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Supplemental Information

Yeast PP4 Interacts with ATR Homolog Ddc2-Mec1 and Regulates Checkpoint Signaling

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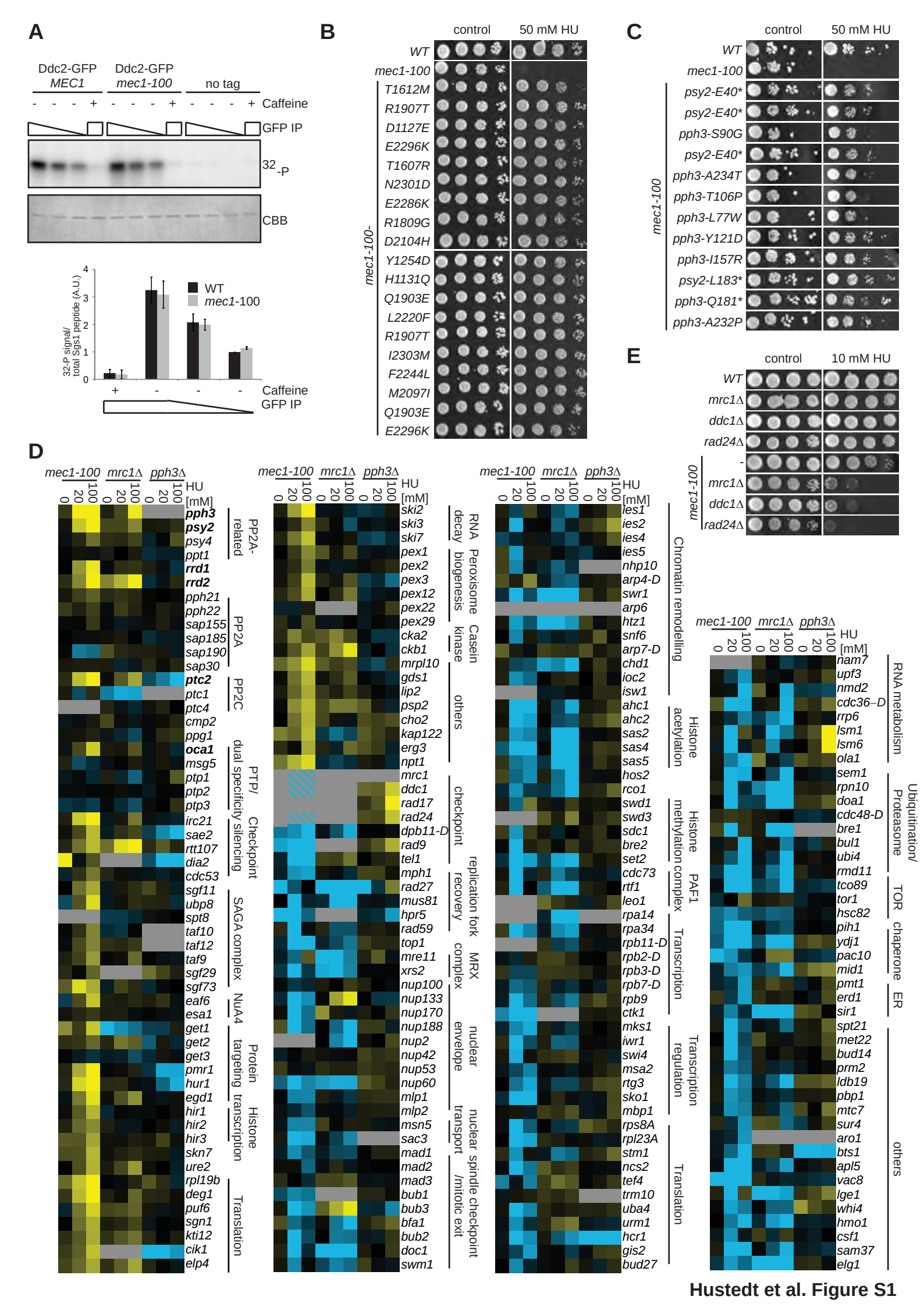
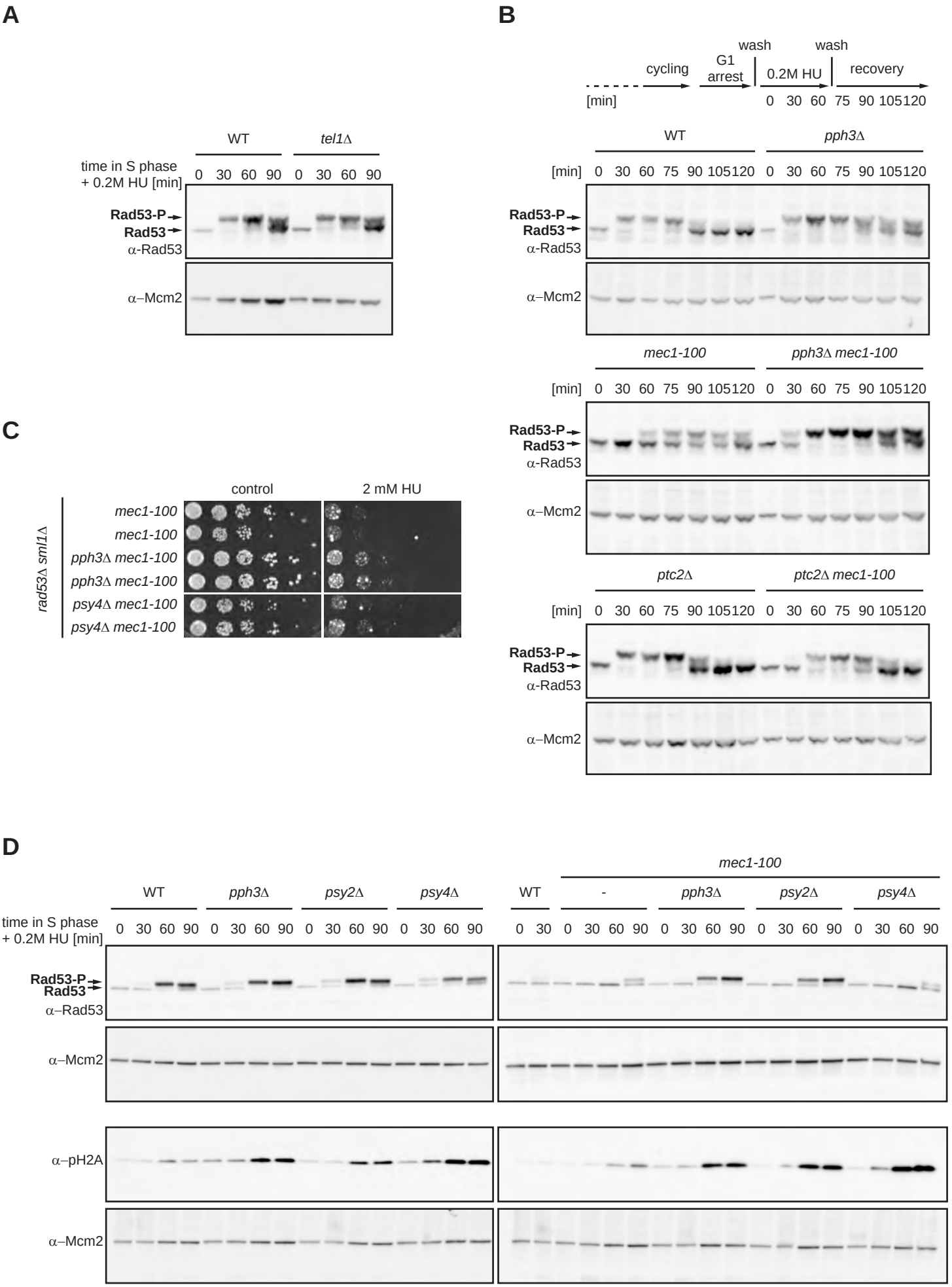
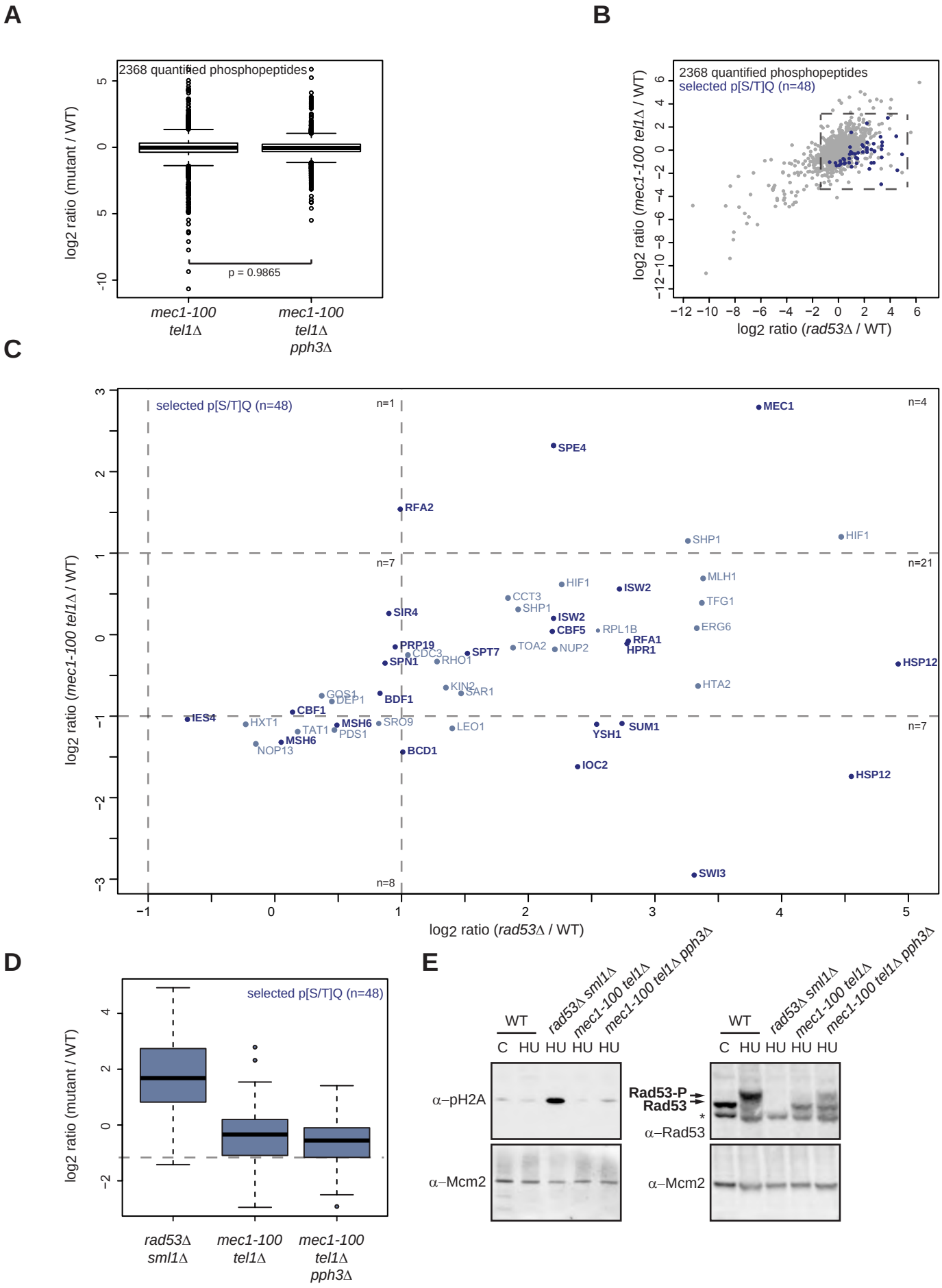


Hustedt et al. Figure S1



Hustedt et al. Figure S2



Hustedt et al. Figure S3

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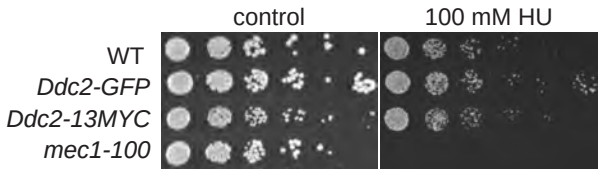

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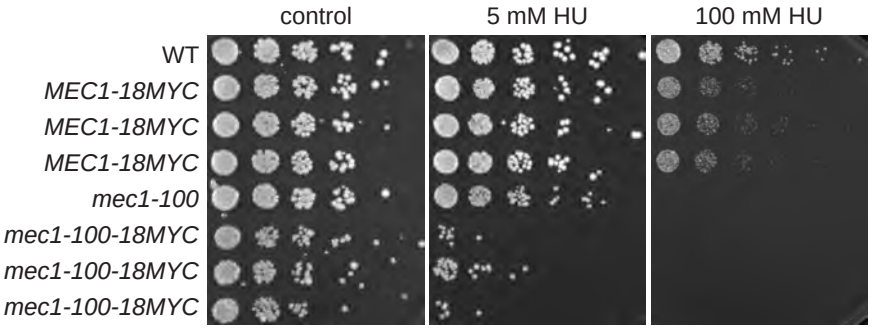

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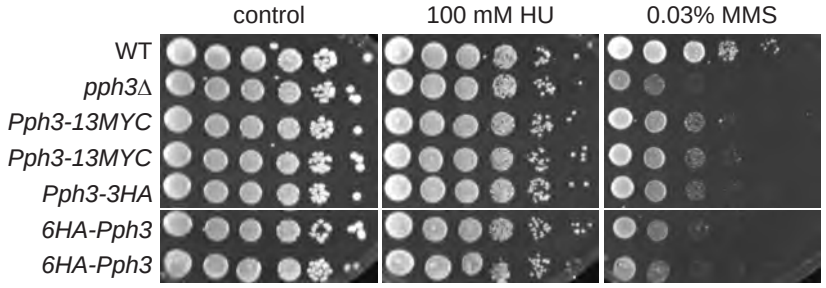

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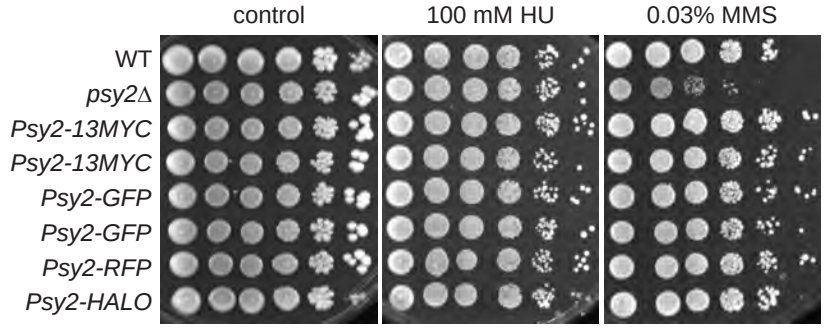

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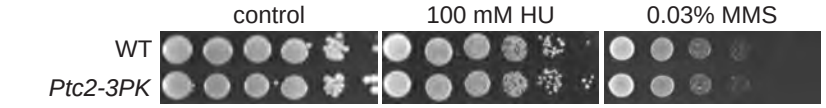

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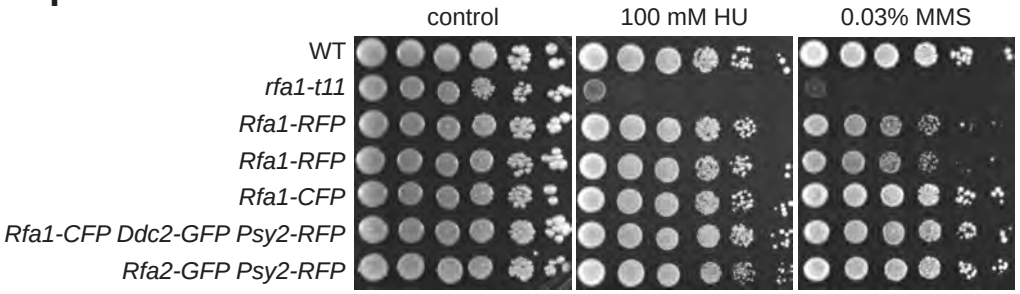
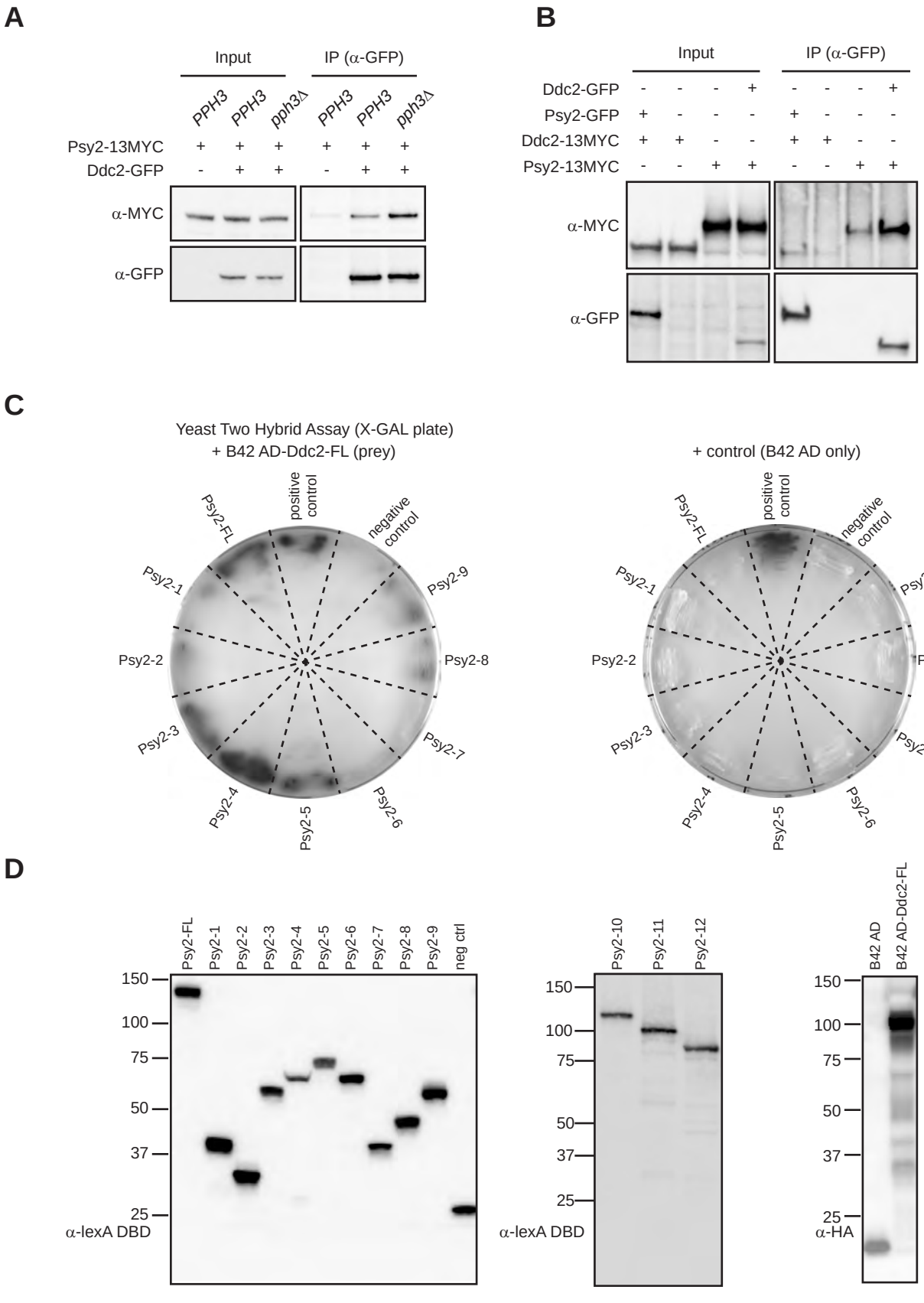
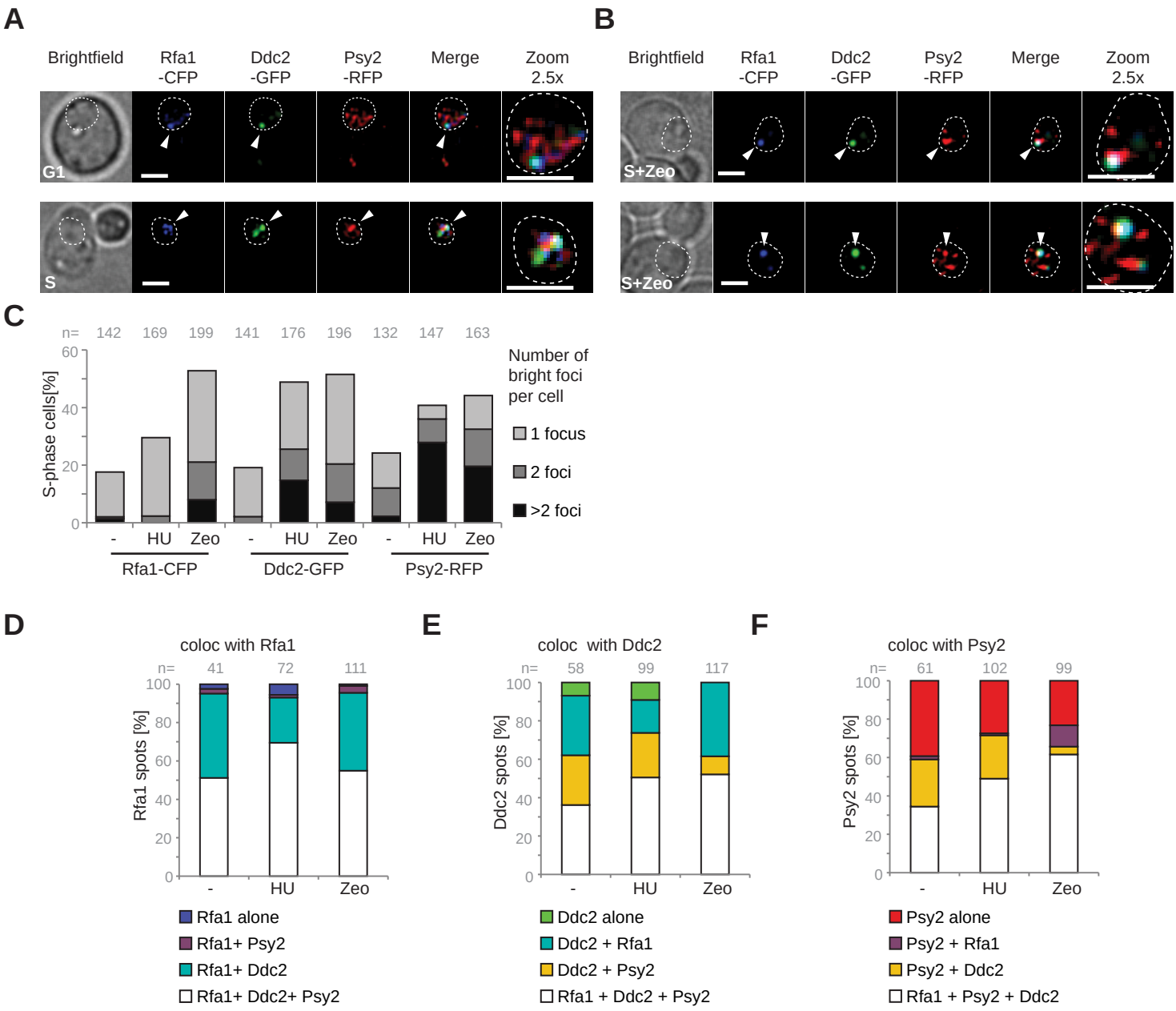


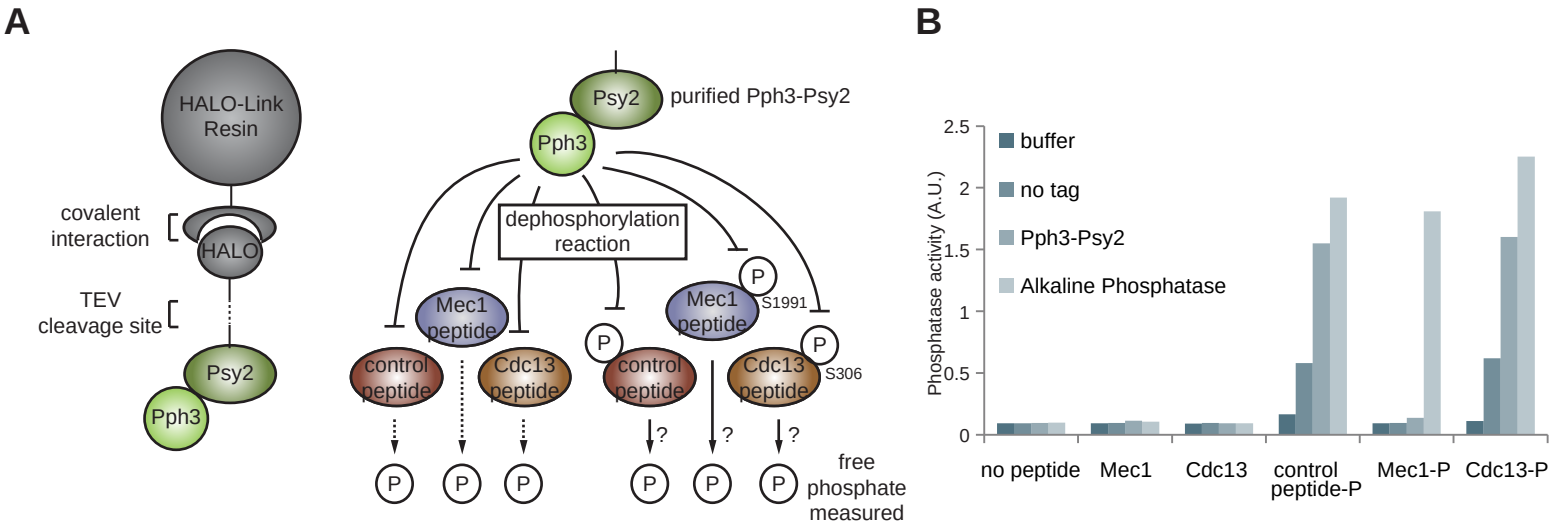
Hustedt et al. Figure S4

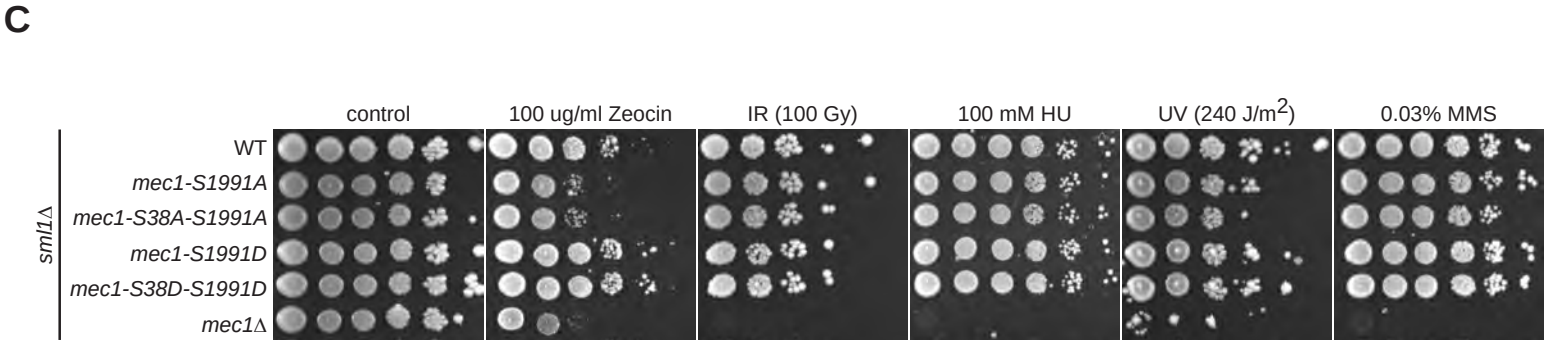


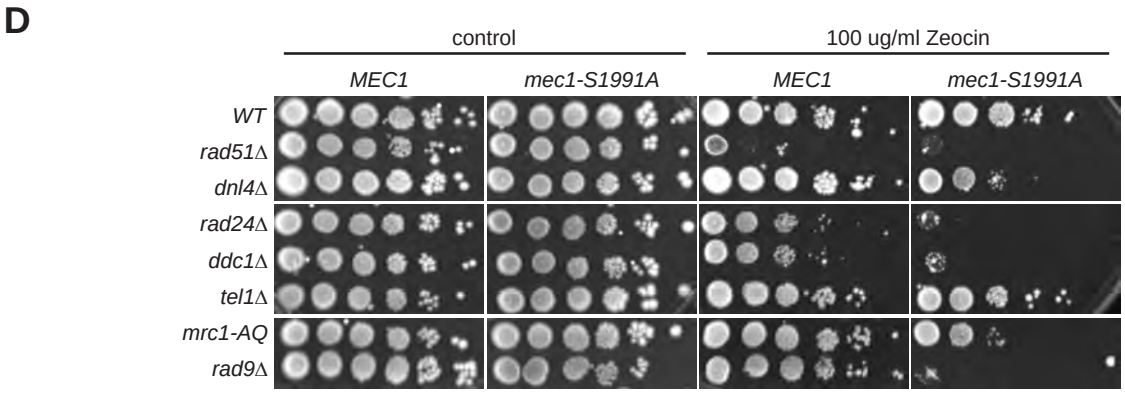
Hustedt et al. Figure S5

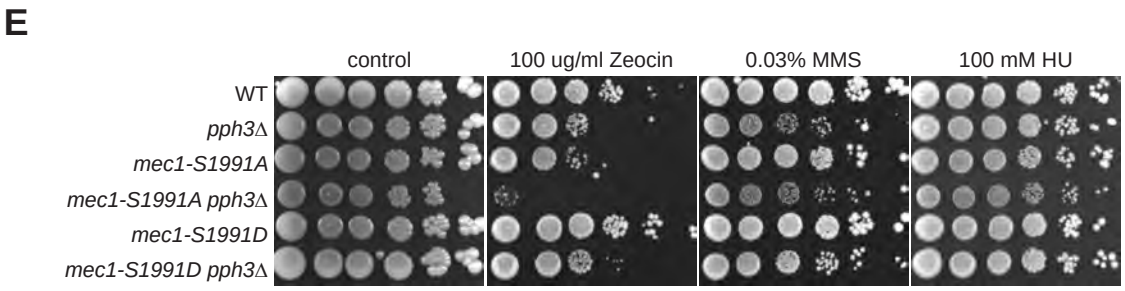


Hustedt et al. Figure S6









Hustedt et al. Figure S7

Supplemental Material

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. The mec1-100/Ddc2 kinase shows robust activity, yet genetic interactions implicate its deficiency in replication checkpoint and fork recovery; related to Figure 1

(A) Kinase assay using anti-GFP IP from *DDC2-GFP* (GA-7268), *DDC2-GFP mec1-100* (GA-7327) or WT (GA-1981) cell extracts. A Sgs1 peptide (aa 404–604, Hegnauer et al., 2010) was used as a substrate in the presence of γ-32P-ATP and was analysed by gel electrophoresis and autoradiography. Where indicated, 30mM caffeine was added to inhibit Mec1. ^{32}P autoradiography and Coomassie Brilliant Blue (CBB) staining are shown. Lower panel shows quantification of ^{32}P signal over CBB signal. Error bars indicate standard deviation of three independent experiments. **(B)** Drop assay of intragenic suppressors. Serial ten-fold dilutions of cultures were plated on YPAD ± 50 mM HU. Relevant genotypes are indicated in the figure. Isogenic strains used were: GA-1981, GA-6583, GA-6769, GA-6410, GA-6412, GA-6416, GA-6418, GA-6422, GA-6466, GA-6468, GA-6470, GA-6472, GA-6480, GA-6510, GA-6514, GA-6518, GA-6522, GA-6529, GA-6533, GA-6537 and GA-6539. **(C)** Drop assay of extragenic suppressors. Cells were treated as in (B). Isogenic strains used were: GA-1981, GA-6583, GA-6575, GA-6596, GA-6577, GA-6598, GA-6600, GA-6601, GA-6603, GA-6605, GA-6606, GA-6610, GA-6608 and GA-6572. Genotypes indicated in figure and in Table S2 Asterisks: STOP codon at indicated residue. **(D)** Genetic interactions with *mec1-100* (genetic interaction score ≤-2 or ≥+2) are shown. Interactions of *mrc1*Δ and *pph3*Δ with the same mutants are shown for comparison. Besides some phosphatase mutants

(*pph3*Δ, *ptc2*Δ, *rrd1*Δ, *rrd2*Δ, *oca1*Δ), *mec1-100* showed positive genetic interactions (yellow) with mutations that dampen the checkpoint response (*rtt107*Δ, *sae2*Δ, *dia2*Δ, *cdc53*Δ, *irc21*Δ), with some of which *pph3*Δ shows synthetic sickness. Negative genetic interactions (blue) of *mec1-100* include chromatin remodelers, nuclear envelope and various transcription regulators. Mutations of additional subunits of multisubunit complexes were included. Hatched areas indicate "no data" in E-MAP, but confirmed negative interaction by Drop Assay (see (E)). D = DAmP allele. **(E)** Serial ten-fold dilutions of cultures were plated on YPAD ± 10 mM HU. Relevant genotypes are indicated. Isogenic strains were: GA-1981, GA-6826, GA-7907, GA-5321, GA-6582, GA-6828 GA-7964 and GA-7209.

Figure S2. Psy4 dephosphorylates H2A, but does not suppress *mec1-100* HU sensitivity; related to Figure 3

(A) Rad53 activation was monitored in WT (GA-1981) and *tel1*Δ (GA-6912) cells. Cells were synchronized in G1 by α-factor arrest and released for indicated times into 0.2M HU before denatured extract preparation and Western blot analysis. **(B)** Cells were treated as in (A) except that after 60 minutes cells were washed and resuspended in medium without HU. Relevant genotypes are indicated. Samples were taken at indicated timepoints and analysed for Rad53 phosphorylation. Relevant genotypes are indicated. Isogenic strains were: GA-1981, GA-7049, GA-7273, GA-6582, GA-7086 and GA-7329. **(C)** Serial five-fold dilutions of saturated *rad53*Δ *sml1*Δ *mec1-100* (GA-7401), *rad53*Δ, *sml1*Δ *mec1-100 pph3*Δ (GA-7377), *rad53*Δ *sml1*Δ *mec1-100 psy4*Δ (GA-8581) cultures (2 cultures derived from 2 single colonies each) were plated on YPAD ± 2 mM HU. **(D)** Rad53 and H2A serine 129 phosphorylation was monitored by treating cells as in (A) and blotting with indicated antibodies. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7049, GA-7391, GA-7383, GA-6582, GA-7086, GA-7393 and GA-7385 cells.

Figure S3. Most S/TQ phosphopeptides are not reduced in *mec1-100* cells on HU; related to Figure 4

(A) Tukey boxplot of indicated abundance ratios of all quantified phosphopeptides. P-value was calculated by one-tailed Wilcoxon signed rank test**. (B)** Abundances (log_2 ratio (mutant/WT)) of all quantified phosphopeptides in *mec1-100 tel1*Δ cells were plotted against abundances in *rad53*Δ *sml1*Δ. Blue marked peptides are phosphorylated on [pS/pT]Q consenses and are (1) specifically downregulated in *mec1-100/tel1Δ* cells (see Figure 4) and/or (2) were at least 2-fold more abundant in *rad53*Δ compared to *mec1-100 tel1*Δ samples (log_2 ratio ≥ 1, $p \leq 0.05$, Student's paired t-test) regardless of the levels in WT sample and/or (3) were previously described as Mec1/Tel1-dependent (Chen et al., 2010) (full list in Table S7). Dotted lines indicate area enlarged in (C). **(C)** Enlarged area of plot shown in (B). Displayed are selected phosphopeptides marked blue in (A). Labels indicated protein names. Bold: phosphopeptides previously described as Mec1/Tel1 specific (Chen et al., 2010). Dotted lines indicate thresholds for up/downregulation. n= number of phosphopeptides that fall between dotted lines. **(D)** Tukey boxplot of indicated ratios of phosphopeptides that match the [pS/pT]Q consensus and were marked blue in (A). **(E)** Samples taken before synchronization (C) and prior protein extraction (HU) (Fig.4B) were analyzed by Western blotting with indicated antibodies.

Figure S4. Functionality of tagged strains used in this study; Related to Figure 5

(A) Serial 5-fold dilutions of cultures were plated on YPAD ± 100 mM HU. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7268, GA-7337 and GA-6582. **(B)** Serial 5-fold dilutions of cultures were plated on YPAD, YPAD + 5 mM HU and YPAD + 100 mM HU. Isogenic strains were GA-1981, GA-7353, GA-7354, GA-7355, GA-6582, GA-7366, GA-7367 and GA7368. **(C)** Serial 10-fold dilutions of cultures were plated on YPAD, YPAD + 100 mM HU and YPAD + 0.03% methyl methanesulfonate (MMS).

Isogenic trains were GA-1981, GA-7049, GA-7852, GA-7853, GA-7865, GA-7966 and GA-7967. **(D)** Cells were treated as in (C). Isogenic strains were GA-1981, GA-7391, GA-7798, GA-7799, GA-8033, GA-8021, GA-8659 and GA-8179 **(E)** Cells were treated as in (C). Isogenic strains were GA-1981 and GA-7923. **(F)** Cells were treated as in (C). Isogenic strains were GA-1981, GA-8396, GA-8687, GA-8688, GA-8694, GA-8695 and GA-8676. Relevant genotypes are indicated.

Figure S5. Ddc2-GFP and Psy2-MYC interaction does not require Pph3, and is detected when tags are swapped and in yeast two-hybrid (Y2H) experiments; Related to Figure 5

(A) Native extracts were prepared from cycling *Psy2-13MYC* (GA-7798), *Psy2-13MYC DDC2-GFP* (GA-7824) and *Psy2-13MYC DDC2-GFP pph3*Δ (GA-7939) cells, subjected to IP with α-GFP and analysis by Western blotting with indicated antibodies. **(B)** Cells were treated as in (A). Strains used were *PSY2-13MYC* (GA-7972), PSY2-13MYC DDC2-GFP (GA-7975), DDC2-13MYC (GA-7337) and *DDC2-13MYC PSY2-GFP* (GA-8034). **(C)** Y2H analysis between full length Ddc2 fused with the B42 transcription activation domain (B42 AD) and an HA-tag and Psy2 fragments fused with the lexA DNA binding domain (DBD) was performed by observing blue color formation on X-GAL plates. negative control: empty lexA-DBD plasmid. positive control: LexA-DBD directly fused with B42-AD. **(D)** Y2H construct expression: Cells were grown in 2% raffinose-containing selective medium to ensure plasmid retention and expression was induced with 2% galactose for 4h prior to denatured extract preparation and Western blot analysis with indicated antibodies.

Figure S6. Ddc2 and Psy2 colocalize and show FRET at spontaneous and Zeocin-induced foci; related to Figure 6

(A) Upper panel: Example of a G1-phase cell with a Ddc2/Rfa1 spot. Lower panel: Example of an S-phase cell with a spontaneous Rfa1/Ddc2/Psy2 focus. Strain used bears *RFA1-RFP DDC2-GFP PSY2-RFP* (GA-8695). Arrowheads indicate spots. Scale Bar = 2μm.

White dashed line: outline of cell nucleus. **(B)** Example images of Zeocin-treated cells, showing a few bright foci per cell. Displayed are examples of cell in which foci of all three fluorophores colocalize. The same strain was used as in (A). Arrowheads indicate foci. **(C)** Cells were treated +/- 0.2M HU or 400 ug/ml Zeocin for 1h prior to fixation. Bright spots per cell were quantified for S-phase cells. The same strain was used as in (A). **(D)** Cells were treated the same as in (C). Colocalization of Ddc2-GFP and/or Psy2-RFP spots with Rfa1-CFP spots was quantified. The same strain was used as in (A). **(E)** Colocalization of Rfa1-CFP and/or Psy2-RFP spots with Ddc2-GFP spots was quantified in the same experiment as (D). **(F)** Colocalization of Rfa1-CFP and/or Ddc2-GFP spots with Psy2-RFP spots was quantified in the same experiment as (D).

Figure S7. Damage sensitivity of the non-phosphorylatable mutant *mec1-S1991A*; related to Figure 7

(A) Setup of Pph3-Psy2 purification using HaloTag (Promega) and Phosphatase Assay **(B)** Phosphatase Assay to test dephosphorylation of Mec1 serine 1991 and Cdc13 serine 306 by Psy2-Pph3. Psy2-Pph3 was purified from *PSY2-HALO* cells (GA-8179) and WT cells (GA-1981, “no tag”) served as a negative control. Purifications were incubated with indicated phosphopeptides for 60 min at 30°C prior measurement of phosphatase activity by release of phosphate. Results are representative of two independent experiments. **(C)** Serial ten-fold dilutions of cultures were plated on YPAD, YPAD + 100 μg/ml Zeocin, YPAD + 100 mM HU, YPAD + 0.03% MMS and on YPAD plates and subsequently treated with γ-irradiation (100 Gy) or UV light (240 J/m^2). Relevant genotypes are indicated in the figure. Isogenic strains (see Table S2) used were: GA-4533, GA-8242, GA-8246, GA-8243, GA-8247 and GA-5286. **(D)** Serial ten-fold dilutions of cultures were plated on YPAD ± 100 μg/ml Zeocin. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-5919, GA-6096, GA-5321, GA-7907, GA-6912, GA-6671, GA-6625, GA-8338, GA-8829, GA-8838, GA-8890,

GA-8867, GA-8340, GA-8835 and GA-8841**(E)** Serial ten-fold dilutions of cultures were plated on YPAD, + 100 μg/ml Zeocin, YPAD + 0.03% MMS and YPAD + 100 mM HU. Relevant genotypes are indicated. Isogenic strains were GA-1981, GA-7049, GA-8338, GA-8873, GA-8342 and GA-8869.

SUPPLEMENTAL TABLES

Table S1: Yeast strains sorted by use in main figures

Figure	Strain
1A	GA-1981, GA-4978 and GA-2529
2B	GA-7049 and GA-7086
2C	GA-6582, GA-7086, GA-7393, GA-7385, GA-7929, GA-7932, GA-7329, GA-7276, GA-7935, and GA-8079
2D	GA-1981, GA-6582, GA-7086, GA-7393, GA-7395, GA-7929, GA-7982, GA-8054, GA-7329, GA-7979, GA-7049, GA-7391, GA-7398, GA-7908, GA-7981, GA-8052, GA-7273 and GA-7977.
2E	GA-1981, GA-6582, GA-7086, GA-7329, GA-7049 and GA-7273
2F	GA-1981, GA-6912, GA-6913, GA-7734, GA-7955, GA-7732 and GA-7953.
3A	GA-1981, GA-7049, GA-7273, GA-6582, GA-7086 and GA-7329
3B	GA-1981, GA-6912, GA-6913, GA-7734, GA-7955, GA-7732 and GA-7953
3C	GA-1981, GA-7049, GA-6582, GA-7086, GA-6913, GA-7734, GA-6912 and GA-7732.
3D	GA-4533, GA-7294, GA-5286, GA-7650, GA-7724, GA-7725 and GA-7707
3E	GA-7373, GA-7375, GA-7401, GA-7377 (two colonies of each) and GA-7709 and GA-7711.
3F	GA-7656, GA-7657, GA-7658, GA-7659, GA-7660, GA-7661, GA-7662 and GA-7663.
4	GA-1981, GA-7373, GA-6913 and GA-7734
5A	GA-7972 and GA-7975
5B	GA-7799, GA-7824, GA-7835 and GA-7827
5C	GA-338
6A-D	GA-8695
6F	GA-8656, GA-8705 and GA-8702
7B	GA-7824 and GA-8232
7C	GA-7824 and GA-7835
7D	GA-7268, GA-7327, GA-7945, GA-8232, GA-8484 and GA-8486
7E	GA-4533, GA-8242, GA-8246, GA-8243, GA-8247 and GA-5286
7F	GA-1981, GA-8338, GA-8342 and GA-5975

Table S2: Yeast strains and plasmids used in this study

Strain	genotype	source
GA-181	*MATa, ade2-1, trp1-1, his3-11, -15, ura3-1, leu2-3, -112, can1-100 (W303)*	R. Rothstein
GA-338	*MATa, trp1, his3, ura3, leu2::(pLEU2-lexAop6)(EGY48)*	R. Brent
GA-1981	*MATa, ade2-1 ,trp1-1 ,his3-11 , -15, ura3-1, leu2-3, -112, can1-100* (W303), RAD5+	H.L. Klein
GA-1982	*MATα, ade2-1 ,trp1-1 ,his3-11 , -15, ura3-1, leu2-3, -112, can1-100* (W303), RAD5+	H.L. Klein
GA-2529	GA-181 with *mec1::HIS3, sml1::KanMX*	this study
GA-4533	GA-1981 with *sml1::HIS3*	this study
GA-4978	GA-1981 with *mec1-100::LEU2(HIS3)*	(Hegnauer et al., 2012)
GA-5286	GA-1981 with *mec1::TRP1, sml1::HIS3*	this study
GA-5321	GA-1981 with *rad24::TRP1*	(Hegnauer et al., 2012)
GA-5919	GA-1981 with *rad51::URA3*	this study
GA-5975	GA-1981 with *rad52::TRP1*	this study
GA-6022	*MATa, his3-D1, leu2D0, met15D0, ura3D0, rfa2::RFA2-GFP-HIS3 (BY4741)*	I.Filipuzzi
GA-6096	GA-1981 with *dnl4::HIS3*	(Shimada et al., 2013)
GA-6356	GA-1981 with *mec1-100::LEU2(HIS3) exo1::kanMX*	this study
GA-6410	GA-1981 with *mec1-100-R1907T::LEU2(HIS3)*	this study
GA-6412	GA-1981 with *mec1-100-D1127E::LEU2(HIS3)*	this study
GA-6416	GA-1981 with *mec1-100-E2296K::LEU2(HIS3)*	this study
GA-6418	GA-1981 with *mec1-100-T1607R::LEU2(HIS3)*	this study

GA-6422	GA-1981 with *mec1-100-N2301D::LEU2(HIS3)*	this study
GA-6466	GA-1981 with *mec1-100-E2286K::LEU2(HIS3)*	this study
GA-6468	GA-1981 with *mec1-100-R1809G::LEU2(HIS3)*	this study
GA-6470	GA-1981 with *mec1-100-D2104H::LEU2(HIS3)*	this study
GA-6472	GA-1981 with *mec1-100-Y1254D::LEU2(HIS3)*	this study
GA-6480	GA-1981 with *mec1-100-H1131Q::LEU2(HIS3)*	this study
GA-6510	GA-1981 with *mec1-100-Q1903E::LEU2(HIS3)*	this study
GA-6514	GA-1981 with *mec1-100-L2220F::LEU2(HIS3)*	this study
GA-6518	GA-1981 with *mec1-100-R1907T::LEU2(HIS3)*	this study
GA-6522	GA-1981 with *mec1-100-I2303M::LEU2(HIS3)*	this study
GA-6529	GA-1981 with *mec1-100-F2244L::LEU2(HIS3)*	this study
GA-6533	GA-1981 with *mec1-100-M2097I::LEU2(HIS3)*	this study
GA-6537	GA-1981 with *mec1-100-Q1903E::LEU2(HIS3)*	this study
GA-6539	GA-1981 with *mec1-100-E2296K::LEU2(HIS3)*	this study
GA-6572	GA-1981 with *mec1-100::LEU2(HIS3), pph3-A232P*	this study
GA-6575	GA-1981 with *mec1-100::LEU2(HIS3), psy2-E40**	this study
GA-6577	GA-1981 with *mec1-100::LEU2(HIS3), psy2-E40**	this study
GA-6582	GA-1981 with *mec1-100::natMX*	this study
GA-6583	GA-1982 with *mec1-100::natMX*	this study
GA-6596	GA-1981 with *mec1-100::LEU2(HIS3), psy2-E40**	this study
GA-6598	GA-1981 with *mec1-100::LEU2(HIS3), pph3-S90G*	this study
GA-6600	GA-1981 with *mec1-100::LEU2(HIS3), pph3-A234T*	this study
GA-6601	GA-1981 with *mec1-100::LEU2(HIS3), pph3-T106P*	this study
GA-6603	GA-1981 with *mec1-100::LEU2(HIS3), pph3-L77W*	this study
GA-6605	GA-1981 with *mec1-100::LEU2(HIS3), pph3-Y121D*	this study
GA-6606	GA-1981 with *mec1-100::LEU2(HIS3), pph3-I157R*	this study
GA-6608	GA-1981 with *mec1-100::LEU2(HIS3), pph3-Q181**	this study
GA-6610	GA-1981 with *mec1-100::LEU2(HIS3), psy2-L183**	this study
GA-6625	GA-1981 with *rad9::TRP1*	this study
GA-6635	GA-1982 with *mec1-100::LEU2(HIS3) exo1::kanMX*	this study
GA-6636	GA-1982 with *mec1-100::LEU2(HIS3)*	this study
GA-6671	GA-GA-1981 with *mrc1-AQ::LEU2(HIS3)*	this study
GA-6769	GA-1981 with *mec1-100-T1612M::LEU2(HIS3)*	this study
GA-6826	GA-1981 with *mrc1::TRP1*	this study
GA-6828	GA-1981 with *mec1-100::natMX, mrc1::TRP1*	this study
GA-6912	GA-1981 with *tel1::URA3*	this study
GA-6913	GA-1981 with *tel1::URA3, mec1-100::natMX*	this study
GA-7049	GA-1981 with *pph3::HIS3*	this study
GA-7086	GA-1981 with *mec1-100::natMX, pph3::HIS3*	this study
GA-7209	GA-1981 with *mec1-100::natMX, rad24::TRP1*	this study
GA-7268	GA-1981 with *ddc2::DDC2-GFP-kanMX*	this study
GA-7273	GA-1981 with *ptc2::URA3*	this study
GA-7276	GA-1981 with *ptc3::HIS3, mec1-100::natMX*	this study
GA-7294	GA-1981 with *pph3::kanMX, sml1::HIS3*	this study
GA-7327	GA-1981 with *ddc2::DDC2-GFP-kanMX, mec1-100::natMX*	this study
GA-7329	GA-1981 with *ptc2::URA3, mec1-100::natMX*	this study
GA-7337	GA-1981 with *ddc2::DDC2-13MYC-URA3*	this study
GA-7353	GA-1981 with *mec1::LEU2-18MYC-MEC1*	this study
GA-7354	GA-1981 with *mec1::LEU2-18MYC-MEC1*	this study
GA-7355	GA-1981 with *mec1::LEU2-18MYC-MEC1*	this study
GA-7366	GA-1981 with *mec1::LEU2-18MYC-mec1-100::natMX*	this study
GA-7367	GA-1981 with *mec1::LEU2-18MYC-mec1-100::natMX*	this study
GA-7368	GA-1981 with *mec1::LEU2-18MYC-mec1-100::natMX*	this study
GA-7373	GA-1981 with *rad53::URA3, sml1::kanMX*	this study
GA-7375	GA-1981 with *rad53::URA3, sml1::kanMX, pph3::HIS3*	this study
GA-7377	GA-1981 with *rad53::URA3, sml1::kanMX, pph3::HIS3, mec1-100::natMX*	this study
GA-7383	GA-1981 with *psy4::hphMX*	this study
GA-7385	GA-1981 with *psy4::hphMX, mec1-100::natMX*	this study
GA-7391	GA-1981 with *psy2::URA3*	this study
GA-7393	GA-1981 with *psy2::URA3, mec1-100::natMX*	this study
GA-7395	GA-1981 with *psy2::URA3, mec1-100::natMX, pph3::HIS3*	this study
GA-7398	GA-1982 with *psy2::URA3, pph3::HIS3*	this study
GA-7401	GA-1981 with *rad53::URA3, sml1::kanMX, mec1-100::natMX*	this study
GA-7650	GA-1981 with *mec1::TRP1, sml1::HIS3, pph3::kanMX*	this study
GA-7656	GA-1981 with *rad53::URA3, sml1::kanMX, chk1::TRP1*	this study

GA-7657	GA-1982 with *rad53::URA3, sml1::kanMX, chk1::TRP1*	this study
GA-7658	GA-1981 with *rad53::URA3, sml1::kanMX, chk1::TRP1, pph3::HIS3*	this study
GA-7659	GA-1982 with *rad53::URA3, sml1::kanMX, chk1::TRP1, pph3::HIS3*	this study
GA-7660	GA-1981 with *rad53::URA3, sml1::kanMX, chk1::TRP1, mec1-100::natMX*	this study
GA-7661	GA-1982 with *rad53::URA3, sml1::kanMX, chk1::TRP1, mec1-100::natMX*	this study
GA-7662	GA-1981 with *rad53::URA3, sml1::kanMX, chk1::TRP1, mec1-100::natMX, pph3::HIS3*	this study
GA-7663	GA-1982 with *rad53::URA3, sml1::kanMX, chk1::TRP1, mec1-100::natMX, pph3::HIS3*	this study
GA-7707	GA-1981 with *mec1::TRP1, tel1::URA3, sml1::HIS3, pph3::kanMX*	this study
GA-7709	GA-1981 with *mec1::TRP1, rad53::URA3, sml1::kanMX*	this study
GA-7711	GA-1981 with *mec1::TRP1, rad53::URA3, sml1::kanMX, pph3::HIS3*	this study
GA-7724	GA-1981 with *mec1::TRP1, tel1::URA3, sml1::HIS3*	this study
GA-7725	GA-1982 with *mec1::TRP1, tel1::URA3, sml1::HIS3*	this study
GA-7732	GA-1981 with *tel1::URA3, pph3::HIS3*	this study
GA-7734	GA-1981 with *tel1::URA3, pph3::HIS3, mec1-100::natMX*	this study
GA-7798	GA-1981 with *psy2::PSY2-13MYC-URA3*	this study
GA-7799	GA-1981 with *psy2::PSY2-13MYC-URA3*	this study
GA-7824	GA-1981 with *ddc2::DDC2-GFP-kanMX, psy2::PSY2-13MYC-URA3*	this study
GA-7827	GA-1981 with *ddc2::DDC2-GFP-kanMX, psy2::PSY2-13MYC-URA3, mec1::TRP1, sml1::HIS3*	this study
GA-7835	GA-1981 with *ddc2::DDC2-GFP-kanMX, psy2::PSY2-13MYC-URA3, mec1-100::natMX*	this study
GA-7852	GA-1981 with *pph3::PPH3-13MYC-URA3*	this study
GA-7853	GA-1981 with *pph3::PPH3-13MYC-URA3*	this study
GA-7865	GA-1981 with *pph3::PPH3-3HA-URA3*	this study
GA-7907	GA-1981 with *ddc1::URA3*	this study
GA-7908	GA-1981 with *rrd1::HIS3*	this study
GA-7923	GA-1981 with *ptc2::PTC2-3PK-LEU2*	this study
GA-7929	GA-1981 with *rrd1::HIS3, mec1-100::natMX*	this study
GA-7932	GA-1981 with *rrd2::kanMX, mec1-100::natMX*	this study
GA-7935	GA-1981 with *oca1::URA3, mec1-100::natMX*	this study
GA-7939	GA-1981 with *DDC2-GFP-kanMX, psy2::PSY2-13MYC-URA3, pph3::HIS3*	this study
GA-7945	GA-1981 with *DDC2-GFP-kanMX, mec1-100::natMX, pph3::HIS3*	this study
GA-7953	GA-1981 with *tel1::URA3, ptc2::HIS3*	this study
GA-7955	GA-1981 with *tel1::URA3, ptc2::HIS3, mec1-100::natMX*	this study
GA-7964	GA-1981 with *mec1-100::natMX, ddc1::URA3*	this study
GA-7966	GA-1981 with *pph3::6HA-PPH3*	this study
GA-7967	GA-1981 with *pph3::6HA-PPH3*	this study
GA-7972	GA-1981 with *psy2::PSY2-13MYC-URA3, ptc2::PTC2-3PK-LEU2*	this study
GA-7975	GA-1981 with *psy2::PSY2-13MYC-URA3, ptc2::PTC2-3PK-LEU2, ddc2::DDC2-GFP-kanMX*	this study
GA-7977	GA-1981 with *ptc2::URA3, pph3::HIS3*	this study
GA-7979	GA-1981 with *ptc2::URA3, pph3::HIS3, mec1-100::natMX*	this study
GA-7981	GA-1981 with *rrd1::HIS3, pph3::HIS3*	this study
GA-7982	GA-1981 with *rrd1::HIS3, pph3::HIS3, mec1-100::natMX*	this study
GA-8021	GA-1982 with *psy2::PSY2-GFP-kanMX*	this study
GA-8033	GA-1981 with *psy2::PSY2-GFP-kanMX*	this study
GA-8034	GA-1981 with *psy2::PSY2-GFP-kanMX, ddc2::DDC2-13MYC-URA3*	this study
GA-8052	GA-1981 with *rrd1::HIS3, psy2::URA3*	this study
GA-8054	GA-1981 with *rrd1::HIS3, psy2::URA3, mec1-100::natMX*	this study
GA-8079	GA-1981 with *glc7-132, mec1-100::natMX*	this study
GA-8179	GA-1981 with *psy2::PSY2-TEV-HALO-kanMX*	this study
GA-8232	GA-1981 with *DDC2-GFP-kanMX, psy2::PSY2-13MYC-URA3, mec1-S1991A::natMX*	this study
GA-8242	GA-1981 with *mec1-S38A::natMX, sml1::HIS3*	this study
GA-8243	GA-1981 with *mec1-S38D::natMX, sml1::HIS3*	this study
GA-8246	GA-1981 with *mec1-S38A-S1991A::natMX, sml1::HIS3*	this study
GA-8247	GA-1981 with *mec1-S38D-S1991D::natMX, sml1::HIS3*	this study
GA-8338	GA-1981 with *mec1-S1991A::natMX*	this study
GA-8340	GA-1981 with *mec1-S1991A::natMX, tel1::URA3*	this study
GA-8342	GA-1981 with *mec1-S1991D::natMX*	this study
GA-8396	GA-1981 with *rfa1-t11*	this study
GA-8484	GA-1981 with *ddc2::DDC2-GFP-kanMX, mec1-D2243E(kd1), sml1::kanMX*	this study
GA-8486	GA-1981 with *ddc2::DDC2-GFP-kanMX, rad53::URA3, sml1::HIS3*	this study
GA-8581	GA-1981 with *rad53::URA3, sml1::kanMX, psy4::hphMX, mec1-100::natMX*	this study
GA-8656	GA-1981 with *ddc2::DDC2-GFP-kanMX, psy2::PSY2-RFP-URA3*	this study
GA-8659	GA-1981 with *psy2::PSY2-RFP-URA3*	this study
GA-8676	GA-1981 with *psy2::PSY2-RFP-URA3, rfa2::RFA2-GFP-HIS3*	this study
GA-8687	GA-1981 with *rfa1::RFA1-RFP-HIS3*	this study

GA-8688	GA-1981 with *rfa1::RFA1-RFP-HIS3*	this study
GA-8694	GA-1981 with *rfa1::RFA1-eCFP-hphMX*	this study
GA-8695	GA-1981 with *psy2::PSY2-RFP-URA3, ddc2::DDC2-GFP-kanMX, rfa1::RFA1-eCFP-hphMX*	this study
GA-8702	GA-1981 with *rfa1::RFA1-RFP-HIS3, psy2::PSY2-GFP-kanMX*	this study
GA-8705	GA-1981 with *rfa1::RFA1-RFP-HIS3, ddc2::DDC2-GFP-kanMX*	this study
GA-8829	GA-1981 with *mec1-S1991A::natMX, rad51::URA3*	this study
GA-8835	GA-1981 with *mec1-S1991A::natMX, mrc1-AQ::LEU2(HIS3)*	this study
GA-8838	GA-1981 with *mec1-S1991A::natMX, dnl4::HIS3*	this study
GA-8841	GA-1981 with *mec1-S1991A::natMX, rad9::TRP1*	this study
GA-8867	GA-1981 with *mec1-S1991A::natMX, ddc1::URA3*	this study
GA-8869	GA-1981 with *mec1-S1991D::natMX, pph3::HIS3*	this study
GA-8873	GA-1981 with *mec1-S1991D::natMX, pph3::HIS3*	this study
GA-8890	GA-1981 with *mec1-S1991A::natMX, rad24::TRP1*	this study

plasmid No.	plasmid name	source
#286	*pRS426*	(Mumberg et al., 1995)
#359	*pSH18-34*	(Golemis et al., 2011)
#361	*pSH17-4*	(Golemis et al., 2011)
#965	*pGAL-lexA*	(Bjergbaek et al., 2005)
#993	*pJG4-6*	(Golemis et al., 2011)
#2221	*pKU2-rfa1-t11*	(Soustelle et al., 2002)
#2745	*pGAL-EcoRI*	(Schar et al., 2004)
#2842	*pEG203+NLS*	this study
#3307	*pGAL-lexA-PSY2*	this study
#3308	*pJG4-6-DDC2*	this study
#3367	*Yeplac195+PPH3*	(O'Neill et al., 2007)
#3368	*Yeplac195+pph3-H112N*	(O'Neill et al., 2007)
#3493	*pcDNA3.1-GFP*	Antoine Peters
#3518	*pcDNA3.1-PP4R3A-GFP*	this study
#3525	*pDEST12.2-MYC-ATRIP*	this study
#3588	*pcDNA3.1-PP4R3B-GFP*	this study
#3592	*pGAL-lexA-PSY2-2(aa25-129)*	this study
#3593	*pGAL-lexA-PSY2-3(aa25-350)*	this study
#3594	*pGAL-lexA-PSY2-4(aa25-447)*	this study
#3595	*pGAL-lexA-PSY2-5(aa25-502)*	this study
#3616	*pEG203+NLS+PSY2*	this study
#3617	*pEG203+NLS+PSY2-1(aa1-129)*	this study
#3618	*pEG203+NLS+PSY2-2(aa25-129)*	this study
#3619	*pEG203+NLS+PSY2-3(aa25-350)*	this study
#3620	*pEG203+NLS+PSY2-4(aa25-447)*	this study
#3621	*pEG203+NLS+PSY2-5(aa-25-502)*	this study
#3622	*pEG203+NLS+PSY2-6(aa-334-720)*	this study
#3623	*pEG203+NLS+PSY2-7(aa560-720)*	this study
#3629	*pEG203+NLS+PSY2-8(aa130-350)*	this study
#3630	*pEG203+NLS+PSY2-9(aa560-end)*	this study
#3649	*pGAL-lexA-PSY2-10(Δ aa25-129)*	this study
#3650	*pGAL-lexA-PSY2-11(Δ aa130-350)*	this study
#3651	*pGAL-lexA-PSY2-12(Δ aa25-350)*	this study
#3653	*pGAL-lexA-PSY2-8(aa130-350)*	this study

Table S3: Query strains and library used for E-MAP

separate Excel file

Table S4: Genetic interaction data

separate Excel file

Table S5: All quantified phosphopeptides

separate Excel file

Table S6: Mec1-100 dependent phosphopeptides

separate Excel file

Table S7: Selected SQ/TQ phosphopeptides

separate Excel file

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Yeast materials, microscopy, phosphoproteomics, phosphatase assay, and E-MAP

Yeast strains and plasmids are described in Tables S1/S2. If not stated otherwise cells were cultured at 30°C in YPAD medium using standard procedures. For Y2H analysis, fragments of *PSY2* were fused to the lexA DNA binding domain (#3616 - #3623, #3629,#3630) and *DDC2* was fused to the B42 transcription activation domain of pJG4-6 (#993), resulting in pJG4-6-DDC2 (#3308). WT cells (GA-338/EGY48) containing the lacZ reporter pSH18-34 (#359), the bait and the prey were streaked on X-GAL plates (Golemis et al., 2011) and incubated 1-2 days at 30°C. β–galactosidase assays were performed as previously described (Hegnauer et al., 2012) using pGAL-lexA Psy2 constructs (#3307, #3592-#3595, #3649-#3651, #3653).

Enzyme assays, recovery and drop assay, Rad53 and H2A phosphorylation, FACS

Mec1 kinase assay was performed as described (Hustedt and Shimada, 2014). A re-combinant domain of Sgs1 (Sgs1 aa404-604) was used as substrate (Hegnauer et al., 2012). Recovery and drop assays, FACS analysis and Rad53 and H2A phosphorylation analysis were done as described previously (Hustedt and Shimada, 2014). Phosphatase assays are described below.

Immunoprecipitation (IP)

Anti-GFP IP was carried out as described for kinase assays, except that the lysis buffer was supplemented with protease and phosphatase inhibitors (Complete protease inhibitors (Roche), 1mM phenylmethylsulfonyl fluoride, PhosSTOP phosphatase inhibitors (Roche), 0.1 mM Na_3VO_4, 1 mM NaF and 10 mM NaPP) and bead-bound protein complexes were washed three times with lysis buffer prior elution with 0.2 M glycine. Eluates were neutralized with Tris/HCl and analysed by Western blotting.

Immunoprecipitation for mammalian cells was essentially performed the same, except that cells were harvested 48h post transfection by scraping off the plate into PBS, and washed once with PBS before snap-freezing pellets in liquid nitrogen.

Nuclease treatment was performed in a modified lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM $MgCl_2$ and 0.5% NP-40) supplemented with protease and phosphatase inhibitors. Cleared lysates were incubated with 5 µl RNAseA (Sigma) and 5 µl benzonase

(Invitrogen) for 30 min on ice prior to immunoprecipitation. After 1h incubation at 4°C with antibody-coupled beads, lysates were recovered, DNA was isolated by phenol/chloroform extraction and analyzed by agarose gel electrophoresis and SYBR SAFE (Invitrogen) staining. Bead-bound protein complexes were analyzed as described above.

Antibodies for Western blotting

Antibodies used were: Monoclonal α-Rad53 antibody (custom made by Genscript), goat αMcm2 antibody (Santa Cruz) and rabbit αH2A phospho-serine 129 antibody (custom made by Sigma Genosys), mouse α-GFP (Roche), rabbit α-GFP, (Invitrogen), rabbit α-MYC (Santa Cruz), rabbit α-Rfa1 (Agrisera), rabbit α-PK (Novus Biologicals), rabbit α-Mec1 (custom made by SDIX), rabbit α Mec1 phospho-serine 1991 (custom made by Genscript), rat α-HA (Roche), rabbit α–lexA (Santa Cruz)

Sequencing of extragenic suppressors

Genomic DNA was isolated using Qiagen Genomic Tip100 (Qiagen) or phenol chloroform extraction and NucleoSpin PCR clean-up kit (Macherey-Nagel) in case of GA-6610 and quantified using a Nanodrop 3000 (Thermo Scientific) PicoGreen Assay. 50 ng of each individual sample were processed for library generation using Illumina's Nextera DNA sample preparation protocol, and barcoded as described for Illumina's TruSeq Dual Index Sequencing primers (Illumina). The samples were pooled at equimolar concentrations and sequenced using a single-end 75-base reads (50-base reads for GA-6610) on the Illumina's HiSeq2000 platform. The sequence data had >95% alignment, using BWA, to a reference S288C genome (genome build S288C_reference_genome_R62-1-1_20090218, obtained from Saccharomyces Genome Database, www.yeastgenome.org). A sequence of progenitor strains used in the experiment was done for *mec1-100* (GA-4978 and GA-6336) and *mec1-100 exo1*Δ (GA-6356 and GA-6335). By comparison to the progenitor strain sequence, the SNP and indel calls were made in the sequence of the various derived sub-strains. In addition, large regions (>0.1 Megabase) of chromosome amplifications and deletions were assessed in each strain by comparison across all of the strains using read depth information.

High throughput genetic interaction screening

All E-MAP query and array strains are described in Table S3. For *mec1-100*, *sgs1-r1* and *rfa1-t11* query strains a *NAT* resistance cassette was integrated approximately 150 bp

downstream of the gene. Subsequently, *mec1-100* and *sgs1-r1* mutations were introduced by pop-in/pop-out (Reid et al., 2002) with linear DNA fragments engineered by PCR using genomic DNA from already mutated strains (Hegnauer et al., 2012; Paciotti et al., 2001) as a template. *rfa1-t11* was created by transforming *NheI*-linearized plasmid #2221.

Double mutants of query strains and library strains were created as described (Tong et al., 2004). Colony sizes were quantified using HT Colony Grid Analyzer (version 1.1) and genetic interaction scores were computed using the E-MAP toolkit (version 2.0) as previously described (Collins et al., 2006). A stringent QA/QC pipeline was employed to identify and remove (i) strains with a high error of measurement, and (ii) incorrectly deleted strains as identified through linkage analysis (Collins et al., 2010; Collins et al., 2006). In addition, all 1525 array mutants were pooled, genomic DNA extracted, and finally hybridized to a microarray containing probes covering tag sequences from the yeast deletion collection as described (Hegnauer et al., 2012). Strains with an intensity of <800 arbitrary fluorescent units were excluded from further analysis. In total, 214 array strains were dropped for further analysis. This included *ufo1*Δ, for which a genetic interaction with *mec1-100* could not be confirmed despite recreating the mutation, most likely indicating mislabeling of this strain.

Mec1 phosphorylation

Immunoprecipitation to map phosphorylation sites in Ddc2-Mec1 was carried out starting from 1 liter cultures treated with 0.2M HU for 1h. Cells were harvested and washed once with PBS. Pellets were weighed, resuspended in 1ml /g PBS and dropped into liquid nitrogen. Droplets were subjected to three rounds of bead-milling using Mixer Mill MM 400 (Retsch). The frozen cell powder was resuspended in an equal volume of cold lysis buffer and Anti-GFP IP was performed as described above.

Eluted protein samples were treated with trypsin overnight at 37°C after reduction and alkylation with tris(2-carboxyethyl)phosphine (TCEP) and iodoacetic acedic acid. The TFA acidified tryptic peptides (final concentration 0.1%) were separated on an Agilent 1100 nanoLC system (Agilent Technologies) coupled to an LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Scientific). The LC system was equipped with a Peptide CapTrap column (Michrom BioResources, Inc.) and a capillary column with integrated nanospray tip (75 µm i.d. x 100 mm, Swiss BioAnalytics AG) filled with MagicC18 (5 µm, Michrom Bioresources, Inc.). Elution was performed with a gradient starting with 2% solvent B and

continued with 2 to 10% solvent B in 3 min and 10 to 40% solvent B in 80 min at a flow rate of 400 nL/ min. Solvent A consisted of 0.1% formic acid/ 2% acetonitrile, solvent B was composed of 0.1% formic acid/ 80% acetonitrile. The mass spectrometer operated in positive mode using the top 15 DDA method. MS scans were acquired at a resolution of 60, 000 over a range of m/z 350 to 1200. Singly charged ions were rejected from MSMS fragmentation. Peptides were identified searching SwissProt (version 2011-08) using Mascot Distiller 2.3 and Mascot 2.3.0.2 (Matrix Science) considering acetylation at protein N-terms, deamidation at asparagine and glutamine, oxidation at methionine and phosphorylation at serine as well as at threonine. Two missed cleavage sites were allowed. Results were compiled in Scaffold 3.0 (Proteome Software).

Microscopy

Fluorescence microscopy used an Olympus IX81 microscope equipped with a Yokogawa CSU-X1 scan head, a Evolve 512 Delta EMCCD camera, a ASI MS-2000 Z-piezo stage and a PlanApo x1000 NA 1.45 total internal fluorescence microscope oil objective. Cells where grown from an overnight culture in synthetic complete media complemented with all amino acids until they reached approximately 5 x 10^6 cells/ml. They were treated for 1 hour + 400 µg/ml Zeocin, + 0.2 M HU or left untreated. Cells where then fixed in 4% PFA for 30 seconds washed three times with PBS and finally resuspended in PBS. Images were acquired on 2% agarose pads. The lasers used to excite the different fluorophores are as follows: 445 nm for CFP (eCFP), 491 nm for GFP and 561 nm for RFP.

For foci number quantification 4 color Z-stacks were obtained taking 20 slices at 200 nm intervals. Exposure times were: 50 ms GFP, 100 ms CFP, 150 ms RFP, 10 ms brightfield. The EMCCD gain was set to approximately 600 in all cases except for the brightfield where it was set to 50. Images were deconvolved using Huygens Remote Manager v3.0.3. The deconvolution algorithm used was classic maximum likelihood estimate with a signal/noise ratio of 5, automatic background estimation and 30 iterations. “Bright foci” were counted and defined as foci that have clear borders. Thresholding was applied to help see foci over background nuclear signal and done with Fiji. We note that in the case of Ddc2 many smaller less bright foci were present.

For colocalization analysis single slice 4 channel images were acquired and processed as above except the exposure of CFP was increased to 150 ms and RFP to 250 ms. CFP, GFP

and RFP were channel aligned using Huygens Pro software. Colocalization was scored when foci either completely overlapped or partially overlapped.

For FRET, the donor GFP was excited by light at 488 nm and the emission signals are collected using filters that allow selective detection of either donor or acceptor signals. Fluorescence bleed-through of the donor signal into the acceptor channel, or the reverse, is quantified by imaging strains that contain only donor or acceptor proteins. Automated subtraction of bleed-through signal, yields the significant FRET value.

Sensitized emission FRET images for each series where acquired on the same day. Four channel images were acquired: GFP channel, FRET channel, RFP channel as well as brightfield, even for donor only (GFP) and acceptor only strains. FRET was calculated using the PixFRET Fiji plugin (Feige et al., 2005). Spectral bleed through was calculated on using donor only (*DDC2-GFP* (GA-7268), *PSY2-GFP* (GA-8033), *RFA2-GFP* (GA-6022)) and acceptor only strains (*PSY2-RFP* (GA-8659), *RFA1-RFP*(GA-8687)). The PixFRET parameters used where 1.0 Gaussian blur, 1.0 Threshold and Output of FRET/sqrt(Donor* Acceptor). The donor only and acceptor only parameters for PixFRET are as follows and were acquired by a montage of 10 donor only or 10 acceptor only images: Ddc2-GFP, a = 2.328, b = 0.00047, linear; Psy2-RFP, a = 0.48385, -b = 0.00004 linear; rfa1-RFP, a= 1.27947, b = -0.00011, linear; Psy2-GFP, a = 0.03595, constant; Rfa2-GFP, a = 0.31318, constant. FRET values were calculated from the mean intensity of the NFRET image of each focus.

Phosphoproteomics

Cells were grown in synthetic medium. 50 ml cultures were grown to an OD_{600}=0.75, arrested in G1 using α-factor, and released into 75 ml synthetic medium containing 0.2M HU until the appearance of small buds (45 min). Proteins were extracted as previously described (Bodenmiller and Aebersold). In brief, proteins were denatured and precipitated by adding a final 6% trichloracetic acid to the cultures and incubating on ice for 30 min. Precipitates were collected by centrifugation and washed three times with ice-cold acetone. Pellets were resuspended in 800 µl urea buffer (8M Urea, 50 mM ammonium bicarbonate, 5 mM EDTA and PhosSTOP phosphatase inhibitors (Roche)) and a corresponding volume of silica beads was added. Extracts were prepared by five round of bead beating. Supernatant was collected, another 800 µl urea buffer added to beads followed by five rounds of bead beating and pooling of supernatants.

Peptide generation and phosphopeptide enrichment

Extracts in 8 M urea, 50 mM ammonium bicarbonate, and 5 mM EDTA had a protein concentration of about 5.2 mg/ ml. 150 µl 200 mM HEPES were added to 1.5 ml of each of the twelve extracts. Reduction and alkylation of cysteines were performed by adding of 160 µl 45 mM DTT for 30 min followed by adding of 180 µl 100 mM iodoacetamide for another 30 min (in the dark), both at room temperature. Before adding of 20 µl of 1 mg/ ml LysC (Wako, Japan) the extracts were twofold diluted to keep a final HEPES concentration of 20 mM. First digest was performed overnight at 25°C. After diluting the extracts 2-fold, 100 µl of 0.5 mg/ ml trypsin were added and the second digest was performed at 37°C overnight. Before phosphopeptide enrichment the digests were desalted using SepPak C18 columns (Waters). The eluates were dried down in a SpeedVac (Thermo Scientific).

The digests were reconstituted in 150 µl 2.5% triflouroacetic acid (TFA)/ 80% acetonitrile, saturated with phthalic acid and 30 min incubated with 1.5 mg TiO2 beads (Inertsil Titansphere 5µm, GL Science, Japan) using Mobitec tubes (MoBiTec, Germany). The beads were thoroughly washed 4 times with 200 µl 2.5% TFA/ 80% acetonitrile. Phospho-peptides were eluted with 100 µl 0.3 M NH4OH and 100 µl 0.3 M NH4OH/ 30% acetonitrile. The pH of the eluates was lowered to about 3 by adding 4 µl TFA before drying down in a SpeedVac. The final desalting step was performed on Oligo R3 media (Life technologies) immobilized on C18 GELoader pipette tips (Proxeon).

LC/MS/MS analyses of enriched phosphopeptides

The LC/MS/MS analyses were performed on an Easy-nLC 1000 pump coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) using a Digital PicoView ion source (New Objective). The peptides were separated on a New Objective analytical column (75 µm x 25 cm, Reprosil, 3 µm) with a gradient from 2 to 30% solvent B in 110 min, 30 to 50% solvent B in 30 min and 50 to 80% solvent B in 5 min. Solvent A consisted of 0.1% formic acid in water, solvent B of 0.1% formic acid in acetonitrile. The flow rate was 200 nL/ min. The dried TiO2 eluates were dissolved in 40 µl 0.1% TFA/ 2% acetonitrile and the peptide concentrations determined with a Qubit fluorimeter and the Qubit protein assay kit (Life technologies). The injection volumes were adapted accordingly for 1 µg peptides on column.

Data were acquired in a Top25 data dependent analysis mode using three different charge (z) rejection settings: positive charged ions are considered for MSMS scans with either $z > 1$,

z = 2 or with z > 2. A different charge selection mode was deployed for each of the replicates. MS scans were acquired at a resolution of 60, 000 over a range of m/z 350 to 1200.

Data evaluation with Progenesis-LC

The twelve raw files were loaded into Progenesis-LC and automatically aligned. The alignments were manually corrected were needed. Finally, the alignment scores were 82.5% and better. Features with two charges and more than two isotopes, features with three to six charges having more than three isotopes and spectra with a limit fragment count of 150 were considered for a database search using MASCOT 2.3. Peptides were identified searching the Saccharomyces Genome Database (SGD), version Sept. 2011) considering the following settings: Carbamidomethylation at cysteines as fixed modification, deamidation at asparagine and glutamine, oxidation at methionine, acetylation at the protein N-terms and phosphorylation at serine, threonine and tyrosine as variable modifications, two missed cleavage sites, a peptide mass tolerance of 7 ppm and a fragment mass tolerance of 0.6 Da, trypsin as enzyme, allowing the cleavage after arginine and lysine also if followed by a proline. Finally, in Progenesis-LC features were kept if they had a MASCOT ion score greater than 15 and were identified as phosphopeptides without any other modifications except acetylation at protein N-terminals. The normalization was performed considering only those phosphopeptides. The final feature data list was exported into Excel. Ratios “mutant versus wild-type” were calculated from the average of the normalized abundances of the three replicates and the probability of a Student’s t-test was determined accordingly. Only phosphopeptides with a probability of 0.95 were considered for further evaluation. The phosphorylation localization probabilities were determined using phosphoRS within Proteome Discoverer (version 1.4.1.14, Thermo Fisher Scientific). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaino et al., 2014) via the PRIDE partner repository with the dataset identifier PXD001492 and null (http://www.ebi.ac.uk/pride).

Phosphatase Assay

1l of late-log phase WT cells (GA-1981) or *PSY2-HALO* (GA-8179) cells were harvested and washed once with PBS. Pellets were weighed, resuspended in 1ml /g buffer 1 (50 mM HEPES pH7.5, 150 mM NaCl, 2mM EDTA) and dropped into liquid nitrogen. Droplets were subjected to three rounds of bead-milling using Mixer Mill MM 400 (Retsch). The frozen cell

powder was resuspended to 1ml/g of cold buffer 2 (50 mM HEPES pH7.5, 150 mM NaCl, 1mM DTT, 0.01% NP-40, and protease inhibitors: 1 mM PMSF, 300 µg/ml benzamidine, 1 µg/ml pepstatin, 0.5 µg/ml leupeptin, 40µg/ml bestatin, 2µM E64 and 50 µg/ml TLCK), and cleared by centrifugation. Halo-Link resin (Promega) was washed five times with buffer 2, 1ml resin was added to 7 ml cleared lysates and incubated at 4°C overnight. Resin-bound protein complexes were washed once with buffer 3 (buffer 2 + 1mM EDTA), four times with buffer 3 lacking protease inhibitors and 2 times with phosphatase buffer (25mM TRIS pH7.5, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.01% Brij-35). Resin-bound protein complexes were finally resuspended in 250 µl Phosphatase buffer + TEV protease and incubated at 4°C overnight. Eluates were separated from resin by centrifuging through micro spin columns (Biorad). 35 µl of eluates were incubated with 10 µM of peptide (positive control: RRA(pT)VA (provided with Ser/Thr phosphatase assay kit (Promega)), Cdc13-p: Biotin-GGGKSYIQ(pS)QTPERK-amide, Cdc13: Biotin-GGGKSYIQSQTPERK-amide (both gifts from D. Durocher), Mec1-p: VK(pS)ITSRSGKSLEKC and Mec1: VKSITSRSGKSLEKC (both synthesized by Genscript)) in phosphatase buffer. Reactions also contained a final 5 mM manganese chloride. Release of phosphate was measured using a colorimetric Ser/Thr phosphatase assay kit (Promega). Phosphate leads to the proportional accumulation of a green dye, measured quantitatively as light absorption at 600 nm.

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