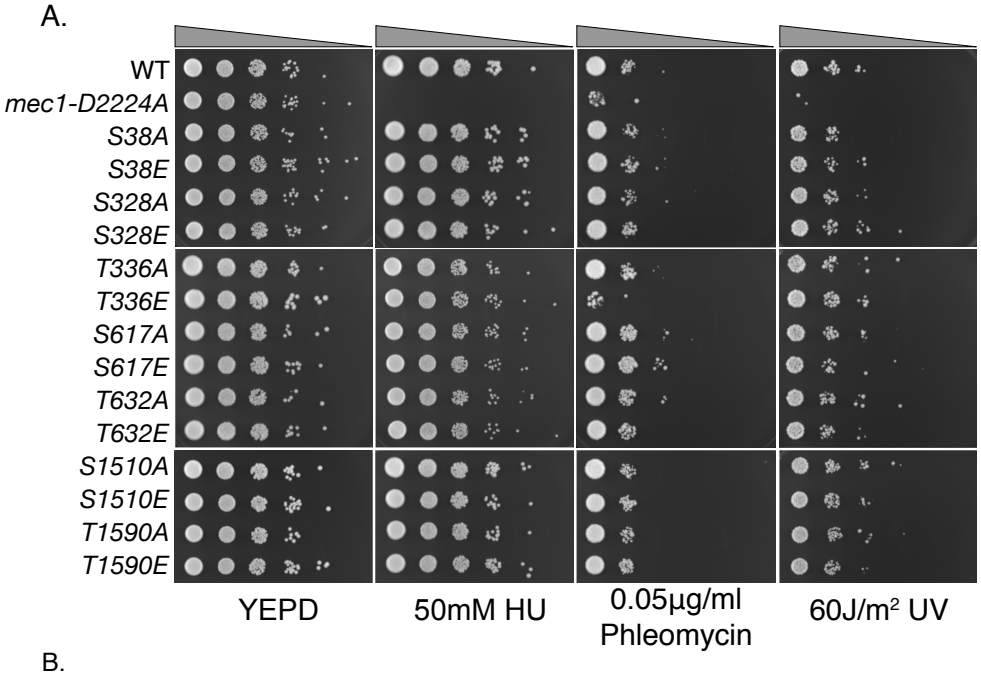
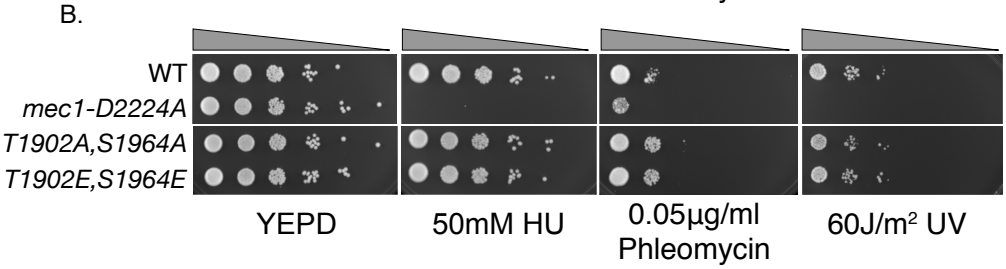
A.


WT
mec1-D2224A
S38A
S38E
S328A
S328E
T336A
T336E
S617A
S617E
T632A
T632E
S1510A
S1510E
T1590A
T1590E
YEPD
50mM HU
0.05µg/ml Phleomycin
60J/m² UV

B.


WT
mec1-D2224A
T1902A,S1964A
T1902E,S1964E
YEPD
50mM HU
0.05µg/ml Phleomycin
60J/m² UV

C.

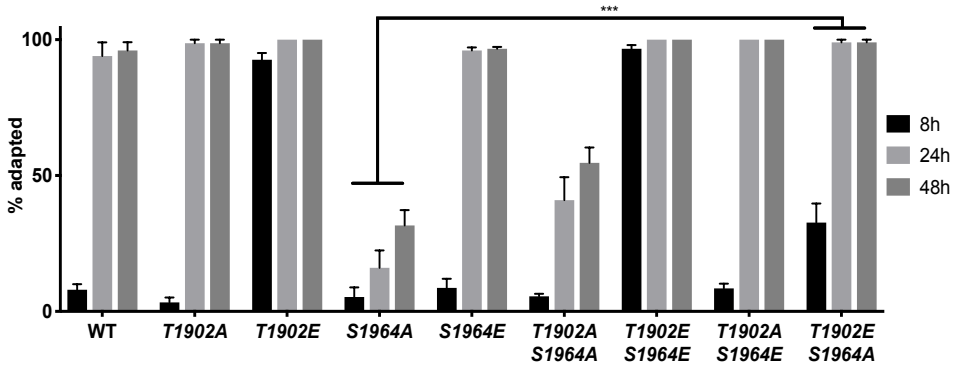
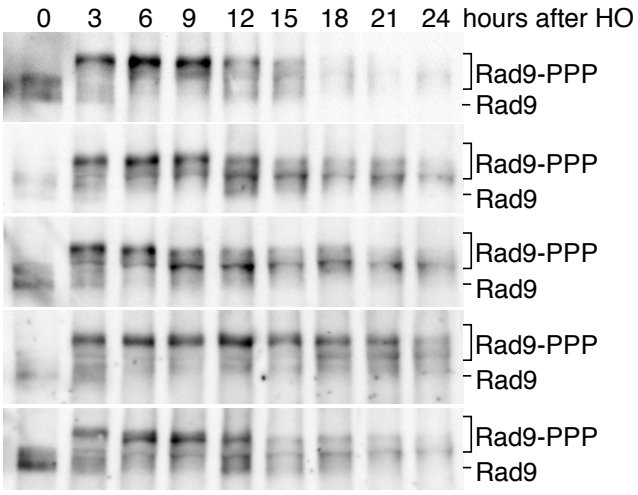

***
100
50
0
% adapted
8h
24h
48h
WT
T1902A
T1902E
S1964A
S1964E
T1902A S1964A
T1902E S1964E
T1902A S1964E
T1902E S1964A

Figure Supplement 1. (Related to Figure 1).

A. Sensitivity of Mec1 SQ/TQ mutants and kinase-dead *mec1-D2224A* to DNA damaging reagents, HU, phleomycin and UV. The WT and *mec1-D2224A* control results also appear on Figure 2B, because all Mec1 SQ/TQ mutants have been screened for drug sensitivity side by side by using the same control strains. **B.** Sensitivity of *mec1-T1902A,S1964A* and *mec1-T1902E,S1964E* mutants to DNA damaging reagents HU, phleomycin and UV. **C.** Cell cycle arrest and adaptation in Mec1 T1902 and S1964 single and double mutants measured by adaptation assay. Error bars represent SEM. *** $p<0.001$.

A.


0 3 6 9 12 15 18 21 24 hours after HO
WT
T1902A
T1902E
S1964A
S1964E
Rad9-PPP
Rad9

B.

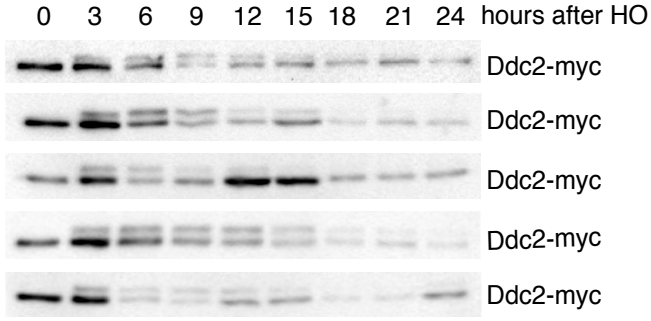

0 3 6 9 12 15 18 21 24 hours after HO
WT
T1902A
T1902E
S1964A
S1964E
Ddc2-myc

Figure Supplement 2. (Related to Figure 1).

A. Rad9 phosphorylation in Mec1 T1902 and S1964 site mutants following a single DSB. Samples were collected as previously described and blotted for Rad9. **B.** Ddc2 phosphorylation in Mec1 T1902 and S1964 site mutants following a single DSB. Samples were blotted for myc for the detection of Ddc2-myc.

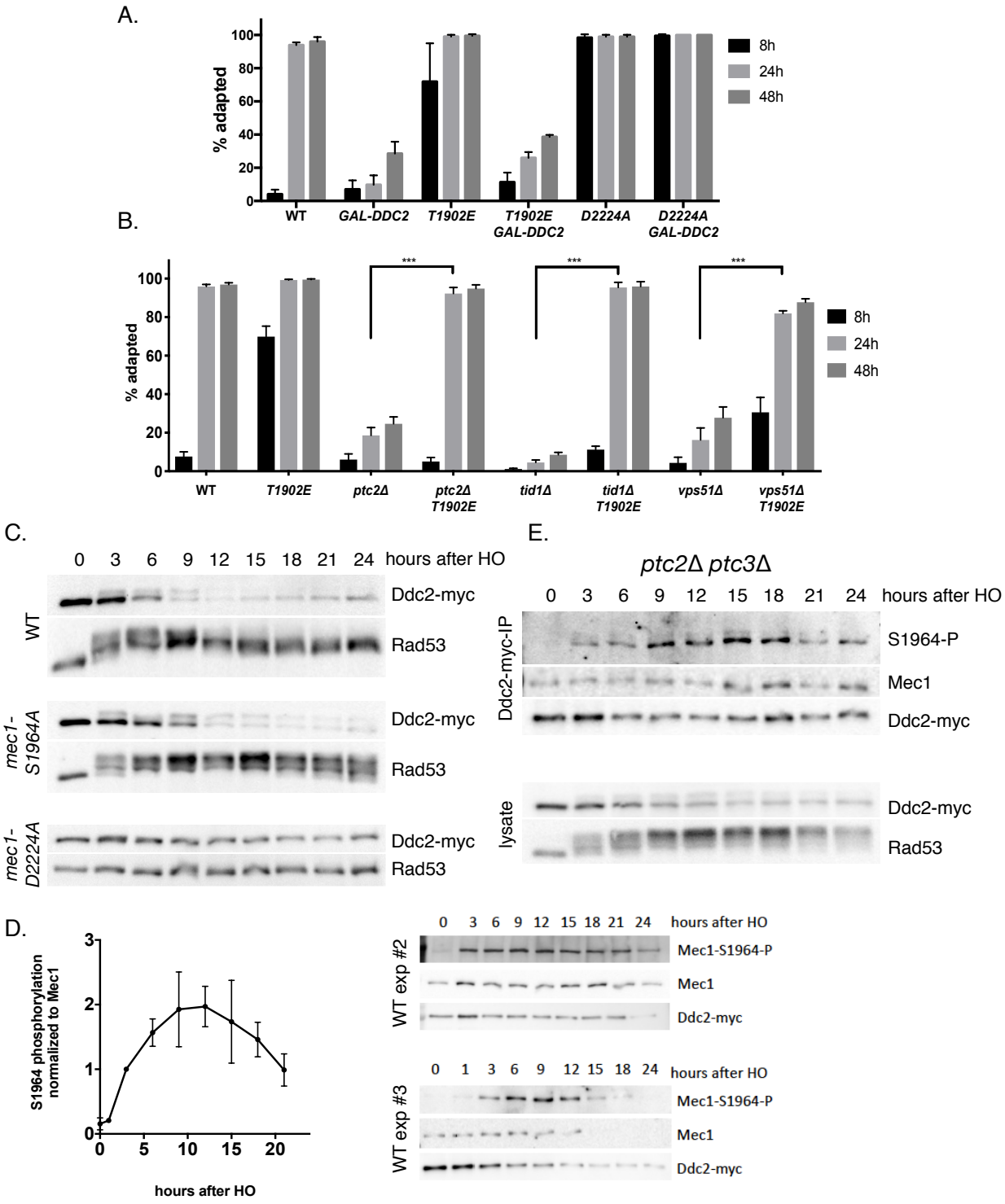

A.
% adapted
100
80
60
40
20
0
8h
24h
48h
WT
GAL-DDC2
T1902E
T1902E GAL-DDC2
D2224A
D2224A GAL-DDC2
B.
% adapted
***
***
***
WT
T1902E
ptc2Δ
ptc2Δ T1902E
tid1Δ
tid1Δ T1902E
vps51Δ
vps51Δ T1902E
C.
0 3 6 9 12 15 18 21 24 hours after HO
WT
Ddc2-myc
Rad53
mec1-S1964A
mec1-D2224A
D.
S1964 phosphorylation normalized to Mec1
hours after HO
E.
ptc2Δ ptc3Δ
Ddc2-myc-IP
S1964-P
Mec1
lysate
WT exp #2
Mec1-S1964-P
WT exp #3

Figure Supplement 3. (Related to Figure 3).

A. Cell cycle arrest and adaptation in WT and *mec1-T1902E* strains after *GAL-DDC2* overexpression determined by adaptation assay. Error bars represent SEM. **B.** Cell cycle arrest and adaptation in WT, *ptc2*Δ, *tid1*Δ, *vps51*Δ and *mec1-T1902E* double mutants. Error bars represent SEM. *** p<0.001. **C.** Ddc2 and Rad53 hyperphosphorylation in WT, *mec1-D2224A* and *mec1-S1964A* mutants following the induction of a DSB. Cell lysate samples were collected side by side with the pulldown samples (Figure 3B) and blotted for myc to infer the phosphorylation status of Ddc2, and for Rad53. **D.** Mec1-S1964 phosphorylation following a DSB normalized to Mec1 protein levels after Ddc2-myc immunoprecipitation from 3 independent experiments. Also see Figure 3B for the rest of the raw data used for this quantification. See Figure 3C for Mec1-S1964 phosphorylation normalized to Ddc2 protein levels. **E.** Mec1-S1964 phosphorylation in the *ptc2*Δ *ptc3*Δ strain following the induction of a DSB, after Ddc2-myc immunoprecipitation. Samples collected from the cell lysates simultaneously were blotted for Rad53 and Ddc2.

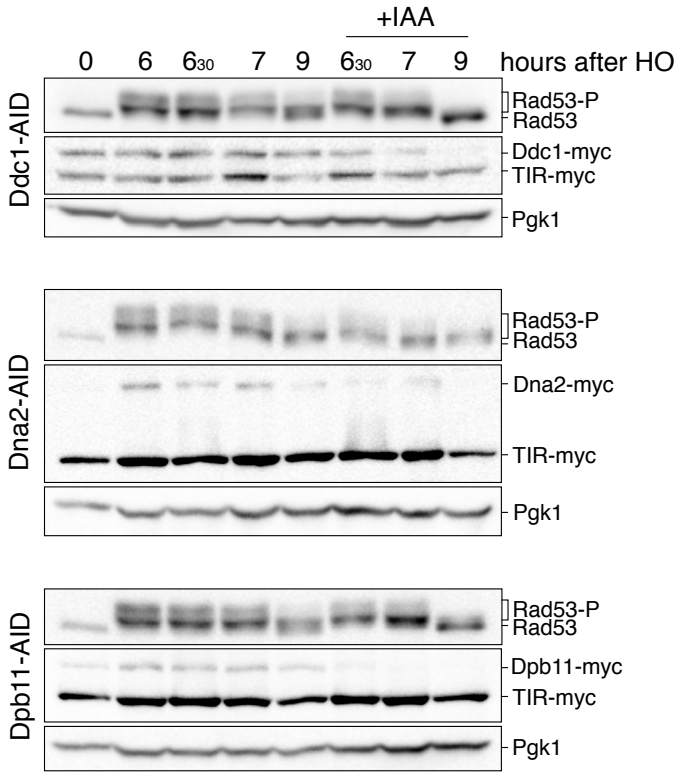
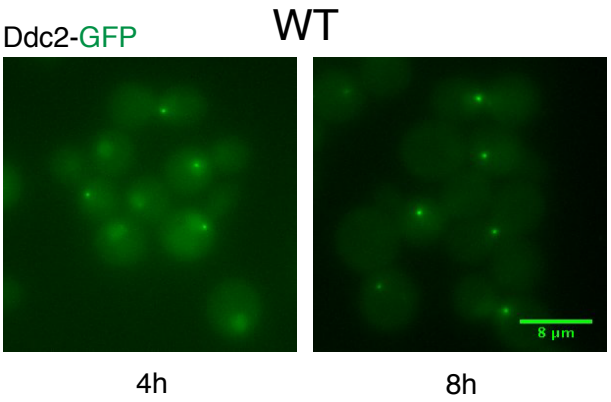
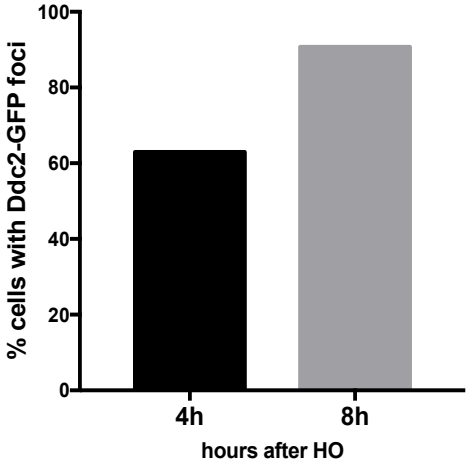

+IAA
0
6
6₃₀
7
9
6₃₀
7
9
hours after HO
Ddc1-AID
Rad53-P
Rad53
Ddc1-myc
TIR-myc
Pgk1
Dna2-AID
Rad53-P
Rad53
Dna2-myc
TIR-myc
Pgk1
Dpb11-AID
Rad53-P
Rad53
Dpb11-myc
TIR-myc
Pgk1

Figure Supplement 4. (Related to Figure 4).

Cultures of Ddc1-AID-myc, Dpb11-AID-myc or Dna2-AID-myc were grown, and DNA damage was induced at 0h by the addition of galactose. 6h later, the cultures were split into two, and one part was treated with IAA to allow the degradation of AID-tagged proteins. Samples were collected and blotted for Rad53 phosphorylation to infer checkpoint activity, for myc to confirm the degradation of the protein of interest, for TIR1 and Pgk1 as a loading control.


Ddc2-GFP
WT
8 µm
4h
8h


% cells with Ddc2-GFP foci
100
80
60
40
20
0
4h
8h
hours after HO

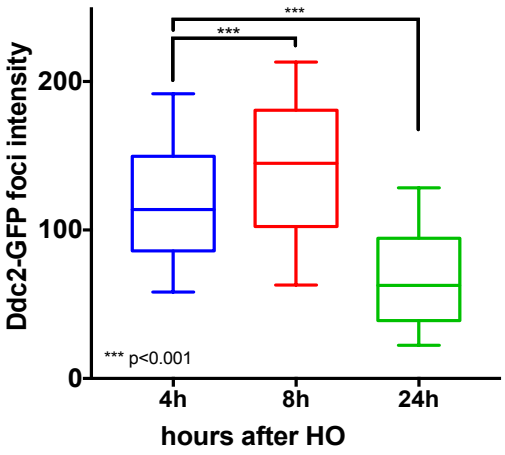

Ddc2-GFP foci intensity
200
100
0
***
***
*** p<0.001
4h
8h
24h
hours after HO

Figure Supplement 5. (Related to Figure 5)

Ddc2-GFP localization in WT strain, 4h and 8h after the induction of a single DSB. The intensity of the Ddc2-GFP signal and the percentage of the cells that show detectible foci were quantified for more than 300 cells. The 8h and 24h quantifications shown here for comparison with 4h time point data also appear on Figure 5A and Figure 5C, as all these experiments were carried out simultaneously. For the Ddc2-GFP foci intensity quantification, error bars represent 10-90% of the data set. *** $p<0.001$.

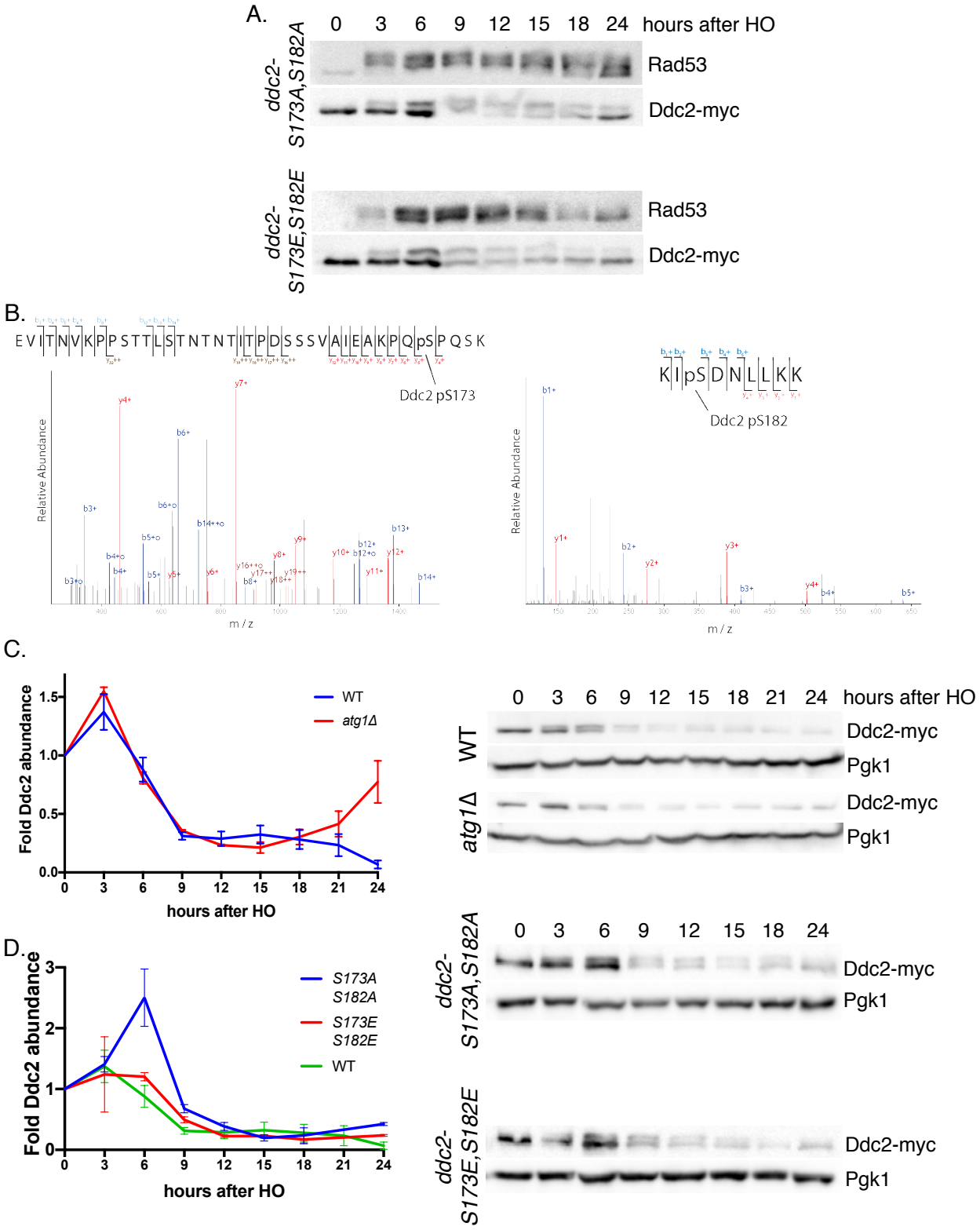

A.
0 3 6 9 12 15 18 24 hours after HO
ddc2-S173A,S182A
Rad53
Ddc2-myc
ddc2-S173E,S182E
Rad53
Ddc2-myc
B.
E V I T N V K P P S T T L S T N T N T I T P D S S S V A I E A K P Q pS P Q S K
Ddc2 pS173
Relative Abundance
m / z
K I pS D N L L K K
Ddc2 pS182
Relative Abundance
m / z
C.
Fold Ddc2 abundance
hours after HO
WT
atg1Δ
0 3 6 9 12 15 18 21 24 hours after HO
WT
Ddc2-myc
Pgk1
atg1Δ
Ddc2-myc
Pgk1
D.
Fold Ddc2 abundance
hours after HO
S173A S182A
S173E S182E
WT
0 3 6 9 12 15 18 24
ddc2-S173A,S182A
Ddc2-myc
Pgk1
ddc2-S173E,S182E
Ddc2-myc
Pgk1

Figure Supplement 6 (Related to Figure 5 and 6).

A. Ddc2 and Rad53 phosphorylation following a single DSB in *ddc2-S173A,S182A* and *ddc2-S173E,S182E* mutants. Samples were blotted for Rad53, and for myc, for the detection of Ddc2-myc. **B.** MS^2 spectra for the Ddc2 S173 and S182 phosphopeptides. For the full list of the peptides, see SI Table 6. **C.** Ddc2 abundance in WT and *atg1*Δ strains after the induction of a single DSB. Error bars represent SEM. Ddc2 abundance in *atg1*Δ was quantified from two independent experiments. **D.** Ddc2 abundance in *ddc2-S173A,S182A* and *ddc2-S173E,S182E* mutants following a DSB. Error bars represent SEM. Ddc2 abundance in *ddc2-S173A,S182A* and *ddc2-S173E,S182E* strains was quantified from two independent experiments.

SI Table 1. Plasmids used in this study. Related to Star Methods.

bRA89	[pPGK1::CaCas9::HPHMX BplI]
bRA90	[pPGK1::CaCas9::LeuMX BplI]
pKH41	[pMEC1::Leu2]
pGM009	bRA90 targeting CACGGATCCATCTTCTTCTC (to mutate Mec1 S38)
pGM010	bRA90 targeting ACCACTTCCCAATTGACCAG (to mutate Mec1 S328)
pGM011	bRA90 targeting TTATTAGTCAACTGCGTGAA (to mutate Mec1 T336)
pGM012	bRA90 targeting ACAATCTCAAAAATTGCCAG (to mutate Mec1 S617 and T632)
pGM014	bRA90 targeting TAATGGATCCTCGTTTGAGA (to mutate Mec1 T1590)
pGM015	bRA90 targeting AGCATTGTCCCACTTATATT (to mutate Mec1 T1902)
pGM016	bRA90 targeting GCACTAGAAACTAGATCATG (to mutate Mec1 S1964)
pGM020	bRA90 targeting ATACATAGACTTTAGCATCC (to mutate Mec1 S1510)
pGM021	bRA90 targeting GTTGGCCATATATTAGGTCT (to mutate Mec1 D2224)
pGM024	bRA90 targeting TTAGTCACAAAAAGTCCCTG (to delete Vps51 ORF)
pGM032	bRA89 targeting GCACTAGAAACTAGATCATG (to mutate Mec1 S1964)
pGM033	[pMEC1-S1964A::LEU2]

pGM047	bRA89 targeting AACTACTGGGAAAACATTCG (to mutate Rad53 K227A)
pGM048	bRA90 targeting AACTACTGGGAAAACATTCG (to mutate Rad53 K227A)
pGM053	bRA89 targeting ATAATCCTATTTGGGTTTAG (to mutate Ddc2 S182)
pGM054	bRA90 targeting ATAATCCTATTTGGGTTTAG (to mutate Ddc2 S182)
pGM055	bRA89 targeting ATTGCAACTGAGGACGAATC (to mutate Ddc2 S173)
pGM056	bRA90 targeting ATTGCAACTGAGGACGAATC (to mutate Ddc2 S173)
pGM059	bRA89 targeting GTTTGTAATGCTGCTGATGA (to mutate Ddc2 T29 and T40)
pGM060	bRA90 targeting GTTTGTAATGCTGCTGATGA (to mutate Ddc2 T29 or T40)
pGM061	bRA89 targeting TCCATAGTGACATAATCAAT (to mutate Ddc2 S636)
pGM062	bRA90 targeting TCCATAGTGACATAATCAAT (to mutate Ddc2 S636)
pGM069	bRA90 targeting ATTGGTGCTAGAGGTTACAC (to delete Arg5,6 ORF)
pGM070	bRA90 targeting AATGGAAGGCTAGAAGCGCA (to delete Arg5,6 ORF)

SI Table 2. The ssDNA templates used in this study for CRISPR/Cas9-mediated gene targeting. Related to Star Methods.

GM74 (for Mec1 S38A)	GGTGCAGATTAAAAAAGTGCCCTCGGATCCATCTTCTGCTCAGGAGTACGCCAAGAGTTTAAAGATCCTGAACACCCTCA
GM75 (for Mec1 S38E)	GGTGCAGATTAAAAAAGTGCCCACGGATCCATCTTCTGAACAGGAGTACGCCAAGAGTTTAAAGATCCTGAACACCCTCA
GM78 (for Mec1 S328A)	CTCAAAATGCTTTGTAGACCACTTTCCTATCGACCAGTGGGCAGATTTTGCTCAAAGTGAACATTTTCCGTTCACGCAGT
GM79 (for Mec1 S328E)	CTCAAAATGCTTTGTAGACCACTTTCCTATCGACCAGTGGGCAGATTTTGAACAAAGTGAACATTT TCCGTTCACGCAGT
GM82 (for Mec1 T336A)	GTGGGCAGATTTTTCACAAAGTGAACATTTTCCGTTCGCTCAGTTGACTAATAAAGCTCTCTCGATTGTATATTTTGATT
GM83 (for Mec1 T336E)	GTGGGCAGATTTTTCACAAAGTGAACATTTTCCGTTCGAACAGTTGACTAATAAAGCTCTCTCGATTGTATATTTTGATT
GM86 (for Mec1 S617A)	AAGATGAACACACTGCCACGCTGATAAAGTTTCTACAAGCTCAAAAATTACCAGTAGTGAAAGAAAATTTAGTCATTGCT
GM87 (for Mec1 S617E)	AAGATGAACACACTGCCACGCTGATAAAGTTTCTACAAGAACAAAAATTACCAGTAGTGAAAGAAAATTTAGTCATTGCT
GM88 (for Mec1 T632A)	AAGTTTCTACAATCTCAAAAGTTACCTGTAGTGAAAGAAAATTTAGTCATTGCTTGGGCTCAATTAACATTGACGACT TCTAATGATGTATTTGATACAC
GM89 (for Mec1 T632E)	AAGTTTCTACAATCTCAAAAGTTACCTGTAGTGAAAGAAAATTTAGTCATTGCTTGGGAACAATTAACATTGACGACT TCTAATGATGTATTTGATACAC
GM96 (for Mec1 T1590A)	TATTGCGAAAGCTTTAATTGCAATATCTAATGAGGATCCATTAAGGGCTCAAAAATACATCCACAATTCCTTTAGGCTTA
GM97 (for Mec1 T1590E)	TATTGCGAAAGCTTTAATTGCAATATCTAATGAGGATCCATTAAGGGAACAAAAATACATCCACAATTCCTTTAGGCTTA
GM100 (for Mec1 T1902A)	GAAGCGCTGCAGCATTGTCCGACATACATTTGGTACTTTGTTTTGGCTCAGTTGTTATCTAGGTTATTACATTCTCATCA
GM101 (for Mec1 T1902E)	GAAGCGCTGCAGCATTGTCCGACATACATTTGGTACTTTGTTTTGGAACAGTTGTTATCTAGGTTATTACATTCTCATCA
GM104 (for Mec1 S1964A)	GCATATTTTAGAAAAGTATAGACAACATGCTCAAAATCCTCATGATCTGGTTTCATCCGCATTGGATTTAACGAAAGCAT
GM105 (for Mec1 S1964E)	GCATATTTTAGAAAAGTATAGACAACATGAACAAAATCCTCATGATCTGGTTTCATCCGCATTGGATTTAACGAAAGCAT
GM184 (for Mec1 S1510A)	GACCCCAAAACTACAACAAGGATGCTTAAGTCAATGTATGACCACCAATTGTATGCTCAAATAATATCGAACTCTTCGTT
GM185 (for Mec1 S1510E)	GACCCCAAAACTACAACAAGGATGCTTAAGTCAATGTATGACCACCAATTGTATGAACAAATAATATCGAACTCTTCGTT
GM189 (for Mec1 D2224A)	AGATCTTACGCCGTCATGGCAATGGTTGGACATATCTTGGGTCTTGGTGCTAGGCACTGTGAAAACATATTACTAGATAT

GM333 (for Mec1 Q1903N)	GAAGCGCTGCAGCATTGTCCGACATACATTTGGTACTTTGTTTTGACTAATTTGTTATCTAGGTTATTACATTCTCATCA
GM334 (for Mec1 Q1964N)	GCATATTTTAGAAAAGTATAGACAACATTCGAATAATCCTCATGATCTGGTTTCATCCGCATTGGATTTAACGAAAGCAT
GM337 (for Rad53 K227A)	AAGAAAGCCATTGAAAGAACTACTGGGAAAACTTTTGCGGTGGCTATTATAAGTAAACGCAAAGTAATAGGCAATATGGA
GM356 (for Ddc2 S182A)	AAAAAACGTAAGATAGCTGATAATTTACTGAAAAAAAATATGGTTCCCCTTAACCCAAATCGTATTATTCCCGATGAAAC
GM357 (for Ddc2 S182E)	AAAAAACGTAAGATAGAAGATAATTTACTGAAAAAAAATATGGTTCCCCTTAACCCAAATCGTATTATTCCCGATGAAAC
GM359 (for Mec1 T1902D)	GAAGCGCTGCAGCATTGTCCGACATACATTTGGTACTTTGTTTTGGATCAGTTGTTATCTAGGTTATTACATTCTCATCA
GM360 (for Mec1 S1964D)	GCATATTTTAGAAAAGTATAGACAACATGATCAAAATCCTCATGATCTGGTTTCATCCGCATTGGATTTAACGAAAGCAT
GM380 (for Ddc2 T29A)	GAATTAGGCACCAGGCCTCCAAGGTTTGCTCAAATACCGCCATCATCAGCCGCCTTGCAAACACAAATTCCCACTACTTT
GM381 (for Ddc2 T29E)	GAATTAGGCACCAGGCCTCCAAGGTTTGAACAAATACCGCCATCATCAGCCGCCTTGCAAACACAAATTCCCACTACTTT
GM384 (for Ddc2 T40A)	CCTCCAAGGTTTACTCAAATACCGCCATCATCAGCCGCCTTGCAAGCTCAAATTCCCACTACTTTGGAGGTTACAACGAC
GM385 (for Ddc2 T40E)	CCTCCAAGGTTTACTCAAATACCGCCATCATCAGCCGCCTTGCAAGAACAAATTCCCACTACTTTGGAGGTTACAACGAC
GM388 (for Ddc2 S636A)	CAATTGCGTGAAGAACAAATCAAGCAGGTAGAAGCTCAATTAATTATGTCACTATGGAGGTTTCTCGTATGCCAAACCGA
GM389 (for Ddc2 S636E)	CAATTGCGTGAAGAACAAATCAAGCAGGTAGAAGAACAATTAATTATGTCACTATGGAGGTTTCTCGTATGCCAAACCGA
GM404 (for Ddc2 T29A,T40A)	TTAGGCACCAGGCCTCCAAGGTTTGCTCAAATACCGCCATCATCAGCCGCCTTGCAAGCTCAAATTCCCACTACTTTGGA
GM405 (for Ddc2 T29E, T40E)	TTAGGCACCAGGCCTCCAAGGTTTGAACAAATACCGCCATCATCAGCCGCCTTGCAAGAACAAATTCCCACTACTTTGGA
GM426 (to delete Arg5,6)	AGAAAGAGAGATCTGAAGTGAGAAATAAGTCTGCATCATTTCACTGTGTATAGTAGATCTGCAGCTCAGTCACATGAGAC