

Expanded View Figures

Figure EV1. Refers to Fig. 1: Detailed mutagenesis analysis of the Zwint helix.

(A) Multiple sequence alignment of Kre28 sequences from different yeast species. Conserved residues are colored according to the Clustal color scheme. Positions of smaller Zwint helix deletions and alanine substitutions of conserved residues are marked. (B) Western blot analysis of different Kre28/Zwint deletion or point mutants. Extracts of log-phase yeast cells were analyzed, Pgk1 blot serves as a loading control. (C, D) Serial dilution assays of haploid yeast strains with different Kre28 Zwint helix deletions or point mutations of conserved residues. Strains with the indicated genotypes were serially diluted and spotted on YEPD or YEPD + benomyl plates and incubated at the indicated temperatures.

	Δ77-90										Δ91-102										Δ103-113										Q120																			
	I80										K90										L100										R110										Q120									
<i>Saccharomyces cerevisiae</i>	F	V	N	P	K	I	L	S	A	K	I	K	E	F	N	K	L	M	E	L	L	K	L	T	Y	L	E	Q	E	T	L	D	Y	F	F	R	F	T	L	S	S	T	-	K	P	L	Q			
<i>Candida glabrata</i>	L	I	D	I	K	Q	L	E	V	K	S	R	E	L	D	S	L	M	D	L	L	R	K	L	Y	W	R	E	S	L	D	L	F	L	K	Y	T	I	N	S	D	A	E	Q	V	P				
<i>Kluyveromyces lactis</i>	Q	C	D	P	S	E	I	N	L	P	L	E	K	L	S	W	T	N	S	L	K	Q	I	H	L	E	L	E	S	I	D	N	F	L	R	Y	A	I	P	S	D	Q	T	I	L	N				
<i>Saccharomyces kudriavzevii</i>	Y	I	N	P	R	I	L	G	V	K	I	K	E	F	N	N	L	M	E	L	L	K	L	T	Y	L	E	Q	E	T	L	D	Y	F	F	R	F	T	L	S	S	T	-	K	P	L	Q			
<i>Ashbya gossypii</i>	H	I	D	P	R	G	I	A	V	S	V	Q	Q	F	K	Q	L	A	E	V	L	K	V	T	H	L	E	Q	E	T	L	D	N	F	L	R	Y	T	I	S	D	N	D	Q	L	L	D			
<i>Vanderwaltozyma polyspora</i>	A	I	D	P	S	L	I	D	E	K	I	K	Q	S	N	N	F	V	E	L	L	K	L	T	H	L	E	Q	E	A	L	D	Y	F	L	R	Y	T	I	S	S	T	-	N	T	L	E			
<i>Zygosaccharomyces rouxii</i>	V	I	D	P	S	G	F	Q	E	K	I	D	T	F	V	E	L	V	E	L	L	K	V	T	H	L	E	Q	E	T	L	D	N	F	L	R	Y	T	I	S	S	S	-	N	L	L	Q			
<i>Zygosaccharomyces parabalii</i>	I	V	D	P	T	C	L	K	E	K	I	D	T	F	A	K	L	V	E	L	L	K	L	T	H	L	E	Q	E	T	L	D	Y	F	L	R	Y	T	I	S	S	T	-	N	L	L	Q			
																				↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓							
																				<i>kre28</i> ^{4A}										<i>kre28</i> ^{3A}										<i>kre28</i> ^{6A}										

Western blot analysis of Kre28-6xFlag and Pgk1 expression. The top panel shows Kre28-6xFlag levels, and the bottom panel shows Pgk1 levels. The strains are WT, Δ Zwint, Δ 77-90, Δ 91-102, Δ 103-113, 4A, 3A, and 6A. Molecular weight markers (MW) are indicated on the left in kDa (70, 55, 35). The anti-Flag antibody detects Kre28-6xFlag, and the anti-Pgk1 antibody detects Pgk1.

Figure 1: Spot assay of Kre28WT and kre28 mutants. The figure shows four panels of spot assays. The first panel (YEPD, 16 °C) shows growth for all strains. The second panel (YEPD, 30 °C) shows growth for all strains. The third panel (YEPD + benomyl, 30 °C) shows growth for Kre28WT and kre28 Δ Zwint, but no growth for kre28^{4A}, kre28^{3A}, and kre28^{6A}. The fourth panel (YEPD, 37 °C) shows growth for all strains.

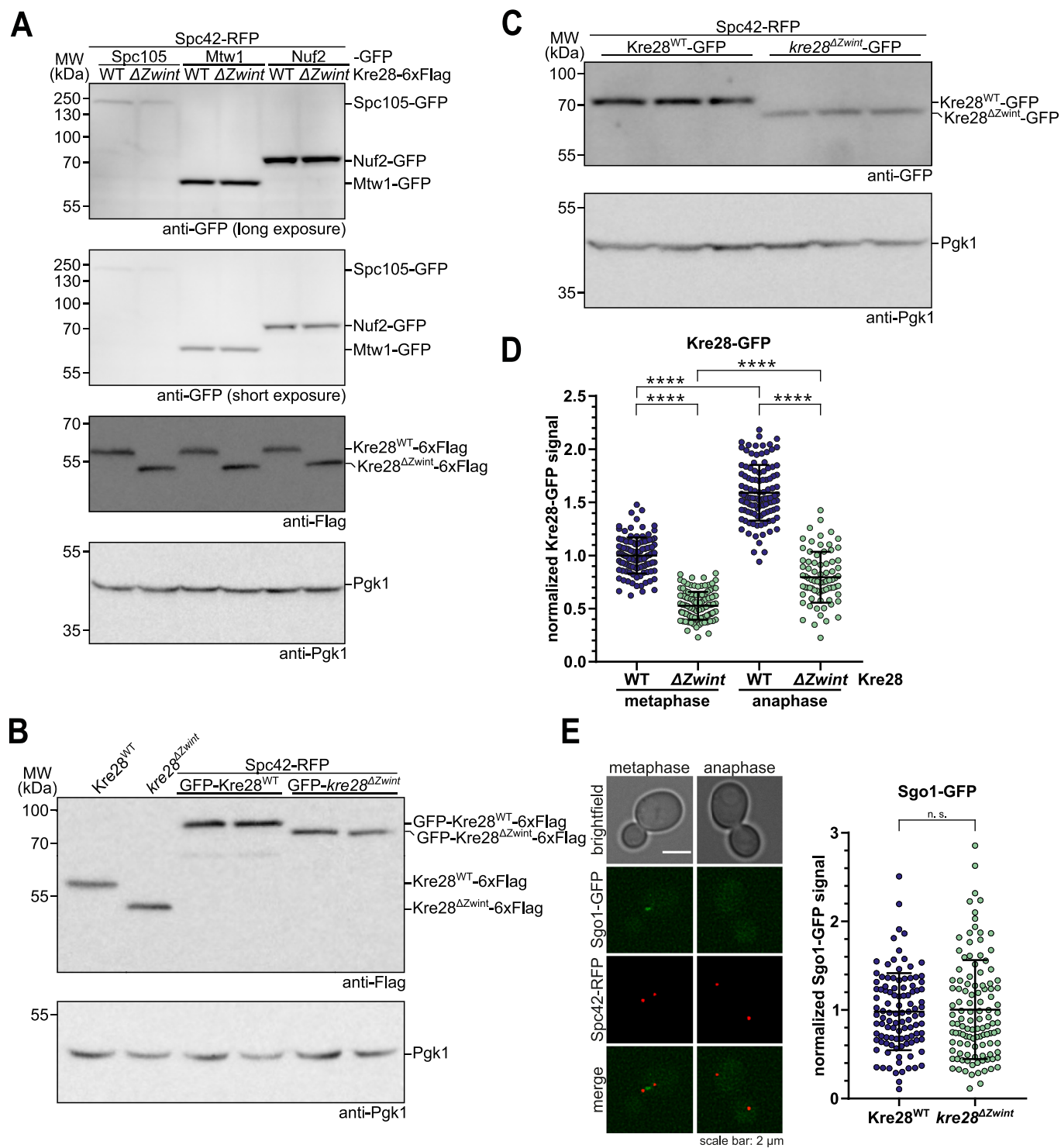




Figure EV2. Refers to Fig. 3: Western blot analysis of GFP-tagged KMN subunits and live-cell microscopy of Sgo1-GFP.

(A) Western blot analysis to compare the expression of different GFP-tagged KMN subunits, in Kre28 wild-type and mutant cells. Pgk1 served as loading control. (B) Western blot analysis of expression level of N-terminal GFP-tagged Kre28 in wild-type and mutant form. (C) Western blot analysis of C-terminal GFP-tagged Kre28 in wild-type and mutant form. (D) Quantification of Kre28-GFP fluorescence signals at metaphase and anaphase kinetochores. Strains with either Kre28^{WT}-GFP or kre28^{ΔZwint}-GFP were analyzed by live-cell microscopy. The C-terminus of Spc42 was fused to RFP to label the position of the mitotic spindle. The mean of metaphase signal intensities in the wild-type strain was defined as 1 and all other data was normalized to this value. Means $\pm$ standard deviation are plotted. *P* values were calculated with a Kruskal-Wallis test, and displayed as follows: *****P* < 0.0001. Exact *P* values for (E): in all cases ****<0.0001. *n* > 100 kinetochore clusters were analyzed for each condition. Representative data from two independent experiments is shown. (E) Live-cell microscopy for analysis of Sgo1-GFP kinetochore localization in a Kre28^{WT} or kre28^{ΔZwint} strain background. Representative images of a metaphase and an anaphase cell are shown on the left and quantification of Sgo1-GFP signals in cells with short mitotic spindles is shown on the right. Data was normalized to the mean value measured for the Kre28^{WT} strain. Mean values $\pm$ standard deviation are plotted. A Mann-Whitney test was used to test for statistical significance.

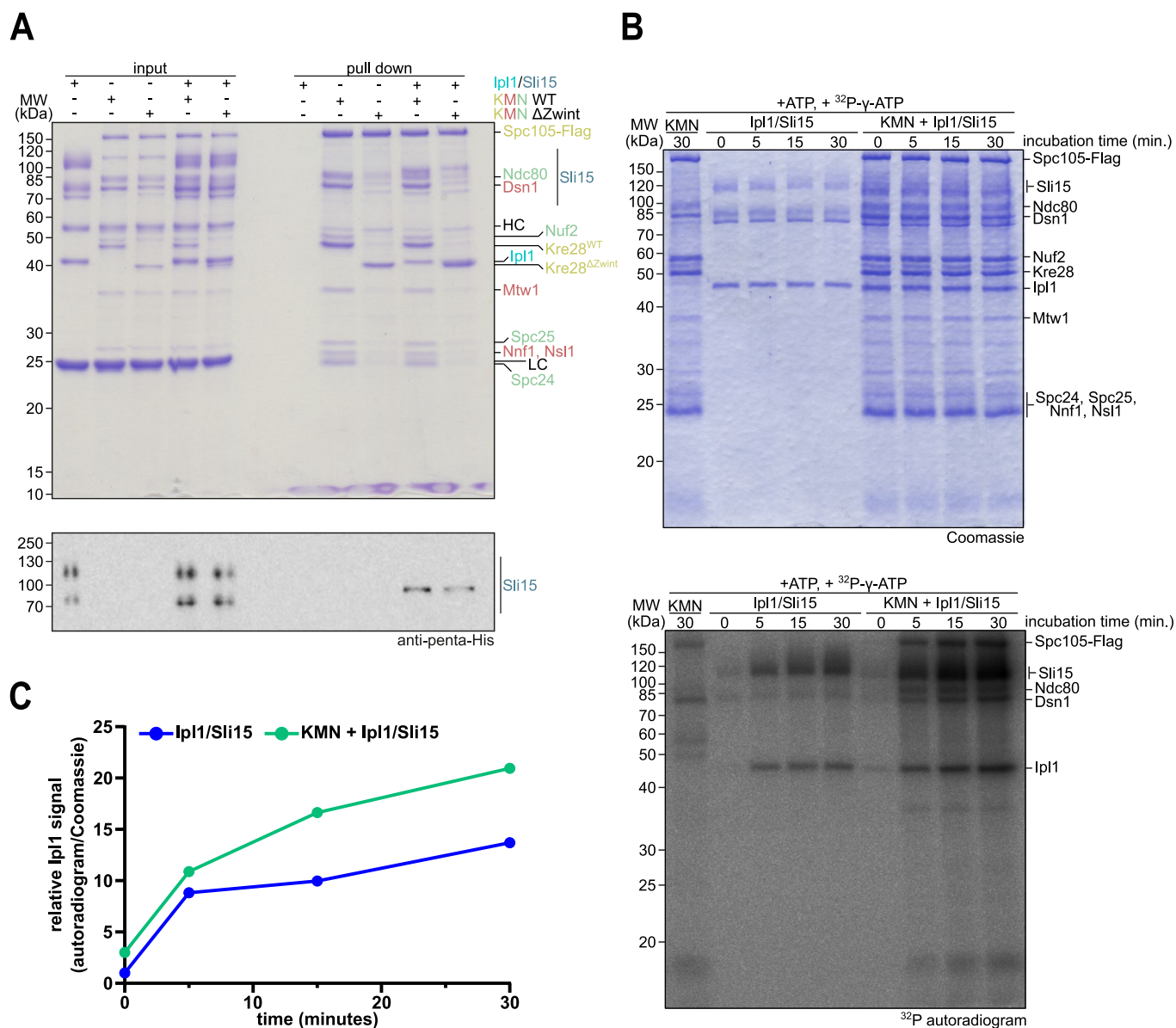
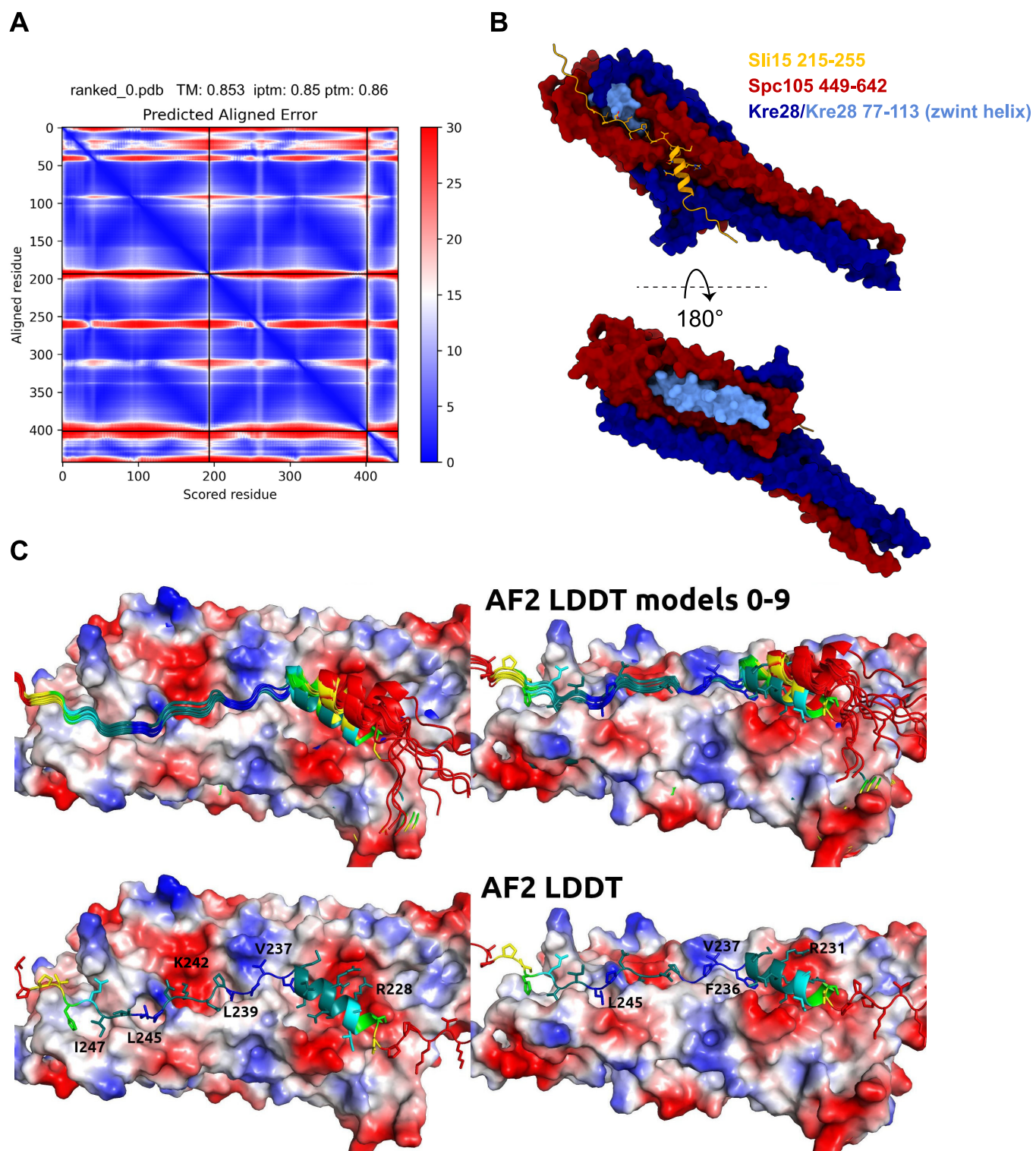


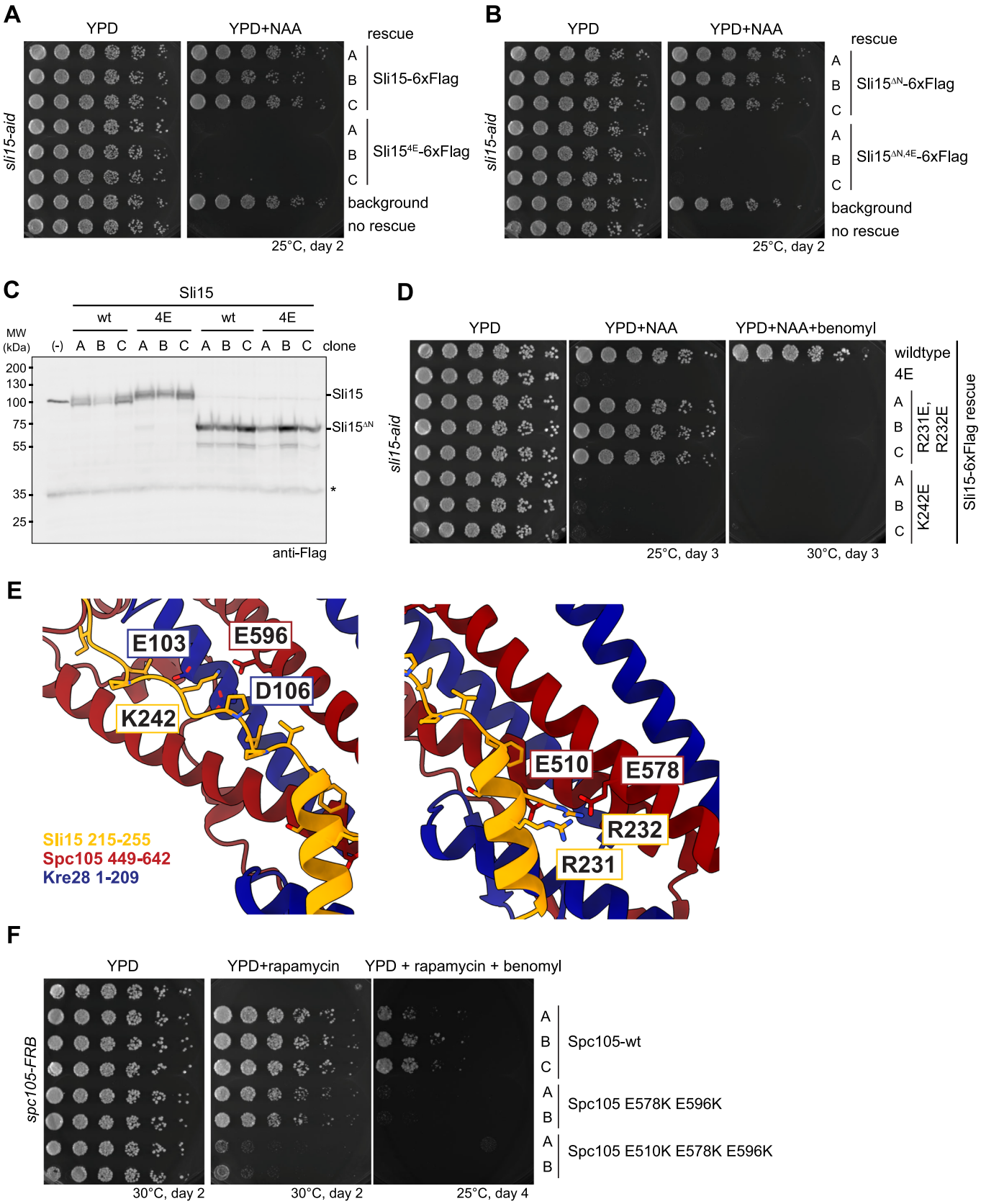
Figure EV3. Refers to Fig. 5: Pull-down experiment and in vitro kinase reaction with Ipl1/Sli15 and KMN.

(A) Pull-down assays to analyze binding of Ipl1/Sli15 to KMN. KMN with either Kre28^{WT} or Kre28 ^{Δ Zwint} was purified from Sf9 cells and immobilized on M2 anti-Flag beads. Loaded beads were incubated with buffer or recombinant Ipl1/Sli15, afterwards proteins bound to the beads were eluted with 3xFlag peptide. Input and pull-down samples were analyzed by SDS-PAGE and Coomassie staining or western blot to detect 6xHis-tagged Sli15. (B) In vitro kinase reaction with Ipl1/Sli15 or Ipl1/Sli15 in the presence of recombinant KMN analyzed over time. Coomassie-stained gel at the top, corresponding autoradiograph at the bottom. For control a KMN only sample taken 30 min after incubation with radioactive ATP is shown in the first lane. (C) Quantification phosphorylated Ipl1 signal over time alone (blue curve) or in the presence of KMN (green curve).



**Figure EV4. Refers to Fig. 7.**

(A) PAE plot of the top-ranked model of the Spc105 (449–642)-Kre28 (1–208)- Sli15 (215–255) complex. Fragment boundaries are indicated by black lines, blue are favorable scores, red are unfavorable. (B) Position of the Kre28 Zwint helix (light blue) in the top-ranked model. Note that only a part of the Zwint helix is involved in the binding interface with Sli15, while the majority of the helix is on the opposite side of the Spc105/Kre28 bundle. (C) Top row: Superposition of the ten top-ranked models of the Sli15 peptide on Spc105/Kre28. pLDDT scores for the Sli15 peptides are indicated with blue and green showing favorable, and red unfavorable scores. The Spc105/Kre28 surface is colored by electrostatic potential. Bottom: Details of the top-ranked model, with key side chains of the Sli15 peptide indicated as sticks. Coloring by pLDDT scores as above.



**Figure EV5. Refers to Fig. 7.**

(A) Serial dilution assay testing full-length Sli15 wild-type and -4E rescue constructs in a *sli15-aid* system. Three clones for wild type and mutant are tested. Background indicates a strain lacking *sli15-aid*, no rescue indicates a *sli15-aid* strain without a Sli15 rescue construct. (B) Serial dilution assay testing Sli15-ΔN wild-type and -4E rescue constructs in a *sli15-aid* system. Three clones for each version are tested. Background indicates a strain lacking *sli15-aid*, no rescue indicates a *sli15-aid* strain without a Sli15 rescue construct. (C) Western Blot analysis of Sli15-Flag rescue constructs tested in A and B. Asterisk denotes unspecific band. (D) Serial dilution assay testing the indicated Sli15 rescue constructs in a *sli15-aid* system. Three clones for each version (Sli15 R231E R232E, or Sli15 K242E) are tested. (E) Left side: Details of the predicted binding site surrounding Sli15 K242 (orange) with interacting residues in Kre28 (dark blue) or Spc105 (dark red) highlighted. Right side: Details of the predicted binding site surrounding Sli15 residues R231 and R232. (F) Serial dilution assay testing the effects of Spc105 mutations predicted to affect the Sli15-binding site. Spc105 rescue constructs are tested in an Spc105-FRB anchor-away system in which upon rapamycin addition the endogenous Spc105 protein is removed from the nucleus. Top row: control lacking a rescue construct. Three clones for wild-type rescue constructs, and two clones of each mutant are tested.