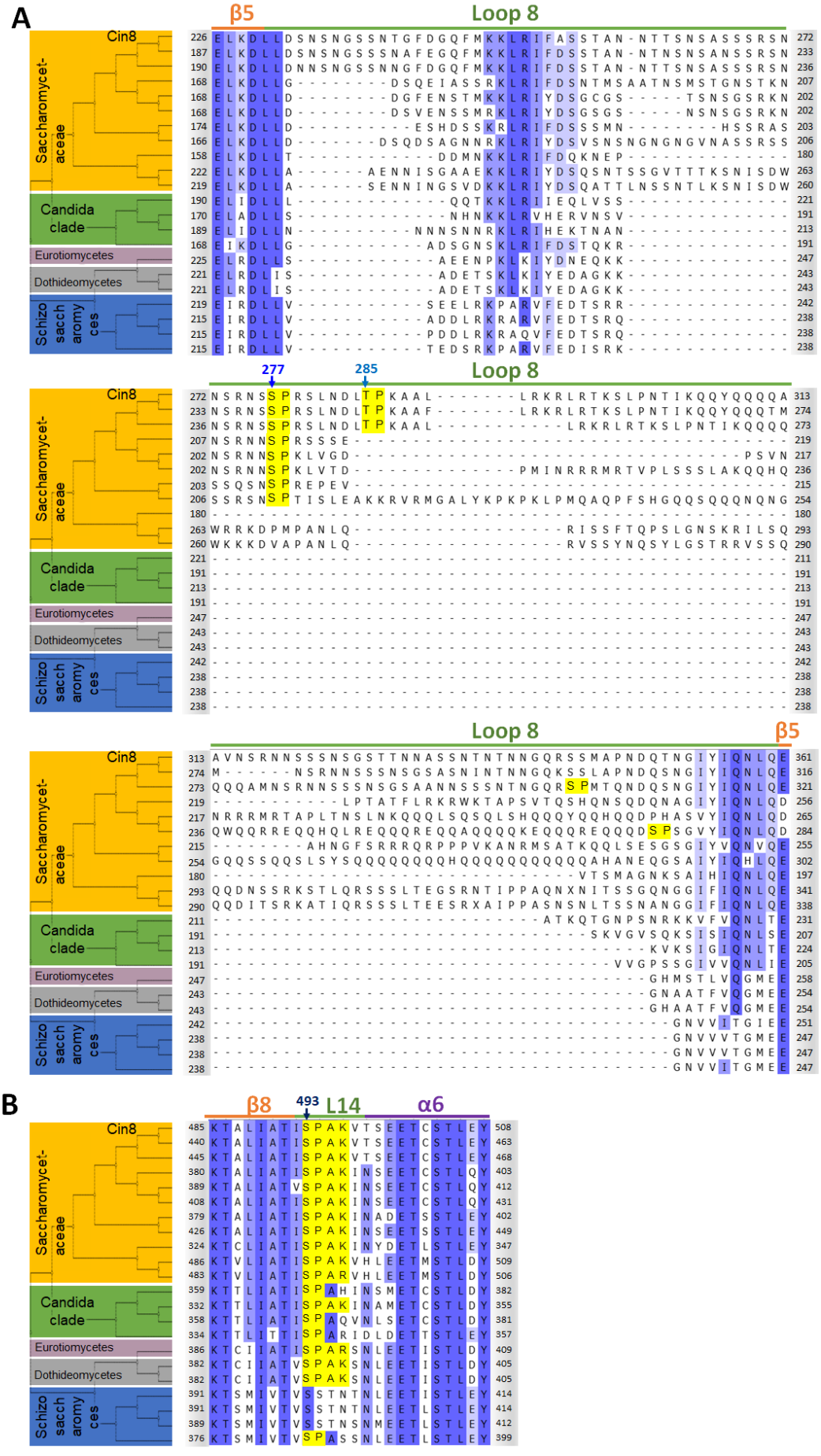
Running title: Evolution of phosphoregulation of kinesin-5 by Cdk


A
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277
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Dothideomycetes
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Figure S1 – Multiple sequence alignment of members of the kinesin-5 family.
(A) Loop-8 region and **(B)** beta-8, loop-14 and alpha-6 regions – location of the native Cdk1 phosphorylation consensuses S277, T285 and S493 in Cin8. Alignment was performed by Unipro UGENE alignment tool using MUSCLE algorithm; degree of identity in the sequence is indicated by blue highlight according to 50% threshold. Secondary structure is annotated according to [1]. The different clades are indicated on the left. Saccharomycetaceae clade (orange), Candida clade (green), Eurotiomycetaes clade (dark pink), Dothideomycetes clade (gray) and Schizosaccharomyces clade (blue). Native Cdk1 phosphorylation sites in Cin8 are annotated (277, 285 and 493). The species from top to bottom: *Saccharomyces cerevisiae (Cin8), Saccharomyces arboricola, Saccharomyces kudriavzevii, Torulaspora delbrueckii, Zygosaccharomyces bailii, Zygosaccharomyces rouxii, Eremothecium gossypii, Kluyveromyces marxianus, Cyberlindnera fabianii, Hanseniaspora uvarum, Hanseniaspora opuntiae, Lodderomyces elongisporus, Candida orthopsilosis, Candida albicans, Meyerozyma guilliermondii, Aspergillus nidulans, Pseudogymnoascus destructans, Pseudogymnoascus verrucosus, Schizosaccharomyces pombe, Schizosaccharomyces cryophilus, Schizosaccharomyces octosporus, Schizosaccharomyces japonicas*.

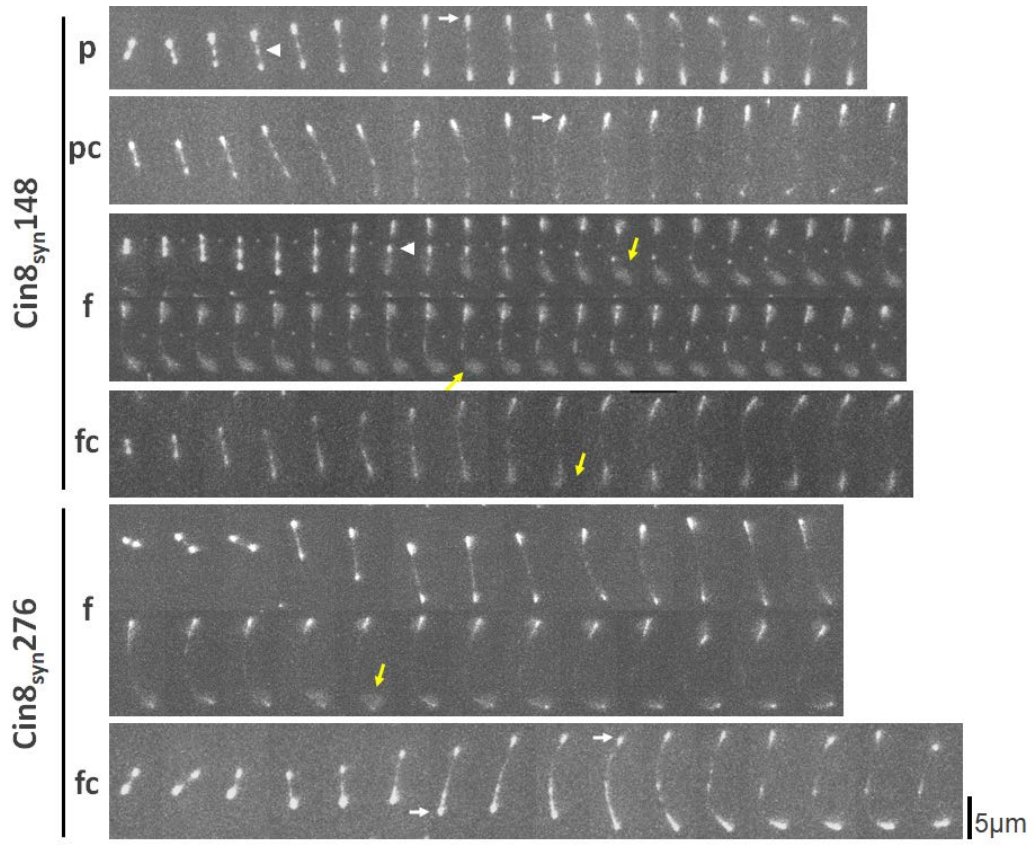

Figure S2: Time-lapse images of anaphase spindle elongation of synthetic Cin8 phospho-variants tagged with 3GFP.
Time-lapse images of synthetic mutants $Cin8_{syn}148$ and $Cin8_{syn}276$ tagged with 3GFP in *cin8Δ* strains; p – partial; pc – partial control; f – full; fc – full control. Yellow arrows indicate Cin8 detachment from the SPBs, white arrows indicate Cin8 concentration at the SPBs, and white arrowheads indicate Cin8 distribution at the midzone. Time difference between frames: 1 min; scale bar: 5 µm. These images were used for calculation of spindle elongation rates (Figs. 4E and S5) and for Cin8 detachments from the spindle perpendicular to the spindle as a function of time (Figs. S3).

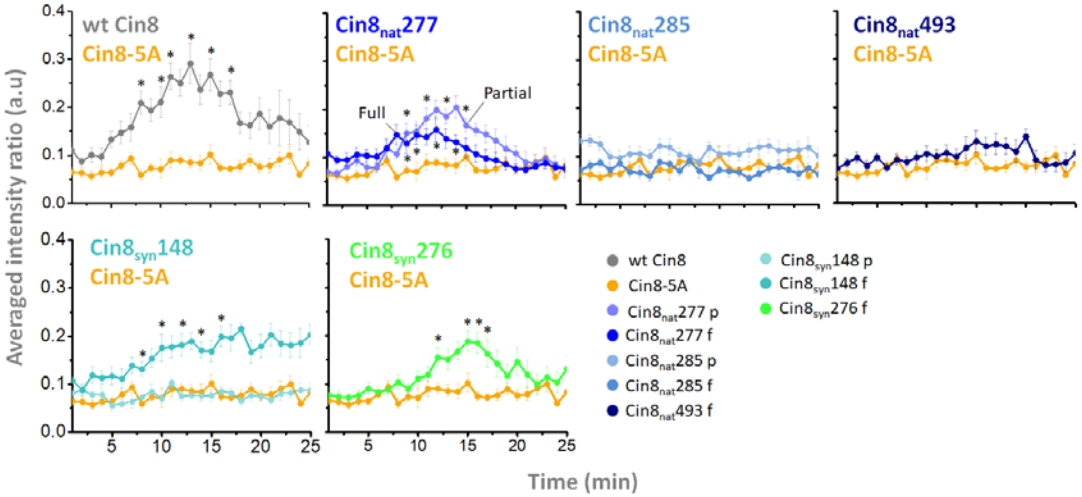

Figure S3: Detachment of the $Cin8_{nat}$ and $Cin8_{syn}$ phospho-variants from the spindle as a function of time during anaphase B.
The different variants, indicated on top, are compared to Cin8-5A (orange) calculated by measurement of intensity perpendicular to the spindle based on images from Fig. S2 (see Materials and Methods). Color coding of the different mutants is indicated on the bottom right. At each time point, the average intensity ratio in arbitrary units ± s.e.m. of 9-12 cells is presented. Significance was determined compared to Cin8-5A. *$p < 0.05$.

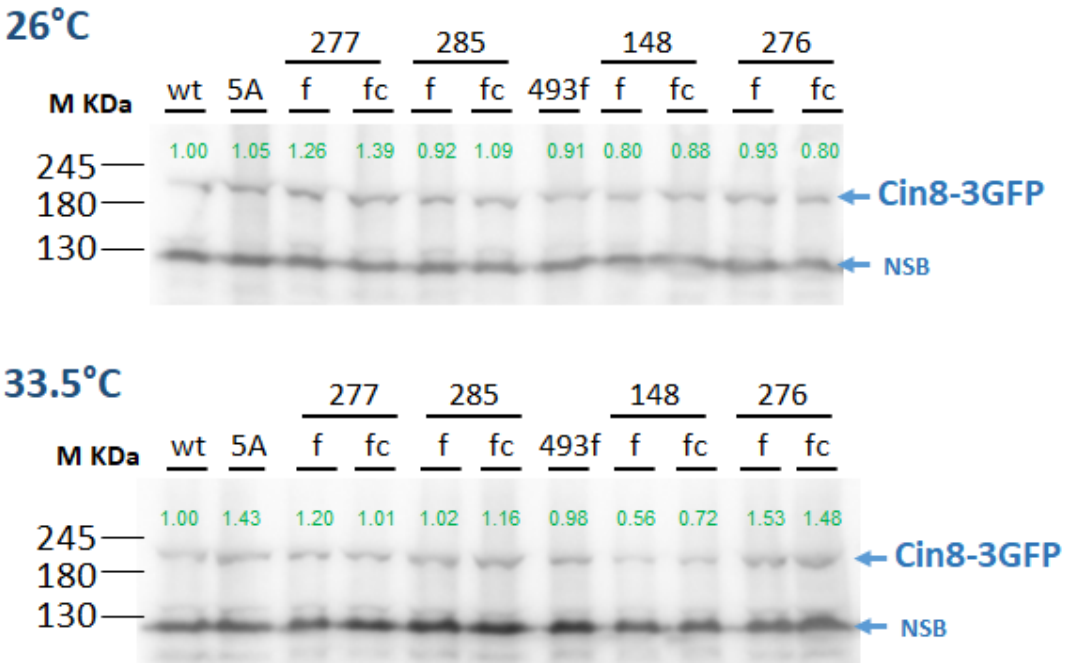

Figure S4: Native and synthetic mutants are stable at all temperatures.
SDS-PAGE followed by an α-GFP-HRP Western blot of native and synthetic mutants (indicated on top) of full Cdk1 phosphorylation consensus sites (f), and their respective controls (fc) tagged with three tandem copies of GFP. Cells expressing the various variants were grown at 26°C and 33.5°C (see Materials and Methods). Arrows point to the Cin8-3GFP and a non-specific band (NSB). Numbers in green indicate the intensity of Cin8 band relative to the intensity of the NSB, normalized to the intensity of wt Cin8-3GFP. Molecular weight ladder is indicated on the left (M, kDa).

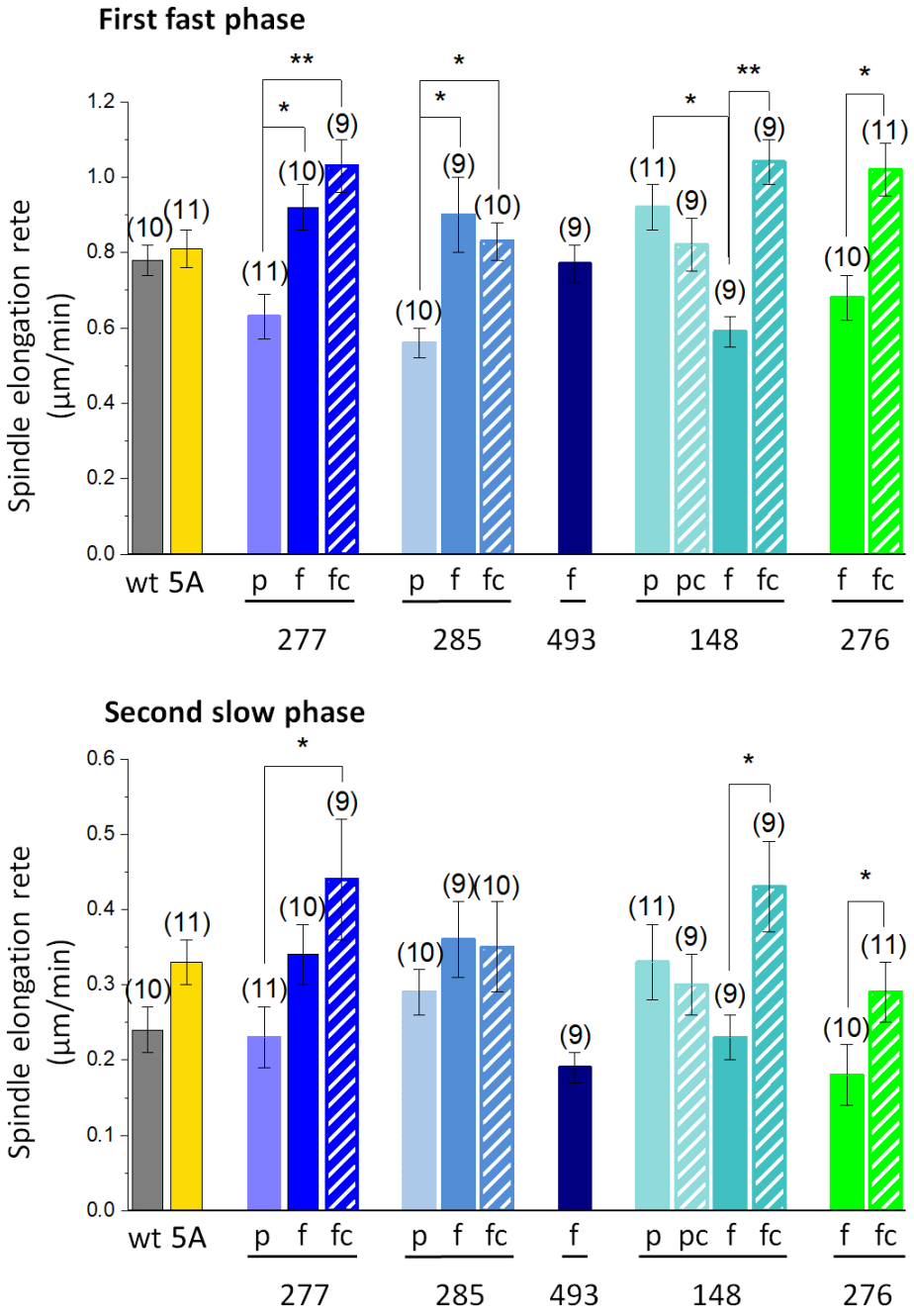

Figure S5: Spindle elongation rates of the first-fast (top) and the second-slow (bottom) phases of the partial, full, and full control of native and selected synthetic variants.
Averages ± s.e.m. of 9-12 cells are shown, number of cells are indicated on top of each column in brackets. Phospho-variants of Cin8 are indicated on the bottom. Measurements were performed for partial (p), partial control (pc), full (f) and full control (fc) Cdk1 phosphorylation consensuses. Significance was determined between the partial, full, and full-control variants of the same Cdk1 site. *$p < 0.05$, **$p < 0.005$.

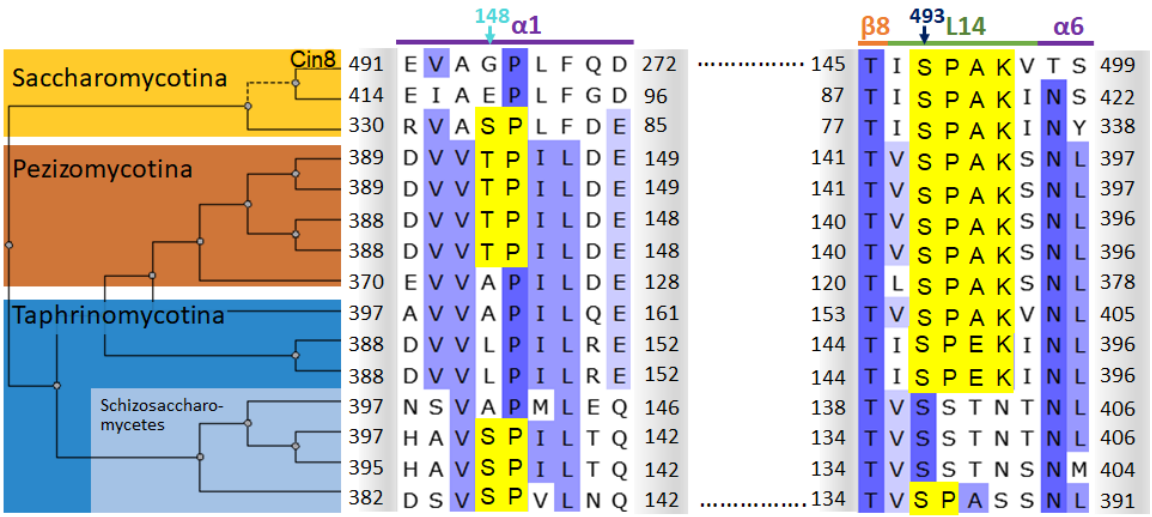

Figure S6: Multiple sequence alignment of members of the kinesin-5 family from three different subphylums of Ascomycota. MSA of the region around position 148 (cyan) and position 493 (blue) in Cin8. Alignment was performed by Unipro UGENE alignment tool using the MUSCLE algorithm; blue highlight indicates >50% threshold. Secondary structure is annotated according to [1]. The different subphylums are indicated on the left. Saccharomycotina (orange), Pezizomycotina (brown), and Taphrinomycotina (blue), the Schizosaccharomycetes class inside the subphylum Taphrinomycotina is highlighted in light blue. The species from top to bottom: *Saccharomyces cerevisiae (Cin8), Saccharomyces arboricola, Zygosaccharomyces rouxii, Cyberlindnera fabianii, Pezoloma ericae, Meliniomyces bicolor, Pseudogymnoascus destructans, Pseudogymnoascus verrucosus, Pseudocercospora fijiensis, Saitoella complicate, Pneumocystis carinii, Pneumocystis murina, Schizosaccharomyces pombe, Schizosaccharomyces cryophilus, Schizosaccharomyces octosporus, Schizosaccharomyces japonicas*.

Table S1: Plasmids 1.

Plasmid name	Genotype
pMA1208 [2]	*CIN8, CYH2, LEU2, CEN*
pVF68 [3]	*CIN8-3GFP, URA3, CEN*
pSAG51	*Cin8-2A-3GFP, URA3, CEN*
pAG30	*Cin8-5A-3GFP, URA3, CEN*
pMP10 [3]	*Cin8 (1-590)-TEV-EGFP-6His*
pMP11 [3]	*Cin8 (1-590)-S277A, T285A, S493A-TEV-EGFP-6His*
pSAG14	*Cin8(1-590)-V99S, S277A, T285A, S493A-TEV-EGFP-6His*
pSAG15	*Cin8(1-590)-V99S, I102K, S277A, T285A, S493A-TEV-EGFP-6His*
pSAG18	*Cin8(1-590)-G148P, S277A, T285A, S493A-TEV-EGFP-6His*
pSAG19	*Cin8(1-590)-G148S, F151P, S277A, T285A, S493A-TEV-EGFP-6His*
pSAG20	*Cin8(1-590)-T170P, S277A, T285A, S493A-TEV-EGFP-6His*
pSAG21	*Cin8(1-590)-S269P, S277A, T285A, S493A-TEV-EGFP-6His*

pSAG22	*Cin8(1-590)-A277P, T285A, S493A-TEV-EGFP-6His*
pSAG23	*Cin8(1-590)-S277A, T285A, S280K, S493A-TEV-EGFP-6His*
pSAG24	*Cin8(1-590)-S277A, A285S, A288K, S493A-TEV-EGFP-6His*
pSAG29	*Cin8(1-590)-T285A, S493A-TEV-EGFP-6His*
pSAG30	*Cin8(1-590)-S277A, T285S, S493A-TEV-EGFP-6His*
pSAG31	*Cin8(1-590)-S277A, T285A-TEV-EGFP-6His*

Table S2: Plasmids 2.
The following plasmids were based on Cin8-5A (pAG30).

Cin8 variant		Plasmid name	Genotype
Cin8$_{syn}$3	partial	pCin8-1	*Cin8-W3S-3GFP, URA3, CEN*
	full	pCin8-2	*Cin8-W3S, S6K-3GFP, URA3, CEN*
Cin8$_{syn}$21	full	pCin8-3	*Cin8-N22P-3GFP, URA3, CEN*
Cin8$_{syn}$39	partial	pCin8-4	*Cin8-M39S-3GFP, URA3, CEN*
	p.cont	pCin8-4SA	*Cin8-M39A-3GFP, URA3, CEN*
	full	pCin8-5	*Cin8-M39S, E42K-3GFP, URA3, CEN*
	f.cont	pCin8-5SA	*Cin8-M39A, E42K-3GFP, URA3, CEN*
Cin8$_{syn}$54	full	pCin8-6	*Cin8-I55P-3GFP, URA3, CEN*
Cin8$_{syn}$68	partial	pCin8-7	*Cin8-V68S-3GFP, URA3, CEN*
	full	pCin8-8	*Cin8-V68S, E71K-3GFP, URA3, CEN*
Cin8$_{syn}$99	partial	pSAG41	*Cin8-V99S-3GFP, URA3, CEN*
	p.cont	pLG84	*Cin8-V99A-3GFP, URA3, CEN*
	full	pCin8-10	*Cin8-V99S, I102K-3GFP, URA3, CEN*
	f.cont	pCin8-10SA	*Cin8-V99A, I102K-3GFP, URA3, CEN*
Cin8$_{syn}$103	full	pCin8-11	*Cin8-G104P-3GFP, URA3, CEN*
Cin8$_{syn}$128	full	pSAG42	*Cin8-V129P-3GFP, URA3, CEN*
	f.cont	pCin8-12SA	*Cin8-T128A, V129P-3GFP, URA3, CEN*
Cin8$_{syn}$134	partial	pCin8-13	*Cin8-G134S-3GFP, URA3, CEN*
	p.cont	pCin8-13SA	*Cin8-G134A-3GFP, URA3, CEN*
	full	pSAG43	*Cin8-G134S, A137K-3GFP, URA3, CEN*
	f.cont	pSAG44	*Cin8-G134A, A137K-3GFP, URA3, CEN*
Cin8$_{syn}$148	partial	pCin8-15	*Cin8-G148S-3GFP, URA3, CEN*
	p.cont	pLG85	*Cin8-G148A-3GFP, URA3, CEN*
	full	pCin8-16	*Cin8-G148S, F151K-3GFP, URA3, CEN*
	f.cont	pCin8-16SA	*Cin8-G148A, F151K-3GFP, URA3, CEN*
Cin8$_{syn}$169	full	pCin8-17	*Cin8-T170P-3GFP, URA3, CEN*
	f.cont	pCin8-17SA	*Cin8-S169A, T170P-3GFP, URA3, CEN*
Cin8$_{syn}$194	partial	pCin8-18	*Cin8-I194S-3GFP, URA3, CEN*
	full	pCin8-19	*Cin8-I194S, V197K-3GFP, URA3, CEN*
	f.cont	pCin8-19SA	*Cin8-I194A, V197K-3GFP, URA3, CEN*

Cin8$_{syn}$268	full	pSAG45	*Cin8-S269P-3GFP, URA3, CEN*
Cin8$_{syn}$276	full	pCin8-23	*Cin8-S277P-3GFP, URA3, CEN*
	f.cont	pCin8-23SA	*Cin8-S276A, S277P-3GFP, URA3, CEN*
Cin8$_{nat}$277	partial	pCin8-24	*Cin8-A277S-3GFP, URA3, CEN*
	full	pCin8-25	*Cin8-A277S, S280K-3GFP, URA3, CEN*
	f.cont	pCin8-25SA	*Cin8-S280K-3GFP, URA3, CEN*
Cin8$_{nat}$285	partial	pCin8-26	*Cin8-A285S-3GFP, URA3, CEN*
	full	pCin8-27	*Cin8-A285S, A288K-3GFP, URA3, CEN*
	f.cont	pCin8-27SA	*Cin8-A288K-3GFP, URA3, CEN*
Cin8$_{syn}$300	partial	pCin8-28	*Cin8-L300S-3GFP, URA3, CEN*
	full	pCin8-29	*Cin8-L300S, T303K-3GFP, URA3, CEN*
Cin8$_{syn}$346	partial	pCin8-30	*Cin8-A346S-3GFP, URA3, CEN*
	full	pCin8-31	*Cin8-A346S, D349K-3GFP, URA3, CEN*
Cin8$_{syn}$402	full	pCin8-32	*Cin8-L403P-3GFP, URA3, CEN*
	f.cont	pCin8-32SA	*Cin8-T402A, L403P-3GFP, URA3, CEN*
Cin8$_{syn}$450	full	pCin8-33	*Cin8-L451P-3GFP, URA3, CEN*
	f.cont	pCin8-33SA	*Cin8-T450A, L451P-3GFP, URA3, CEN*
Cin8$_{syn}$465	full	pCin8-34	*Cin8-I465T-3GFP, URA3, CEN*
Cin8$_{nat}$493	full	pLG86	*Cin8-A493S-3GFP, URA3, CEN*
Cin8$_{syn}$510	full	pCin8-36	*Cin8-K511P-3GFP, URA3, CEN*
	f.cont	pCin8-36SA	*Cin8-S510A, K511P-3GFP, URA3, CEN*
Cin8$_{syn}$518	partial	pCin8-37	*Cin8-K518S-3GFP, URA3, CEN*
	full	pCin8-38	*Cin8-K518S, L521K-3GFP, URA3, CEN*
Cin8$_{syn}$548	full	pLG87	*Cin8-K549P-3GFP, URA3, CEN*
Cin8$_{syn}$700	partial	pCin8-40	*Cin8-Q700S-3GFP, URA3, CEN*
	full	pCin8-41	*Cin8-Q700S, L703K-3GFP, URA3, CEN*
Cin8$_{syn}$849	full	pCin8-42	*Cin8-E849S-3GFP, URA3, CEN*
Cin8$_{syn}$881	full	pCin8-43	*Cin8-E882P-3GFP, URA3, CEN*
	f.cont	pCin8-43SA	*Cin8-S881A, E882P-3GFP, URA3, CEN*
Cin8$_{syn}$899	full	pCin8-44	*Cin8-V900P-3GFP, URA3, CEN*
Cin8$_{syn}$913	full	pCin8-45	*Cin8-L914P-3GFP, URA3, CEN*

Cin8$_{syn}$986	partial	pCin8-46	*Cin8-I986S-3GFP, URA3, CEN*
	p.cont	pCin8-46SA	*Cin8-I986A-3GFP, URA3, CEN*
	full	pCin8-47	*Cin8-I986S, L989K-3GFP, URA3, CEN*
	f.cont	pCin8-47SA	*Cin8-I986A, L989K-3GFP, URA3, CEN*
Cin8$_{syn}$993	full	pCin8-48	*Cin8-M993S-3GFP, URA3, CEN*

Table S3: Yeast strains

Yeast strain	**Genotype**
LGY 620 [3]	*MATa, ura3-52, leu2-3,112, his3-Δ200, lys2-801, ade2-101, cyh2^r, cin8::HIS3, kip1::HIS3, (pMA1208: CIN8, CYH2, LEU2, CEN)*
LGY 727 [3]	*MATa, ura3-52, leu2-3,112, his3-Δ200, lys2-801, ade2-101. cin8::LEU2*

Supplemental references

1. Subbiah S, Harrison SC. A simulated annealing approach to the search problem of protein crystallography. Acta Crystallogr A. 1989; 45 (Pt 5): 337-42.
2. Gheber L, Kuo SC, Hoyt MA. Motile properties of the kinesin-related Cin8p spindle motor extracted from Saccharomyces cerevisiae cells. J Biol Chem. 1999; 274: 9564-72.
3. Goldstein A, Siegler N, Goldman D, Judah H, Valk E, Koivomagi M, et al. Three Cdk1 sites in the kinesin-5 Cin8 catalytic domain coordinate motor. Cell Mol Life Sci. 2017; 28: 017-2523.