Appendix – Table of Contents

Appendix Figure Legends:

Figure S1:

Mus81-Mms4 forms a complex in mitosis with kinases and scaffold proteins, and is a target to phosphorylation by these kinases.

(A) SILAC-based quantification of Mms4^{3FLAG} pulldowns in untagged vs *MMS4^{3FLAG}* cells after G2/M arrest with nocodazole. H/L ratios from two label-switch experiments without ratio count cut-off are plotted. #, as the only protein of the analysis Dpb11 displayed exclusively peptides that were derived from the Mms4^{3FLAG} IP samples, but not the control samples. This experiment is already shown as Fig. S8A in Gritenaite *et al.*, 2014.

(B) Coomassie staining to show running behaviour of peptides used in Fig. 1C. Peptides 1-3 shift down upon increasing phosphorylation, whereas peptides 4-6 display an up-shift.

(C) Kinetic *in vitro* kinase assay. Purified, immobilized Mus81-Mms4 is either mock treated or treated with CDK in a non-radioactive priming step, and incubated with purified DDK (upper panel) or Cdc5 (lower panel). Samples were taken after indicated time points.

(D) Mus81-Mms4 *in vitro* phosphorylation is independent of DKK and/or CDK pre-phosphorylation. Purified, immobilized Mus81-Mms4 is incubated in an *in vitro* kinase assay with purified CDK2/cycA^{N170} (a model CDK), DDK or Cdc5 (lanes 1-4). Additionally, Mus81-Mms4 is incubated with respective kinases after a non-radioactive priming step with DDK (lanes 5-8) or CDK and DDK (lanes 9-12).

Figure S2:

DDK and Cdc5 target Mus81-Mms4 in an interdependent manner.

(A) Formation of the Mus81-Mms4 complex depends on Cdc5 activity. SILAC-based quantification of Mms4^{3FLAG} pulldowns in *WT* vs *cdc5-as1* cells after mitotic arrest with nocodazole and additional treatment with 15 μM CMK for 1 h. Plotted are the H/L ratios of two label-switch experiments.

(B) CDK activity is required for Mms4 hyperphosphorylation. Whole-cell extracts of *WT* and *cdc28-as1* cells arrested in mitosis, titrated with 1NM-PP1 as indicated.

(C) Phosphorylation shift of Mms4 in whole-cell extracts of mitotically arrested *WT* and mutant cells.

(D) Cdc5 association with Mus81-Mms4 is dependent on DDK activity. Mms4^{3FLAG} pulldown as in Fig. 1A. Cells were cultivated and arrested in mitosis at RT. Inhibition of

DDK was achieved by using the *cdc7-1* allele and shifting cells to permissive temperature (38 °C) for the indicated time.

(E) Effect of DDK and Cdc5 mutants on Cdc5 substrates. Phosphorylation of Cdc5 substrates Ulp2 and Scc1 (and as control Mms4) was tested, indicated by their phosphorylation shift in 7% Tris-Acetate gels in untagged, *WT*, *cdc5-as1* and *cdc7Δ* backgrounds. Western blot analysis of Ulp2^{9myc} and Scc1^{9myc} whole-cell extracts from alpha-factor- (G1) or nocodazole-arrested (G2/M) cells. Cdc5 was inhibited by treatment with 15 µM CMK for 1 h.

(F) DDK and Cdc5 association to Mus81-Mms4 is reduced when the DNA damage checkpoint is triggered by DNA damage induction. Mms4^{3FLAG} pulldown as in Fig. 1A, but in G2/M-arrested cells that were untreated or treated with 50 µg/ml phleomycin.

Figure S3:

Summary of Mms4 phosphorylation sites. Shown is the Mms4 primary amino acid sequence. Colours indicate phosphorylation sites on endogenous Mms4 that were affected in SILAC-based mass spectrometry experiments (Fig. 3A-B) by Cdc5 inhibition (blue), *CDC7* deletion (red) or in both backgrounds (green). Serine to alanine exchanges in the *mms4-8A* mutant are boxed. Additional serine to alanine exchanges in the *mms4-12A* mutant are boxed with a dashed line.

Figure S4:

DDK phosphorylation controls activation of Mus81-Mms4 resolvase activity in mitosis.

(A) Endogenous Mus81^{3FLAG}-Mms4 purified from mitotically arrested cells shows increased activity compared to non-phosphorylated recombinant protein expressed in yeast. Left panel: Western blot analysis for quantification of bead-bound protein levels of Mus81 (endogenous and recombinant) compared to increasing amounts of soluble recombinant Mus81. Approx. 5 fmol Mus81^{3FLAG} -Mms4 are used in the assay to cleave 500 fmol nHJ substrate. Right panel: Resolution assay using a nicked HJ substrate and comparing Mus81^{3FLAG}-Mms4 purified from mitotically arrested cells with recombinant, dephosphorylated Mus81^{3FLAG}-Mms4 in similar protein concentration.

(B,C) Interaction of Mus81-Mms4 with other complex factors such as Rtt107 and Cdc5 is salt-labile, but their absence does not influence Mus81-Mms4 activity.

(B) Mms4^{3FLAG} pulldown as in Fig. 1A from mitotically arrested cells, but proteins were washed on beads with either low salt (150 mM NaCl) or high salt buffer (350 mM NaCl).

(C) Left panel: Resolution assay using a nHJ substrate and Mus81^{9myc}-Mms4^{3FLAG} purified from mitotically arrested cells under low salt (150 mM NaCl) or high salt (350 mM NaCl) conditions. Right panel: Western blots samples of anti-myc IPs.

(D,F) Western blot analysis of Mus81^{9myc} IP samples that were used as inputs for the *in vitro* resolution assays of Fig. 4A and C, respectively.

(E) DDK is required for mitotic activation of Mus81-Mms4. Resolution assay using a replication fork (RF) substrate and Mus81^{9myc}-Mms4^{3FLAG} purified from mitotically arrested *bob1-1 (DDK+)* and *bob1-1 cdc7Δ* strains or untagged control cells. Lower panel: Western blots samples of anti-myc IPs.

Figure S5:

Dpb11 interacts with the N-terminal region of Mms4 and its binding is dependent on CDK activity.

(A) Dpb11 binds to a minimal interacting fragment of Mms4 comprising the residues 101-230. Two-hybrid analysis of GAL4-BD fused to Dpb11 and GAL4-AD fusions with Mms4 or Mms4 fragment constructs (left panel). Expression of constructs was verified by western blot analysis (right panel).

(B) CDK activity is required for Dpb11 and Slx4 association with Mus81-Mms4. Mms4^{3FLAG} pulldown as in Fig. 1A, but in G2/M-arrested *WT* and *cdc28-as1* mutant cells treated with 5 μM 1NM-PP1 for 1 h. This figure is from the same experiment as Fig. 2B and therefore as control includes the identical anti-Flag western.

(C) A defect in the Dpb11-Mms4 interaction introduces only a minor defect in Mus81 activation. Resolution assay using a nicked HJ substrate and Mus81^{9myc}-Mms4^{3FLAG} purified from mitotically arrested *WT* or *mms4-S201A* cells. Right panel: Western blots samples of anti-myc IPs.

Figure S6:

The Rtt107 scaffold tethers DDK and Cdc5 to Mus81-Mms4.

(A) Formation of the Mus81-Mms4 complex depends on Rtt107. SILAC-based quantification of Mms4^{3FLAG} pulldowns in *WT* vs *rtt107Δ* cells. Plotted are the H/L ratios of two experiments including label-switch.

(B) Rtt107 binding to Cdc5 and DDK is not affected by the presence of Mus81-Mms4. Rtt107^{3FLAG} pulldown as in Fig. 1A, but in G2/M-arrested *WT* and *mus81Δ* cells.

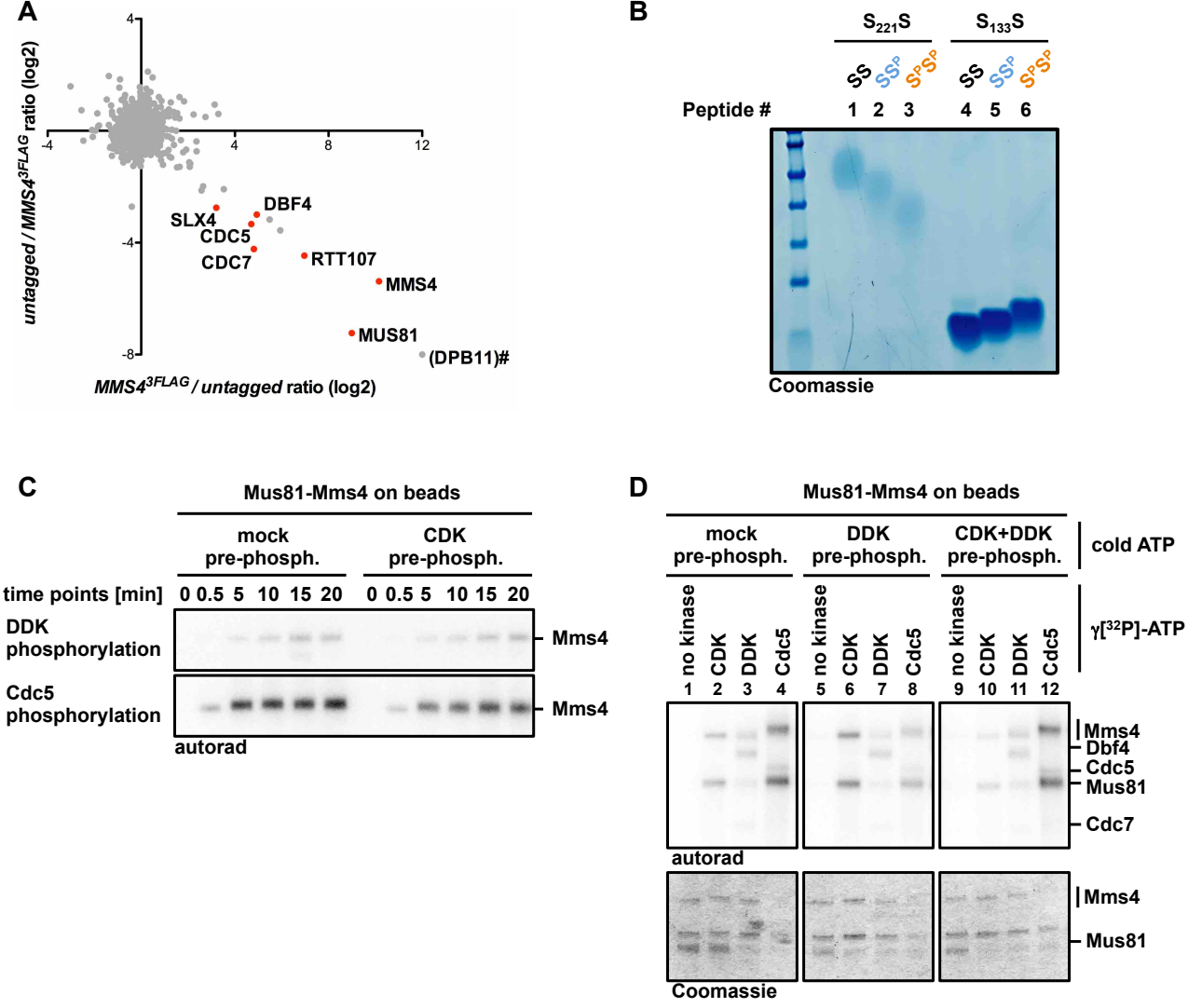
Figure S7:

Rtt107 is required for efficient Mus81-Mms4 activation in mitosis.

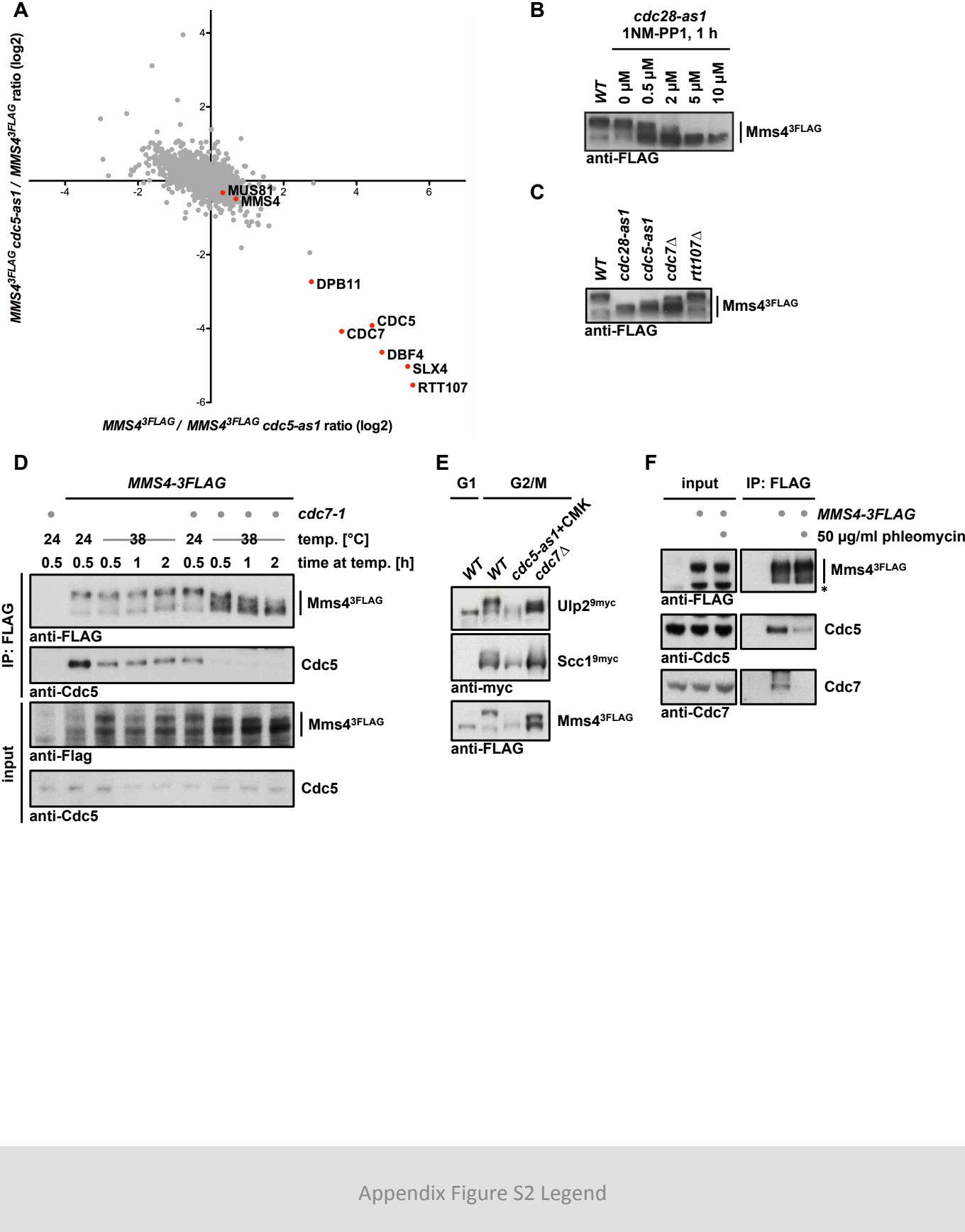
(A,B) Rtt107 influences the phosphorylation of specific Cdc5-dependent phosphorylation sites. SILAC-based MS analysis of Mms4 phosphorylation after purification of endogenously expressed Mus81-Mms4^{3FLAG} **(A)** or of Mus81^{3FLAG}-Mms4$^{His10\text{-}Strep2}$ expressed from the *pGAL1-10* promoter **(B)**.

(C) Western blot analysis of Mus81^{9myc} IP samples that were used as inputs for the *in vitro* for resolution assay of Fig. 7A.

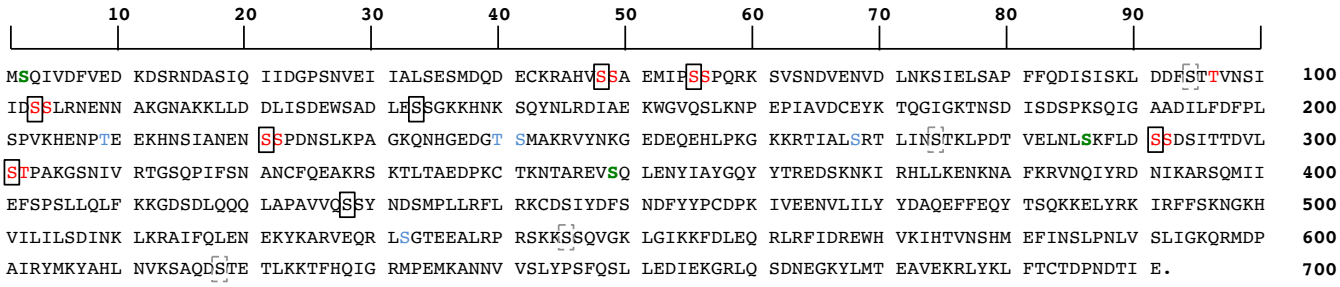
(D) *RTT107* deletion does not lead to a further reduction in Mus81 activity in the *cdc7Δ* background. Resolution assay using a nicked HJ substrate and Mus81^{9myc}-Mms4^{3FLAG} purified from mitotically arrested *bob1-1 cdc7Δ* or *bob1-1 cdc7Δ rtt107Δ* cells. Lower panel: Western blots samples of anti-myc IPs.



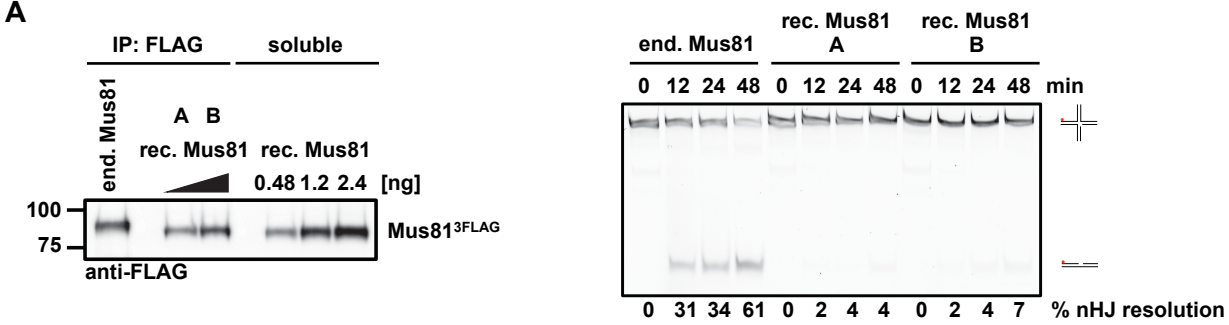
Appendix Figure S1 Legend



Appendix Figure S2 Legend



Appendix Figure S3 Legend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soluble
end. Mus81
A B
rec. Mus81 rec. Mus81
0.48 1.2 2.4 [ng]
100
75
Mus81 3FLAG
anti-FLAG
end. Mus81
rec. Mus81 A
rec. Mus81 B
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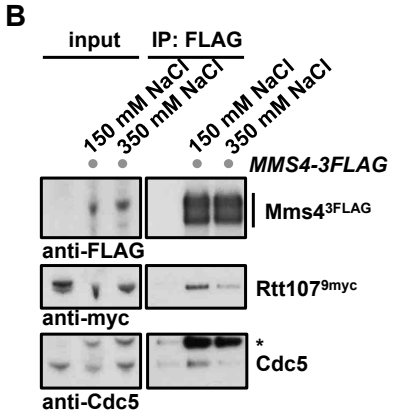
0 31 34 61 0 2 4 4 0 2 4 7 % nHJ resolution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350 mM NaCl
150 mM NaCl
350 mM NaCl
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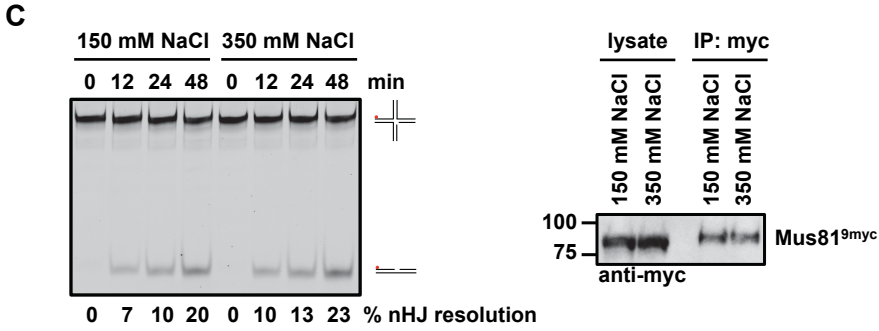
Mms4 3FLAG
anti-FLAG
Rtt107 9myc
anti-myc
*
Cdc5
anti-Cdc5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350 mM NaCl
0 12 24 48 0 12 24 48 min
0 7 10 20 0 10 13 23 % nHJ resolution
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IP: myc
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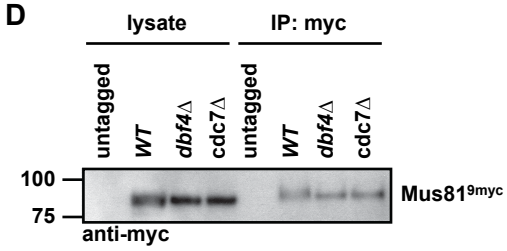
150 mM NaCl
350 mM NaCl
100
75
Mus81 9myc
anti-myc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cdc7Δ
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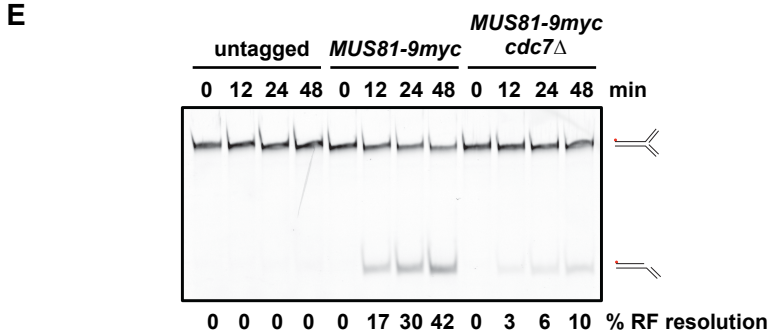
dbf4Δ
cdc7Δ
100
75
Mus81 9myc
anti-myc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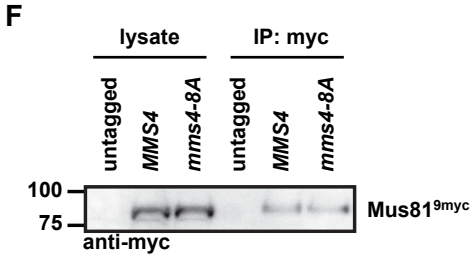
untagged
MUS81-9myc
MUS81-9myc cdc7Δ
0 12 24 48 0 12 24 48 0 12 24 48 min
0 0 0 0 0 17 30 42 0 3 6 10 % RF resolution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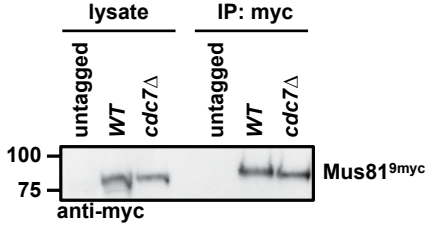
untagged
MMS4
mms4-8A
untagged
MMS4
mms4-8A
100
75
Mus81 9myc
anti-myc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cdc7Δ
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WT
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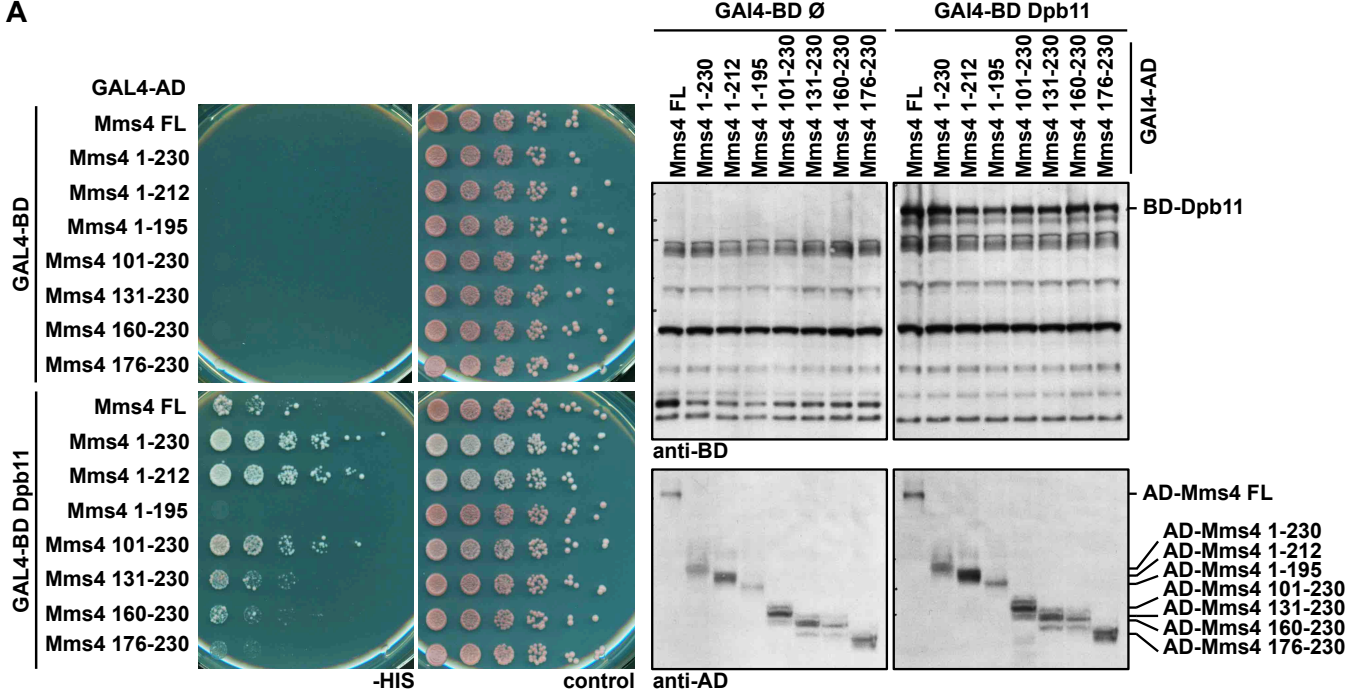
100
75
Mus81 9myc
anti-myc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GAL4-BD
GAL4-BD Dpb11
Mms4 FL
Mms4 1-230
Mms4 1-212
Mms4 1-195
Mms4 101-230
Mms4 131-230
Mms4 160-230
Mms4 176-230
-HIS
control
GAl4-BD Ø
GAl4-BD Dpb11
GAl4-AD
BD-Dpb11
anti-BD
AD-Mms4 FL
AD-Mms4 1-230
AD-Mms4 1-212
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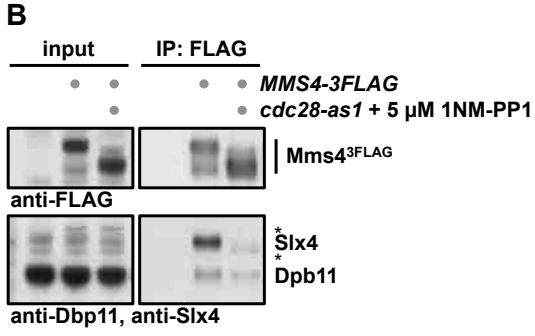
AD-Mms4 176-230
anti-AD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input
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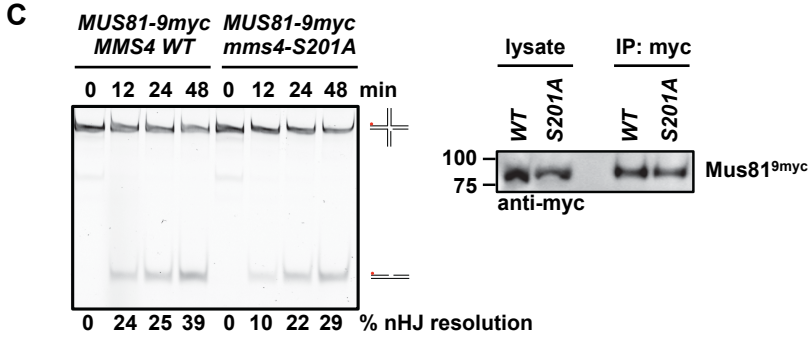
MMS4-3FLAG
cdc28-as1 + 5 μM 1NM-PP1
Mms4 3FLAG
anti-FLAG
Slx4
Dpb11
anti-Dbp11, anti-Slx4


C
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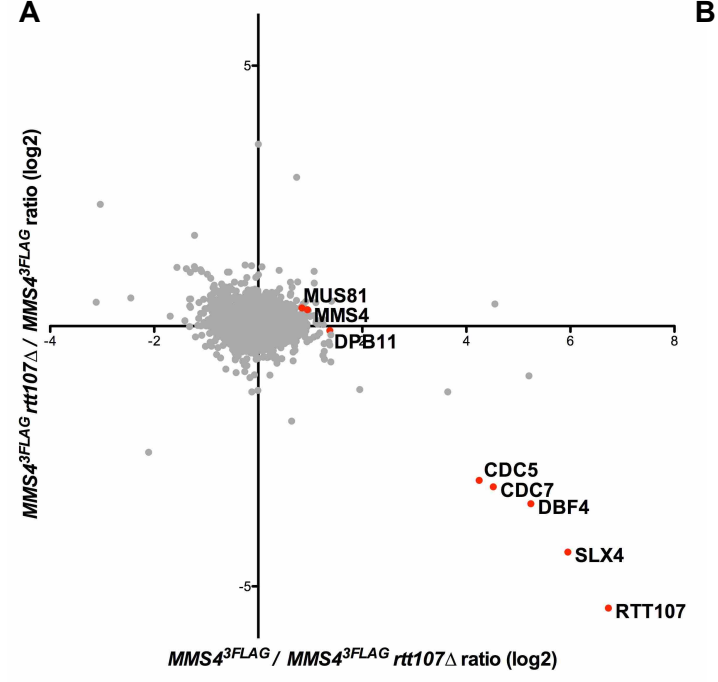
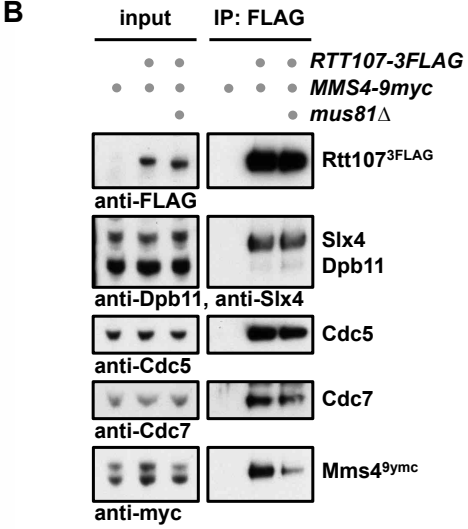
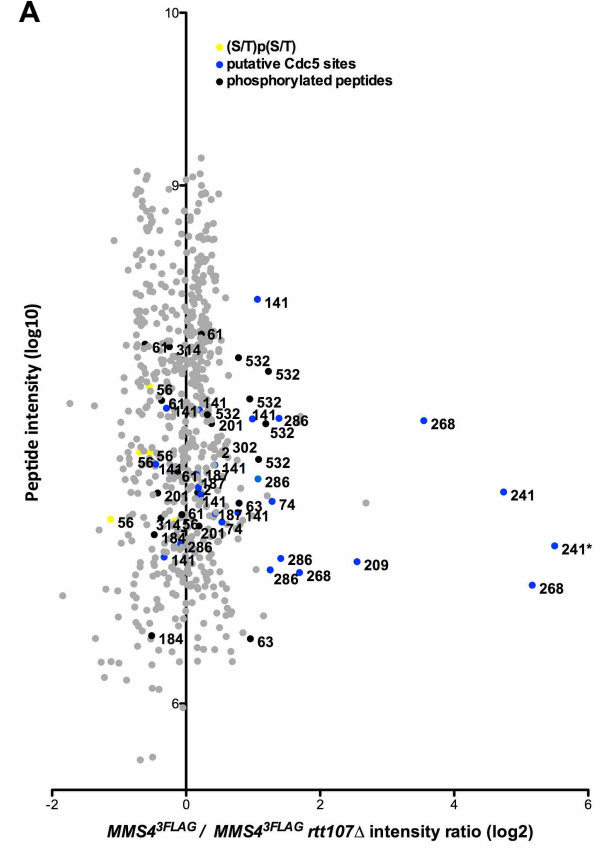
MUS81-9myc mms4-S201A
0 12 24 48 0 12 24 48 min
0 24 25 39 0 10 22 29 % nHJ resolution
lysate
IP: myc
WT
S201A
100
75
anti-myc
Mus81 9myc





Appendix Figure S6 Legend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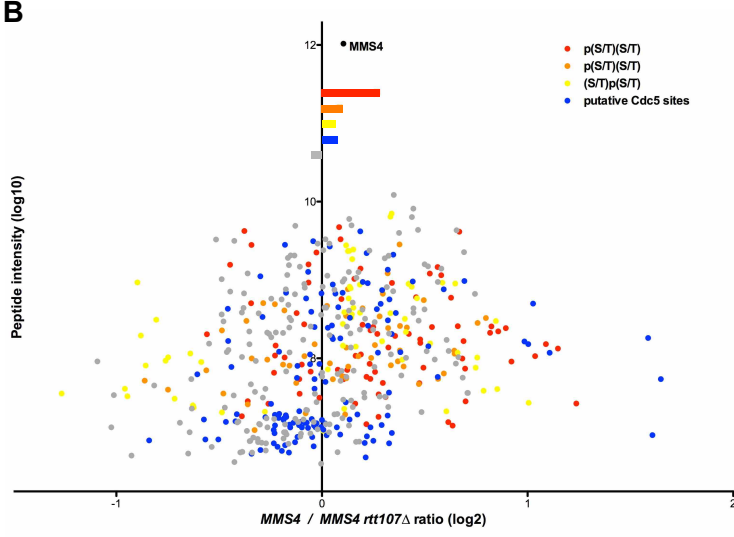
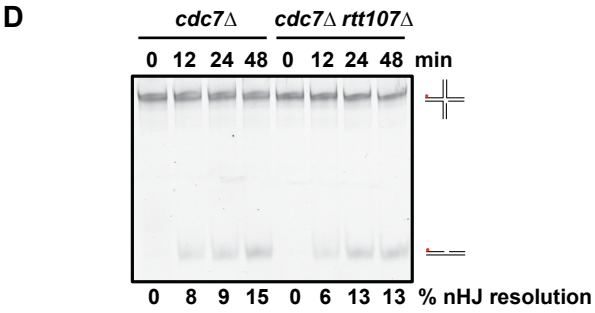
putative Cdc5 sites
phosphorylated peptides
Peptide intensity (log10)
MMS4^3FLAG / MMS4^3FLAG rtt107Δ intensity ratio (log2)


B
MMS4
p(S/T)(S/T)
p(S/T)(S/T)
(S/T)p(S/T)
putative Cdc5 sites
Peptide intensity (log10)
MMS4 / MMS4 rtt107Δ ratio (log2)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cdc7Δ rtt107Δ
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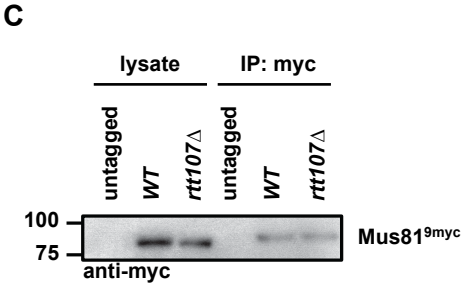
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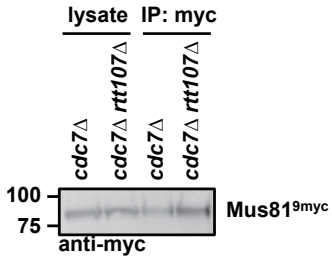
lysate
IP: myc
cdc7Δ
cdc7Δ rtt107Δ
cdc7Δ
cdc7Δ rtt107Δ
100
75
anti-myc
Mus81^9myc

Appendix Table S1. Mms4 phosphorylation sites and their regulation by DDK or Cdc5 as detected by SILAC-based quantitative mass spectrometry (Fig. 3)

Mus81-Mms4 endogenous	Mus81-Mms4 overexpressed
2	2
48	48
49	49
55	55
56	56
61	61
63	63
74	74
86	78**
88*	86
94**	88**
96	94
99	95
103	96
104	99
124**	103
128**	104
133**	124
134**	128
141**	133
156**	134
184*	141
187	156
201	187
209**	201
221*	222*
222*	264
240**	268
241**	274*
268**	280*
286	286
291	291
292	292
294	294
296**	297
297**	301
301	302
302	314
314**	330**
330**	349
349	366
366**	396**
396**	532
532	542

* not measured in *cdc5-as1*
** not measured in *cdc7Δ*
phosphorylation sites affected in *cdc5-as1*
phosphorylation sites affected in *cdc7Δ*
phosphorylation sites affected in *cdc5-as1* and *cdc7Δ* backgrounds

Appendix Supplementary Materials and Methods

Yeast strains and construction

All yeast strains are based on W303 (Thomas & Rothstein, 1989). Genotypes are listed below. All biochemical experiments were performed in a W303-1A *pep4Δ* background. The genetic experiments in Fig. 4D-E, 5C, and EV2A,B,D were performed in a W303 *RAD5+* background to exclude any effect from a partial defect of the *rad5-535* allele. Two-hybrid analyses were performed in the strain PJ69-7A (James *et al.*, 1996).

S. cerevisiae strains were prepared by genetic crosses and transformation techniques. Deletion of particular genes and endogenous protein tagging were performed as described (Knop *et al.*, 1999). Correct integrations were checked by genotyping PCR. Denaturing cell extracts were prepared by alkaline lysis and TCA precipitation. The *mms4* alleles were generated using site-directed mutagenesis and integrated as linear plasmids at the TRP1 locus.

Appendix Table S2. Yeast strains used in this study

Strain	Full genotype	Relevant genotype	Source
MGBY3294	MATa *ade2-1 his3-11 leu2-3,112 trp1Δ2 can1-100 pep4::KanMX bar1::hph-NT1 ura3-52::GAL1,10p-FLAG3-MUS81/GST-His10-Strep2-MMS4::URA3*	*pGAL-FLAG3-MUS81-GST-His10-Strep2-MMS4*	This study (Blanco lab)
YBP388	MATa *ade2-1 ura3-1 his3-11,15 trp1-1 can1-100 leu2-3,112::pep4::LEU2*	*pep4*	Klein lab
YDG208	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100*		This study
YDG291	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 yen1::hph-NT1*	*yen1*	Gritenaite et al., 2014
YDG329	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 sgs1::hph-NT1*	*sgs1*	Gritenaite et al., 2014
YDG355	MATa *RAD5+ ade2-1 ura3-1 his3-11,15 trp1-1 can1-100 mms4::hph-NT1 leu2-3,112::mms4-SS184,201AA::LEU2*	*mms4-SS184,201AA*	Gritenaite et al., 2014
YDG356	MATa *RAD5+ ade2-1 ura3-1 trp1-1 can1-100 mms4::hph-NT1 leu2-3,112::mms4-SS184,201AA::LEU2 his3-11,15::sgs1::HIS3Mx4*	*mms4-SS184,201AA sgs1*	Gritenaite et al., 2014
YDG376	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 yen1::hph-NT1 sgs1::nat-NT2*	*yen1 sgs1*	Gritenaite et al., 2014

YJB82	Mata/Matalpha *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trp1-1/trp1-1 can1-100/can1-100 ade2-n/ade2-I LYS2/lys2::Gal-ISceI his3::NATMX/his3::HPHMX4 met22::kIURA3/MET22*	*diploid*	This study
YJB84	Mata/Matalpha *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trp1-1/trp1-1 can1-100/can1-100 ade2-n/ade2-I LYS2/lys2::Gal-ISceI his3::NATMX/his3::HPHMX4 met22::kIURA3/MET22 rtt107::KanMX/rtt107::KanMX*	*diploid rtt107*	This study
YJB86	Mata/Matalpha *ade2-1/ade2-1 ura3-1/ura3-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 trp1-1/trp1-1 can1-100/can1-100 ade2-n/ade2-I LYS2/lys2::Gal-ISceI his3::NATMX/his3::HPHMX4 met22::::kIURA3/MET22 mms4::KanMX/mms4::KanMX trp1-1:pRS304-Mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA:TRP1/trp1-1:pRS304-Mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA:TRP1*	*diploid mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA*	This study
YLP015	MATa *ade2-1 ura3-1 his3-11,15 can1-100 trp1-1::bar1::TRP1 leu2-3,112::pep4::LEU2 lys1::nat-NT2*	*lys1*	Gritenaite et al., 2014
YLP063	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 cdc5-as1 his3-11,15::pep4::HIS3Mx4 MMS4-3FLAG::hph-NT1*	*MMS4-3FLAG cdc5-as1*	Gritenaite et al., 2014
YLP065	MATa *ade2-1 ura3-1 his3-11,15 can1-100 trp1-1::bar1::TRP1 leu2-3,112::pep4::LEU2 lys1::nat-NT2 MMS4-3FLAG::hph-NT1*	*lys1 MMS4-3FLAG*	This study
YLP070	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 his3-11,15::pep4::HIS3Mx4 lys1::nat-NT2 mms4::KanMx trp1-1::mms4-S184A::TRP1 MMS4-3FLAG::hph-NT1*	*lys1 mms4-S184A-3FLAG*	This study
YLP074	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 his3-11,15::pep4::HIS3Mx4 lys1::nat-NT2 mms4::KanMx trp1-1::mms4-S201A::TRP1 MMS4-3FLAG::hph-NT1*	*lys1 mms4-S201A-3FLAG*	This study
YLP078	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 his3-11,15::pep4::HIS3Mx4 MMS4-3FLAG::hph-NT1 slx4::KanMx*	*MMS4-3FLAG slx4*	Gritenaite et al., 2014

YLP092	MATa *ade2-1 ura3-1 his3-11,15 trp1-1 can1-100 leu2-3,112::pep4::LEU2 RTT107-9myc::hph-NT1*	*RTT107-9myc*	This study
YLP100	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1*	*bob1-1*	This study
YLP111	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 MMS4-3FLAG::KanMx4*	*bob1-1 MMS4-3FLAG*	This study
YLP113	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 cdc7::nat-NT2 MMS4-3FLAG::KanMx4*	*bob1-1 cdc7 MMS4-3FLAG*	This study
YLP121	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 cdc5-as1 his3-11,15::pep4::HIS3Mx4 lys1::nat-NT2 MMS4-3FLAG::hph-NT1*	*lys1 MMS4-3FLAG cdc5-as1*	This study
YLP126	MATa *ade2-1 leu2-3,112 trp1-1 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 cdc7::nat-NT2 MMS4-3FLAG::KanMx4 ura3-1::lys1::URA3*	*lys1 bob1-1 cdc7 MMS4-3FLAG*	This study
YLP128	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 his3-11,15::pep4::HIS3Mx4 cdc7-1*	*cdc7-1*	This study
YLP132	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 his3-11,15::pep4::HIS3Mx4 cdc7-1 MMS4-3FLAG::KanMx*	*cdc7-1 MMS4-3FLAG*	This study
YLP156	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 his3-11,15::pep4::HIS3Mx4 MMS4-3FLAG::hph-NT1 RTT107-9myc::nat-NT2*	*MMS4-3FLAG RTT107-9myc*	This study
YLP164	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3Mx4 rtt107::KanMx trp1-1::lys1::TRP1*	*lys1 MMS4-3FLAG rtt107*	This study
YLP277	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3Mx4 SCC1-9myc*	*MMS4-3FLAG SCC1-9myc*	This study
YLP279	MATa RAD5+ *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 cdc5-as1 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3 SCC1-9myc::KanMx*	*MMS4-3FLAG SCC1-9myc cdc5-as1*	This study
YLP287	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 his3-11,15::pep4::HIS3Mx4 mms4::KanMx trp1-1::mms4-S201A::TRP1 MMS4-3FLAG::hph-NT1 RTT107-9myc::nat-NT2*	*mms4-S201A-3FLAG RTT107-9myc*	This study

YLP339	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA*	This study
YLP341	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 sgs1::nat-NT2*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA sgs1*	This study
YLP350	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 yen1::KanMx*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA yen1*	This study
YLP351	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 sgs1::nat-NT2 yen1::KanMx*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA sgs1 yen1*	This study
YLP344	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3Mx4 dbf4-ΔN66::KanMx*	*MMS4-3FLAG dbf4-ΔN66*	This study
YLP345	MATa *ade2-1 ura3-1 leu2-3,112 trp1-1 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3Mx4 dbf4-ΔN109::KanMx*	*MMS4-3FLAG dbf4-ΔN109*	This study
YLP356	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 MMS4-3FLAG::hph-NT1*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA-3FLAG*	This study
YLP360	MATa *ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 MMS4-3FLAG::hph-NT1 cdc28-as1*	*MMS4-3FLAG cdc28-as1*	This study
YLP367	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3Mx4 trp1-1:: MMS4::TRP1 MMS4-3FLAG::hph-NT1 MUS81-9myc::nat-NT2*	*MMS4-3FLAG MUS81-9myc*	This study

YLP368	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3Mx4 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 MMS4-3FLAG::hph-NT1 MUS81-9myc::nat-NT2*	*mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA-3FLAG MUS81-9myc*	This study
YLP369	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dbf4-ΔN66::KanMx*	*dbf4-ΔN66*	This study
YLP370	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dbf4-ΔN109::KanMx*	*dbf4-ΔN109*	This study
YLP371	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dbf4-ΔN66::KanMx sgs1::hph-NT1*	*dbf4-ΔN66 sgs1*	This study
YLP372	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dbf4-ΔN109::KanMx sgs1::hph-NT1*	*dbf4-ΔN109 sgs1*	This study
YLP374	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100 dbf4-ΔN66::KanMx yen1::hph-NT1*	*dbf4-ΔN66 yen1*	This study
YLP375	MATa *RAD5+ ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 dbf4-ΔN109::KanMx yen1::hph-NT1*	*dbf4-ΔN109 yen1*	This study
YLP438	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3 ULP2-9myc::KanMx*	*MMS4-3FLAG ULP2-9myc*	This study
YLP439	MATa RAD5+ *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 cdc5-as1 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3 ULP2-9myc::KanMx*	*MMS4-3FLAG ULP2-9myc cdc5-as1*	This study
YLP442	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3 trp1-1::mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA::TRP1 MMS4-3FLAG::hph-NT1 lys1::nat-NT2*	*lys1 mms4-SSSSSSSS48,55,103,133,221,291,301,428AAAAAAAA-3FLAG*	This study
YLP444	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3 trp1-1::mms4- S201A::TRP1 MMS4-3FLAG::hph-NT1 MUS81-9myc::nat-NT2*	*mms4-S201A-3FLAG MUS81-9myc*	This study
YLP445	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 trp1-1::MUS81-9myc::TRP1 his3-11,15::bob1-1::HIS3 pep4::hph-NT1 MMS4-3FLAG::KanMx cdc7::nat-NT2 rtt107::kIURA*	*bob1-1 MUS81-9myc cdc7 rtt107*	This study
YLP458	MATa *ade2-1 his3-11,15 can1-100 trp1-1::bar1::TRP1 leu2-3,112::pep4::LEU2 lys1::nat-NT2 ura3-1::pRS306-pGAL1,10-FLAG3-MUS81-His-Strep-MMS4::URA3*	*lys1 pGAL-FLAG3-MUS81-His10-Strep2-MMS4*	This study

YLP459	MATa *ade2-1 trp1-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3 pep4::hph-NT1 lys1::nat-NT2 ura3-1::pRS306-pGAL1,10-FLAG3-MUS81-His-Strep-MMS4::URA3*	*lys1 pGAL-FLAG3-MUS81-His10-Strep2-MMS4*	This study
YLP461	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3 trp1-1::mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA::TRP1 MMS4-3FLAG::hph-NT1*	*mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA-3FLAG*	This study
YLP462	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA::TRP1*	*mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA*	This study
YLP463	MATa *RAD5+ ade2-1 ura3-1 leu2-3,112 his3-11,15 can1-100 mms4::hph-NT1 trp1-1::mms4-SSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAA::TRP1 sgs1::nat-NT2*	*mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA sgs1*	This study
YLP465	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 cdc7::nat-NT2 MMS4-3FLAG::KanMx4 trp1-1::ULP2-9myc::TRP1*	*bob1-1 cdc7 MMS4-3FLAG ULP2-9myc*	This study
YLP466	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 cdc7::nat-NT2 MMS4-3FLAG::KanMx4 trp1-1::SCC1-9myc::TRP1*	*bob1-1 cdc7 MMS4-3FLAG SCC1-9myc*	This study
YLP468	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 mms4::KanMx his3-11,15::pep4::HIS3 trp1-1::mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA::TRP1 MMS4-3FLAG::hph-NT1 MUS81-9myc::nat-NT2*	*mms4-SSSSSSSSSSSS48,55,94,103,133,221,274,291,301,428,545,618AAAAAAAAAAAA-3FLAG MUS81-9myc*	This study
YLP469	MATa *RAD5+ ade2-1 leu2-3,112 trp1-1 can1-100 cdc5-as1 his3-11,15::pep4::HIS3Mx4 lys1::nat-NT2 ura3-1::GAL1,10p-FLAG3-MUS81/His10-Strep2-MMS4::URA3*	*lys1 cdc5-as1 pGAL-FLAG3-MUS81-His10-Strep2-MMS4*	This study
YLP470	MATa *ade2-1 leu2-3,112 trp1-1 can1-100 his3-11,15::bob1-1::HIS3Mx4 pep4::hph-NT1 cdc7::KanMx lys1::nat-NT2 ura3-1::GAL1,10p-FLAG3-MUS81/His10-Strep2-MMS4::URA3*	*lys1 bob1-1 cdc7 pGAL-FLAG3-MUS81-His10-Strep2-MMS4*	This study

YLP471	MATa *ade2-1 his3-11,15 trp1-1 can1-100 leu2-3,112::pep4::LEU2 rtt107::KanMx lys1::nat-NT2 ura3-1::GAL1,10p-FLAG3-MUS81/His10-Strep2-MMS4::URA3*	*lys1 rtt107 pGAL-FLAG3-MUS81-His10-Strep2-MMS4*	This study
YML1601	MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ADE2 MMS4-9myc::KanMx trp1-1::pGAL1-CDC5-GFP::TRP1*	*MMS4-9myc pGAL-CDC5-GFP*	Matos et al., 2013
YML3304	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 trp1-1::MUS81-9myc::TRP1 his3-11,15::bob1-1::HIS3 pep4::hph-NT1 MMS4-3FLAG::KanMx dbf4::nat-NT2*	*bob1-1 MUS81-9myc dbf4*	This study (Matos lab)
YML3306	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 trp1-1::MUS81-9myc::TRP1 his3-11,15::bob1-1::HIS3 pep4::hph-NT1 MMS4-3FLAG::KanMx cdc7::nat-NT2*	*bob1-1 MUS81-9myc cdc7*	This study (Matos lab)
YML3447	MATa *ade2-1 ura3-1 leu2-3,112 can1-100 trp1-1::MUS81-9myc::TRP1 his3-11,15::bob1-1::HIS3 pep4::hph-NT1 MMS4-3FLAG::nat-NT2 rtt107::KanMx*	*bob1-1 MUS81-9myc rtt107*	This study (Matos lab)
YSS3	MATa *ade2-1 ura3-1 trp1-1 leu2-3,112 can1-100 MMS4-3FLAG::hph-NT1 his3-11,15::pep4::HIS3Mx4*	*MMS4-3FLAG*	Gritenaite et al., 2014
YFZ020	MATa *ade2-1 ura3-1 trp1-1 can1-100 his3-11,15::pRS303-CDC5-3FLAG-pGAL1-GAL4::HIS3Mx4 leu2-3,112::pep4::LEU2*	*pGAL-CDC5-3FLAG*	This study
YFZ021	MATa *ade2-1 ura3-1 trp1-1 can1-100 his3-11,15::pRS303-DBF4-CDC7-pGAL1-GAL4::HIS3Mx4 pep4::hph-NT1 DBF4-3FLAG::KanMx leu2-3,112::CDC7-9myc::LEU2*	*pGAL-DBF4-3FLAG-CDC7-9myc*	This study

Antibodies

Proteins were detected using specific antibodies: rabbit-anti-Dpb11 (BPF19, Pfander lab), rabbit-anti-Slx4 (2057, Pfander lab), goat-anti-Cdc5 (sc-6733, Santa Cruz), rabbit-anti-Cdc7 (Diffley lab), rabbit-anti-Clb2 (sc-9071, Santa Cruz), goat-anti-Dbf4 (sc-5705; Santa Cruz), rabbit-anti-FLAG (F7425, Sigma), mouse-anti-myc (05-724, clone 4A6; Millipore), mouse-anti-Gal4-AD (TA-C10; Santa Cruz), mouse-anti-Gal4-BD (RK5C1; Santa Cruz).

FACS analysis

$1x10^7$ - $2x10^7$ cells were harvested by centrifugation and resuspended in 70% ethanol + 50 mM Tris pH 7.8. After centrifugation cells were washed with 1 ml 50 mM Tris pH 7.8 (Tris buffer) followed by resuspending in 520 µl RNase solution (500 µl 50 mM Tris pH 7.8 + 20 µl RNase A (10 mg/ml in 10 mM Tris pH 7.5, 10 mM $MgCl_2$) and incubation for 4

h at 37 °C. Next, cells were treated with proteinase K (200 µl Tris buffer + 20 µl proteinase K (10 mg/ml in 50% glycerol, 10 mM Tris pH 7.5, 25 mM $CaCl_2$) and incubated for 30' at 50 °C. After centrifugation cells were resuspended in 500 µl Tris buffer. Before measuring the DNA content, samples were sonified (5''; 50% CYCLE; minimum POWER) and stained by SYTOX solution (999 µl Tris buffer + 1 µl SYTOX). Measurement was performed using FL1 channel 520 for SYTOX-DNA by BD FACSCalibur system.

Acrylamide gel electrophoresis and western blot analysis

Protein samples were separated by standard SDS-polyacrylamide gel electrophoresis in 4-12% Novex NuPAGE Bis-Tris precast gels (ThermoFisher) with MOPS buffer (50 mM MOPS, 50 mM Tris-base, 1.025 mM EDTA, 0.1% SDS, adjusted to pH 7.7). To resolve phosphorylation shifts of Mms4 in Fig. EV1, and of $Ulp2^{9myc}$ or $Scc1^{9myc}$ (Fig. S2E), protein samples were separated in 7% Novex NuPAGE Tris-Acetate precast gel (ThermoFisher) with Tris-Acetate buffer (50 mM Tris-base, 50 mM Tricine, 0.1% SDS, adjusted to pH 8.24).

After electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham Protran Premium 0.45 µM NC) using a tank blotting system. Membranes were incubated with primary antibodies at 4 °C overnight. Incubation with appropriate secondary antibodies coupled to horseradish peroxidase (HRP) was performed at room temperature for 3 h. Membranes were washed five times for 5 min with western wash buffer (50 mM Tris pH 7.5, 137 mM NaCl, 3 mM KCl, 0.2 % NP-40) and incubated with Pierce ECL western blotting substrate (ThermoFisher) according to the instructions of the manufacturer. Chemiluminescence was detected with a tabletop film processor (OPTMAX, Protec).

Yeast Two-Hybrid analysis

The plasmids used for yeast two-hybrid analysis in this study were based on pGAD-C1 and pGBD-C1. To assay for an interaction between the proteins, respective plasmids were transformed into competent PJ69-7A cells. Transformants were spotted in serial dilution (1:5) either on SC-Leu-Trp plates (control) or on SC-Leu-Trp-His plates (selection) and incubated at 30 °C for 2-3 days. Cells from the control plates were then grown in SC-Leu-Trp to log-phase to take samples for subsequent analysis of the expression of the AD-/BD-fusion proteins by western blot.

Preparation of whole-cell extracts (alkaline lysis/TCA)

Cell pellets were re-suspended in 1 ml pre-cooled H_2O and incubated with 150 µl of freshly prepared lysis solution (1.85 M NaOH, 7.5% beta-mercaptoethanol) at 4 °C for 15 min. Then, the lysate was admixed with 150 µl 55% trichloroacetic acid (TCA) and incubated at 4 °C for 10 min. After centrifugation and careful aspiration of the supernatant, the precipitated proteins were re-suspended in 50 µl HU-buffer (8 M urea, 5% SDS, 200 mM Tris pH 6.8, 1.5% dithiothreitol, traces of bromophenol blue) and incubated at 65 °C for 10 min.

Synchronization of cells

Logarithmic growing cells were synchronized in mitosis by nocodazole (5 µg/ml), in S-phase by HU (200 mM), or in G1-phase by α-factor (5-10 µg/ml). Release from G1 synchronization into S-phase was performed by washing twice in pre-warmed YPD, and suspending cells in pre-warmed YPD with nocodazole, with HU or without chemical.

Drug treatment

DNA damage in liquid cultures was induced by addition of phleomycin to a final concentration of 50 µg/ml.

For solid media, concentrations of methyl methanesulfonate (MMS) were as indicated in the figures. Cells from stationary grown ON cultures were spotted in serial dilution (1:5) and incubated at 30 °C for 2-3 days.

Interaction assays

After cell growth under the indicated conditions, yeast extracts were obtained by freezer mill lysis (Spex Sample Prep) in lysis buffer (100 mM Hepes pH 7.6, 200 mM KOAc, 0.1% NP-40, 10% glycerol, 2 mM b-ME, 100 mM ocadaic acid, 10 mM NaF, 20 mM b-glycerophosphate, 400 µM PMSF, 4 µM aprotinin, 4 mM benzamidin, 400 µM leupeptin, 300 µM pepstatin A). Co-IP was performed for 2 hours with head-over-tail rotation at 4 °C using anti-FLAG agarose resin (Sigma). Non-specific background was removed by six washes and bound proteins were eluted by incubation with 0.5 mg/ml 3X FLAG-peptide (Sigma). The TCA-precipitated eluates were resolved on 4-12% NuPAGE gradient gels (Invitrogen), and analyzed by standard Western blotting techniques.

SILAC-based quantitative mass-spectrometry

For Co-IP experiments followed by mass spectrometry analysis, cells deficient in lysine biosynthesis were grown in synthetic complete (SC) medium supplemented with normal

lysine ("light" medium) or heavy-isotope-labeled lysine (Lys6 or Lys8; "heavy" medium) from Cambridge Isotope Laboratories and arrested in G2/M phase with nocodazole. In SILAC experiments with high-copy expression of *MUS81-MMS4*, overexpression was induced by addition of 2% galactose for 2 h after nocodazole arrest.

Lysates were prepared by harvesting cells in equal amounts after growth under the indicated conditions. After co-IP, eluted proteins from light and heavy cultures were pooled, TCA precipitated and separated on a 4-12% NuPAGE Bis-Tris gel (Invitrogen). The gel was stained with GelCode Blue (Thermo Scientific). The gel lane was excided into ten slices and peptides were analyzed by LC-MS/MS after in-gel Lys-C digestion. Samples were measured on an LTQ-Orbitrap and analyzed using MaxQuant (Cox & Mann, 2008).

For analysis of proteins (Fig. S1A, 2E, S2A, EV3A, 6D, S6A), log2 values of H/L ratios from two label-switch experiments without ratio count cut-off were plotted against each other.

For analysis of phosphorylation sites from endogenous protein levels (Fig. 3A-B, S7A), H/L ratios for Mms4 peptides were calculated from the corresponding H and L intensities of MS evidences and plotted in their log2 values against the log10 values of the peptide's overall intensity. Evidences of non-phosphorylated Mms4 peptides are shown in grey, evidences of phosphorylated peptides are shown in black. Phosphorylated peptides were sorted into categories according to their phosphorylation status. Putative DDK target sites were differentiated into the categories pSpS (red), pSS (orange) or SpS (yellow), in which the respective residues of the (S/T)(S/T) motif were phosphorylated (detected phosphorylation probability >0.7). Phosphorylated peptides matching the Cdc5 consensus site are coloured in blue. Numbers indicate the phosphorylated residue in the depicted peptide. An asterisk marks peptide evidences that contained measured intensity values exclusively in the H or L sample. Their ratio value was set to a fixed value.

For analysis of phosphorylation sites from overexpressed *MUS81-MMS4* (Fig. 3C-D, S7B), log2 values of H/L ratios of Mms4 peptides were plotted against the log10 values of the peptide's intensity. Depicted are phosphorylated peptides only. Peptides were sorted into categories according to their phosphorylation status. Putative DDK target sites were differentiated into the categories pSpS (red), pSS (orange) or SpS (yellow), in which the respective residues of the (S/T)(S/T) motif were phosphorylated (detected phosphorylation probability >0.7). Phosphorylated peptides matching the Cdc5 consensus site are coloured in blue. All other phosphorylated peptides are marked in grey. Bars depict the mean of the ratios of the respective category.

Protein purification

CDK was expressed in *E. coli* BL21 pRIL cells (Agilent). Mus81-Mms4, DDK and Cdc5 were overexpressed in *S. cerevisiae* from a galactose-inducible GAL1-10 promoter. All purification steps were performed on ice or at 4 °C.

Purification of Mus81-Mms4 from S. cerevisiae

^{FLAG3}*MUS81* and $^{GST\text{-}HIS10\text{-}STREP2}$*MMS4* were cloned under the control of the *GAL1,10* bidirectional promoter in a pRS306 derivative plasmid. The resulting vector was linearized with StuI and integrated at the *ura3-1* locus of a W303 *pep4Δ* strain.

The resulting MGBY3294 strain was grown in YP+2% raffinose to mid-log phase at 25 °C and protein expression was induced by addition of 2% galactose. Cells (10 liters at ~2-4x10^7 cells/ml) were harvested, washed and resuspended in a small volume of A500 buffer (40 mM Tris-HCl pH 7.5, 500 mM NaCl, 20% glycerol, 0.1% NP-40, 1 mM DTT) containing phosphatase and protease inhibitors and mechanically disrupted. The frozen lysate was resuspended in 2 volumes of A500, cleared by ultracentrifugation and incubated with anti-FLAG M2 agarose beads (Sigma) for 1 h at 4 °C. After extensive washing of the beads in A500, Mus81-Mms4 was dephosphorylated by treatment with 10,000 units of lambda phosphatase (New England Biolabs) for 30 min at room temperature. Beads were washed in A500 buffer and Mus81-Mms4 was then eluted with 3 volumes of A500 supplemented with 0.5 mg/ml 3X FLAG-peptide (Sigma). The eluate was then adjusted to 5 mM imidazole and proteins were loaded onto a Ni-NTA column (Qiagen). The column was washed with A500 buffer containing increasing concentrations of imidazole up to 50 mM, and finally Mus81-Mms4 was eluted with A500 containing 300 mM imidazole. The eluate was dialyzed extensively against A500, and stored in aliquots at -80 °C. Protein concentrations were determined using the Bradford assay (BioRad) and on Coomassie-stained PAGE gels using BSA as the standard, which also confirmed absence of phosphorylation-dependent electrophoretic migration shifts. Control experiments confirmed the absence of non-specific endo- or exonuclease activities.

Purification of bacterially expressed CDK2/cycA$^{\Delta N170}$

To generate CDK2/cycA$^{\Delta N170}$ complex, ^{GST}CDK2 and ^{His6}cycA$^{\Delta N170}$ were expressed separately. Bacteria with either expression plasmids were grown in 1 l LB medium supplemented with antibiotics to mid-log phase. Both cultures were cooled down on ice for 5 min to increase chaperone expression followed by addition of 1 mM IPTG and incubation for 20 h at 20 °C. Cells were pelleted and resuspended in 40 ml lysis buffer

(300 mM NaCl, 20 mM HEPES pH 7.6, 5 mM β-mercaptoethanol, 0.01% NP-40, 100 µM AEBSF, 1x complete protease inhibitor cocktail EDTA-free) followed by lysis with an EmulsiFlex-C3 system for three rounds at 1,000 bar. Cell debris was spun down at 140,000 g for 45 min. To allow complex formation between both subunits, extracts were pooled and incubated for 45 min. For glutathione affinity chromatography, 1 ml bed volume of equilibrated Glutathione Sepharose beads were added to the extract and incubated for 2 h. Beads were then washed four times with 25 CV Wash Buffer B2 (300 mM NaCl, 20 mM HEPES pH 7.6, 5 mM β-mercaptoethanol, 0.01% NP-40) before elution was achieved by protease cleavage. For this purpose, beads were resuspended in 1 CV wash buffer (150 mM NaCl, 20 mM HEPES pH 7.6, 5 mM β-mercaptoethanol, 0.01% NP-40) and incubated together with 250 U GST-PreScission protease (MPIB Core Facility) for 18 h. The eluate was then adjusted to 300 mM NaCl and 6 mM imidazole for subsequent Ni-NTA affinity chromatography. Here, a bed volume of 1 ml equilibrated Ni-NTA Agarose (Qiagen) was added to the eluate and incubated for 1 h. Beads were subsequently washed four times with 15 CV wash buffer (300 mM NaCl) + 6 mM imidazole and five times with 2 CV wash buffer (300 mM NaCl) + 6 mM imidazole + 5% glycerol. Elution was then performed with wash buffer (300 mM NaCl) + 250 mM imidazole. Fractions containing CDK were pooled and dialyzed by stirring two times against 300 volumes of dialysis buffer (150 mM NaCl, 50 mM HEPES pH 7.6, 0.1% NP-40, 2 mM β-mercaptoethanol, 10% glycerol) for 4 h in a Slide-A-Lyzer Dialysis Casette (Thermo Scientific). Dialysed material was recovered, aliquoted, snap-frozen and stored at -80 °C.

Purification of Cdc5 from S. cerevisiae

YFZ020 was grown in 10 l YP medium + 2% raffinose at 30 °C until mid-log phase before expression was induced by addition of 2% galactose. After 4 h of induction, yeast cells were harvested and washed twice with 250 ml 1 M Sorbitol + 25 mM HEPES pH 7.6. The pellet was resuspended in 1 volume of lysis buffer (500 mM NaCl, 100 mM HEPES pH 7.6, 0.1% NP-40, 10% glycerol, 2 mM β-mercaptoethanol, 400 µM PMSF, 4 µM aprotinin, 4 mM benzamidin, 400 µM leupeptin, 300 µM pepstatin A, 4x complete protease inhibitor cocktail, EDTA-free) and frozen drop-wise in liquid nitrogen. Frozen cell drops were crushed using a freezer/mill system (Spex Sample Prep). Cell powder was thawed on ice and centrifuged at >185,000 g for 1 h. The clear phase was recovered and incubated with 1 ml bed volume of anti-FLAG M2 resin (Sigma) equilibrated in lysis buffer. After 2 h of incubation, the resin was washed five times with 10 CV of wash buffer (500 mM NaCl, 100 mM HEPES pH 7.6, 0.1% NP-40, 10% glycerol, 2 mM β-

mercaptoethanol). Two elution steps were performed by adding 1 CV 0.5 mg/mL 3FLAG peptide in wash buffer and incubation for 30 min. Obtained fractions were pooled, brought to a conductivity of 10 mS/cm (100 mM salt) and subjected to anion exchange chromatography using a MonoQ 5/50 GL column with a salt gradient of 0.1-1 M NaCl over 20 CV. $Cdc5^{3FLAG}$ eluted at a conductivity of ~15 mS/cm. Kinase containing fractions were aliquoted, snap-frozen and stored at -80 °C.

Purification of DDK from S. cerevisiae

DDK was purified as described by Gros *et al.* with modifications (Gros *et al.* 2014). YFZ021 cells were grown in 10 l YP medium + 2% raffinose at 30 °C until mid-log phase before expression was induced by addition of 2% galactose. After 4 h of incubation, yeast cells were harvested and washed twice with 250 ml 1 M Sorbitol + 25 mM HEPES pH 7.6. The pellet was resuspended in 1 volume of lysis buffer (400 mM NaCl, 100 mM HEPES pH 7.6, 0.1% NP-40, 10% glycerol, 2 mM β-mercaptoethanol, 400 µM PMSF, 4 µM aprotinin, 4 mM benzamidin, 400 µM leupeptin, 300 µM pepstatin A, 4x complete protease inhibitor cocktail EDTA-free) and frozen drop-wise in liquid nitrogen. Frozen cell drops were crushed using a freezer/mill system. Cell powder was thawed on ice and centrifuged at >185,000 g for 1 h. The clear phase was recovered and incubated with 1 ml bed volume of anti-FLAG M2 resin (equilibrated in lysis buffer). After incubation for 2 h at 4 °C, the resin was washed six times with 2 CV wash buffer (400 mM NaCl, 100 mM HEPES pH 7.6, 0.1% NP-40, 10% glycerol, 2 mM β-mercaptoethanol). For λ-phosphatase treatment, beads were resuspended in 1 CV wash buffer + 2 mM $MnCl_2$ + 900 U λ-phosphatase (New England Biolabs) and incubated for 1 h at 30 °C in a tabletop thermoshaker. Beads were recovered and bound DDK was eluted twice with 1 CV 0.5 mg/ml 3FLAG peptide in wash buffer for 30 min. Elutions were pooled, concentrated using a Vivaspin 500 MWCO 50.000 (GE healthcare) and fractionated by size exclusion chromatography using a Superdex 200 GL 10/300 column (GE healthare, equilibrated in wash buffer) over 1.2 CV. DDK containing fractions were pooled, brought to a conductivity of 10 mS/cm (100 mM salt) and fractionated by anion exchange chromatography using a MonoQ 5/50 GL column with a salt gradient of 0.1-1 M NaCl over 20 CV. DDK containing fractions eluted at ~24-26 mS/cm and were aliquoted, snap frozen and stored at -80 °C.

In vitro kinase assays

Sequential kinase assays with purified Mus81-Mms4

Kinase assays were performed as described previously (Pfander & Diffley, 2011; Mordes *et al.*, 2008) with minor modifications.

Per reaction 20 pmol Mus81-Mms4 were used as substrate for 10 pmol kinase (CDK2/cyclinA$^{\Delta N170}$, DDK and/or Cdc5) in a 50 µL reaction volume containing 5 µg BSA. For sequential phosphorylation reactions Mus81-Mms4 was immobilized to Glutathione Sepharose 4B resin (GE Healthcare) for 1 h at 4 °C shaking. Beads were washed twice with binding buffer-100 (100 mM Hepes pH 7.6, 100 mM KOAc, 10% glycerol, 0.02% NP-40, 2 mM β-mercaptoethanol) and once with kinase buffer (10 mM HEPES pH 7.6, 100 mM KOAc, 50 mM β-glycerophosphate, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol), and aliquoted. Residual buffer was removed.

Priming phosphorylation reactions were performed by addition of 10 pmol (of each) kinase and started by addition of 2 or 10 mM (Fig. 1B, S1C) ATP. For samples without priming reaction the equivalent volume of added kinase was substituted by kinase buffer. After 30 min at 30 °C in a tabletop shaker beads were washed twice with binding buffer-200 (100 mM Hepes pH 7.6, 200 mM KOAc, 10% glycerol, 0.02% NP-40, 2 mM β-mercaptoethanol), once with binding buffer-100 and once with kinase buffer.

The consecutive kinase reaction was performed by addition of 10 pmol kinase and started by addition of 1 mM ATP + 5 µCi γ[^{32}P]-ATP (PerkinElmer). After incubation for 30 min shaking at 30 °C reactions were stopped by addition of Laemmli sample buffer followed by boiling at 95 °C.

For kinetic analysis of the phosphorylation reactions (Fig. S1C), the second kinase reaction was upscaled to 100 µl and 20 µl samples were taken at indicated time points.

Proteins were separated on NuPAGE Novex 12% Bis-Tris gels (ThermoFisher) and analyzed by autoradiography using a Typhoon FLA 9500 imager (GE healthcare).

Kinase assays using synthetic Mms4 peptides

Kinase reactions were performed with 25 µg desthiobiotin-labelled Mms4 peptide and 10 pmol kinase in kinase buffer (10 mM HEPES pH 7.6, 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 5 mM $Mg(OAc)_2$, 2 mM β-mercaptoethanol) with 100 mM KOAc in a 50 µL reaction volume containing 5 µg BSA. Reactions were started by addition of 1 mM ATP + 5 µCi γ[^{32}P]-ATP. After incubation for 30 min shaking at 30 °C reactions were stopped by addition of Laemmli sample buffer followed by boiling at 95 °C. Proteins were separated on NuPAGE Novex 12% Bis-Tris gels (ThermoFisher) in MES buffer and analyzed by autoradiography using a Typhoon FLA 9500 imager (GE healthcare).

Nuclease assays

5'-Cy3-end-labelled oligonucleotides were used to prepare synthetic nicked Holliday Junctions (nHJ) as described (Rass & West, 2008). Nuclease assays were carried out with immunopurified Mus81^{9myc} of Mus81^{3FLAG} (Fig. S4A) from cells arrested in mitosis with nocodazole. The anti-myc/anti-FLAG immunoprecipitates were extensively washed and mixed with 10 µl reaction buffer (50 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$) containing 30 ng 5'-Cy3-end-labelled nHJs or RFs [11]. Reactions were incubated for the indicated times with gentle rotation at 30 °C and stopped by addition of 5 µl 10 mg/ml proteinase K and 2% SDS, and further incubation at 37 °C for 1 h. Loading buffer was added and fluorophore-labelled products were separated by 10% PAGE, and analyzed using a Typhoon scanner. Substrate cleavage was normalized using the level of immunoprecipitated Mus81^{9myc} as reference.

DSB-induced recombination assay

The DSB-induced recombination assay was performed as described previously (Ho *et al.*, 2010). In brief, diploids were grown in liquid YPAR (YPR + 40 mg/l Adenine) until the cultures reached an OD_{600} of 0.5. Cells were arrested with nocodazole and I-SceI expression was induced by adding galactose to a final concentration of 2%. After 2.5 h cells were plated onto YPAD (YPD + 10 mg/l Adenine), incubated for 3-4 days and then replica plated onto YPAD+Hyg+Nat, YPAD+Hyg, YPAD+Nat, SC-Met, SC-Ura, and SCR-ADE+Gal media to classify recombination events. The different classes depicted arise from repair of DSBs by either short tract or long tract gene conversion which produces ade2-n or ADE+ recombinants, respectively (white class: two short tract conversions; red class: two long tract conversions; red/white class: one short and one long tract conversion). Within the distinct classes CO events are measured by the number of colonies that have rendered both daughter cells homozygous for the HPH and NAT marker.

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