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SUPPLEMENTAL INFORMATION TO:

Lagging strand maturation factor Dna2 is a component of the replication checkpoint initiation machinery

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Materials and Methods

Yeast strains, Plasmids and DNA substrates
All yeast strains except PY310 are derived from PY301 which is a heterozygous diploid from a cross between PY269 and RSY1061. PY310 is derived from a cross between BDY110-1 and PY284. Plasmid pBL176 contains *RAD27* cloned into pRS306 (*LEU2*, 2μ). Plasmid series pBL584 contains either full length *DNA2* (or *DNA2* mutants) cloned into pRS314 (*TRP1*, CEN), having 396 bp upstream of the start codon and 214 bp downstream of the stop codon. Plasmid pBL583 is identical to pBL584 except that it is cloned in pRS316 (*URA3*, CEN). Plasmid series pBL587 contains *DDC1* (or mutants or *ddc1-(1-404^{W352A})-dna2(41-243)* fusion) cloned into pRS315 (*LEU2*, CEN), having 299bp upstream of the start codon and 358bp downstream of the stop codon. The *ddc1-(1-404^{W352A})-dna2(41-243)* fusion construct has aa 1-404 from Ddc1, containing also the W352A mutation, fused to aa 41-243 from *DNA2,* and containing the upstream and downstream regulatory regions from *DDC1*. Plasmid series pBL589 was generated by cloning of a BamH1-Sal1 fragment from pBL587-6 (Supplemental Table 1) into the corresponding sites of pRS424Gal (2μ origin, *TRP1*, Gal1-10). Cloning amino acids 1-499 of Dna2 into the *Eco*R1 and *Xho*1 sites of *E. coli* over-expression vector pGEX6P1 generates plasmid pBL582. Mutations in the DNA2 fragment gives plasmid pBL582-x. Plasmid series pBL581 (pBM2)(Ayyagari et al. 2003) was used for over-expression and purification of wild type or nuclease or helicase dead Dna2. Annealing of ten 28mer oligonucleotides to single stranded pBSK II at approximately equal distance generated decaprimed ssDNA.

Protein purification. To purify His_7-Dna2 protein (or nuclease or helicase dead proteins), series pBL581 plasmids were transformed into yeast strain FM113 (MAT*a ura3-52 trp1-289 leu2-3 112 prb1-1122 prc1-407 pep4-3*). Growth, induction, and extraction were similar to procedures described previously (Bylund et al. 2006). Cells were harvested and resuspended in 1/5th the volume of 5x buffer A (buffer A: 60 mM HEPES-NaOH [pH 7.8], 0.4 M sodium acetate, 0.1 mM EDTA, 0.005% $E_{10}C_{12}$, 50 mM sodium bisulfite, 50 μM pepstatin A, 50 μM leupeptin), then frozen in liquid nitrogen. Frozen cell pellets (80 g) were blended in dry ice powder. All further preparation was carried out at 0-4˚C. After thawing of the lysate, 10% glycerol, 1 mM dithiothreitol (DTT), 0.05 mM phenylmethylsufonyl fluoride (100 mM stock), and 150 mM ammonium sulfate (4 M stock), 0.45 % polymin P (10% stock, pH 7.3) were added to the lysate. The mixture was stirred for 15 minutes, the lysate was cleared at 40,000 × g for 30 minutes, and the supernatant was precipitated with 0.31 g/ml solid ammonium sulfate. The precipitate was collected at 40,000 × g for 30 minutes and then redissolved in buffer A_0 [50mM HEPES (pH 7.8); 0.01% NP40; 10% glycerol and 1mM DTT; subscript designated mM NaCl] supplemented with protease inhibitor cocktail . It was followed with buffer exchange in buffer A_{100}

over a PD10 column, followed by Nickel-NTA affinity chromatography. Two runs over the MonoQ column further purified His_7-Dna2 or nuclease-dead or helicase-dead protein.

The series of pBL582 plasmids, containing Dna2(1-499) were transformed into *E. coli* STL5730 strain. 50ml primary culture was started by inoculating a few colonies into LB broth, with ampicillin, and incubated overnight at 37 ^{0}C. This was used to inoculate 3L of LB broth and the cultures were further grown to an OD of A_{600}=0.5 at 30 ^{0}C with vigorous shaking at 250rpm. Expression was induced with the addition of 1 mM isopropyl β-D-thiogalactopyranoside and cells were allowed to grow further for 4hrs. The cells were harvested and washed once with buffer A_{100} and then resuspended in 50ml buffer A_{100}. To this, protease inhibitor cocktail was added and cells were lysed by sonication. Cell debris was removed by centrifugation at 15,000 rpm for 30 min. The cleared lysate was allowed to batch bind for 2 hour at 4 ^{0}C to 2ml of gluthathione beads. After batch binding, the beads were subjected to four consecutive 50 ml washes with buffer A_{100}. The first 50 ml wash was supplemented with protease inhibitor cocktail and the third wash was supplemented with 300 mM NaCl, 10mM $MgCl_2$ and 1mM ATP. The GST-tagged protein was eluted with reduced glutathione and the peak fractions were pooled and treated with rhinoviral 3C protease overnight at 4 ^{0}C. The resulting protein was further purified over a MonoQ column in buffer A and the peak fractions were stored at -70 ^{0}C until further analysis.

For purification of 9-1-1 containing the Ddc1(1-404^{W352A})-Dna2(41-243) fusion protein, plasmid pBL589 was co-transformed with pBL764 (over-expressing GST-*MEC3* and *RAD17*) into yeast strain FM113 and the protein purification was carried out as described earlier (Majka and Burgers 2003).

Western blot and cell cycle analysis. Yeast cells were synchronized in G1 phase by treating exponentially growing cells with 5 μg/ml alpha factor, which was replenished again after 90 min for a total of 180 minutes. The cells were synchronized in G2 phase by treating cells with nocodazole (20μg/ml for 3h) and the media was supplemented with 1% dimethyl sulfoxide. G1 and G2 synchronized cells were then treated with 4NQO (2μg/ml) for 30 min either at room temperature or at 30 °C. For experiments involving S phase, the G1 synchronized cells were released into S phase for 90min with or without 200mM hydroxyurea and pronase (0.1mg/ml). Protein extracts were prepared by trichloroacetic acid precipitation. These extracts were probed with anti-Rad53, anti-Mrc1 and anti-Rad9 antibodies. Anti-Mrc1 antibody was a kind gift from Stephen J. Elledge (Harvard Medical School) and anti-Rad53 (SC-6749) and anti-Rad9 (SC-6740 and SC-6742) were purchased from Santa Cruz Biotechnology. Anti-Mrc1, anti-Rad53 and anti-Rad9 antibodies were used at 1:5000, 1:1000 and 1:500 dilution respectively, in 5% fat free milk prepared in TBST. Western blots were developed as described previously (Navadgi-Patil et al. 2011), except for the Mrc1 blots which were developed using alkaline phosphatase-conjugated anti-rabbit secondary antibody (Sigma) at 1:10,000 dilution in TBST.

In experiments involving cell cycle analysis, the yeast cells were grown to an OD A_{660}=0.5 and synchronized in G1 as described above. 400μl of G1 synchronized cells were fixed in 1100μl of 95% ethanol overnight at 4^{0}C. The fixed cells were washed once with phosphate buffer saline (PBS) and resuspended in PBS supplemented with RNase A (0.1 mg/ml) and incubated for 2hrs at 37^{0}C. After this, the cells were treated with proteinase K (0.2 mg/ml) and incubation continued for 60 min at 50^{0}C. The cells were washed and resuspended in fresh 1ml PBS supplemented with propidium iodide (10μg/ml). Before analysis cells were briefly sonicated and DNA content of 15-30,000 cells was analyzed using FACS.

Pulsed Field Gel Electrophoresis (PFGE). Yeast strains were synchronized in G1 as described above and cells were either harvested after G1 arrest or they were washed and resuspended in fresh YPD supplemented with 100 mM hydroxyurea. Four hours after hydroxyurea treatment cells were harvested and agarose plugs prepared according to standard techniques. Yeast chromosomes were then resolved onto 1% agarose gel in a PFGE apparatus following manufacturers instructions.

DNA damage sensitivity assay. Ddc1 deletion strain YLL244 was transformed with few members of the plasmid series pBL587 and selected on a synthetic complete minus Leucine plate. Transformants were then grown overnight in liquid synthetic complete media without Leucine. Serial dilution (10 fold) was spotted onto YPD plates and the plates were exposed to UV irradiation of 70J/m^2. The cells were then allowed to grow for 2days at 30^{0}C and photographed.

Tetrad Analysis. Strain PY305 was mated with PY310 and sporulated. The spores of fifteen individual tetrads were then separated onto a YPD plate and their growth was monitored for 5 days at room temperature. Only those tetrads were selected for further study in which (i) the wild-type *DNA2* genotype could be unambiguously assigned to all four spores, and (ii) all spores germinated and underwent at least three cell divisions to give >8 cells.

Supplemental Tables

Supplemental Table 1. Plasmids with *DNA2* or *DDC1* or *RAD27* expressed from their native promoter

Name	Plasmid Backbone	Gene
pBL176	pRS425-*LEU2*-2μ	Wild type *RAD27*
pBL583	pRS316-*URA3*-CEN	Wild type *DNA2*
pBL584	pRS314-*TRP1*-CEN	Wild type *DNA2*
pBL584-2	pRS314-*TRP1*-CEN	*dna2-Δ(1-257)*
pBL584-4	pRS314-*TRP1*-CEN	*dna2-W128A, Y130A*
pBL587	pRS315-*LEU2*-CEN	Wild type *DDC1*
pBL587-1	pRS315-*LEU2*-CEN	*ddc1-W352A*
pBL587-2	pRS315-*LEU2*-CEN	*ddc1-W352A, W544A*
pBL587-3	pRS315-*LEU2*-CEN	*ddc1-W352A, W544A, T602A*
pBL587-4	pRS315-*LEU2*-CEN	*ddc1-(1-404),W352A*
pBL587-9	pRS315-*LEU2*-CEN	*ddc1-T602A*
pBL587-6	pRS315-*LEU2*-CEN	*ddc1-(1-404),W352A--dna2-(41-243)*
pBL587-8	pRS315-*LEU2*-CEN	*ddc1-(1-404),W352A--dna2-(41-243),W128A,Y130A*

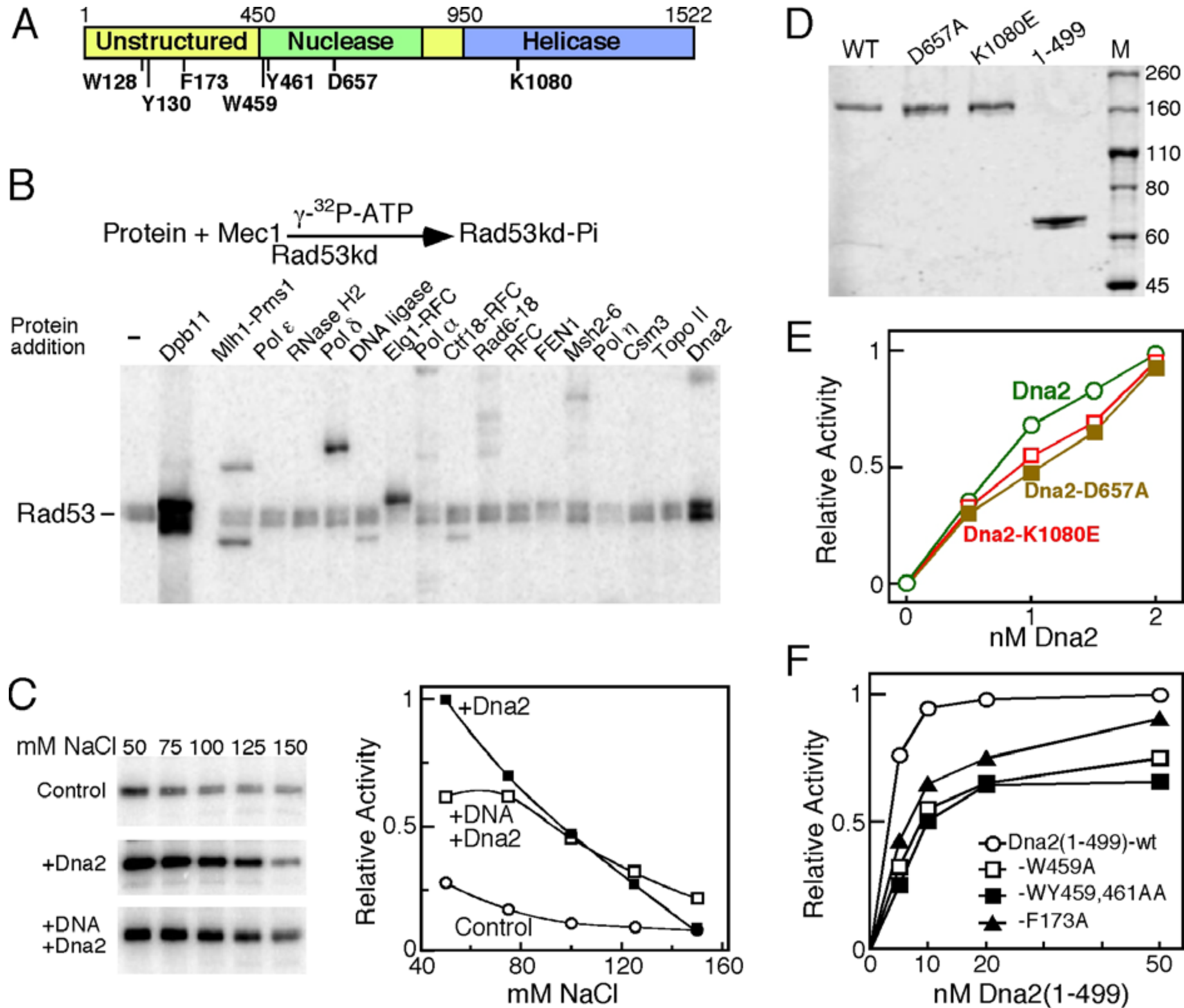
Supplemental Table 2. Plasmids for protein over-expression in yeast or *E. coli*.

Name	Plasmid Backbone	Gene
pBL581	pBM2-*URA3 GAL-UAS*	Wild type *DNA2*, N-term. His_7 tag
pBL581-2	pBM2-*URA3 GAL-UAS*	*dna2-D657A*, N-term. His_7 tag
pBL581-3	pBM2-*URA3 GAL-UAS*	*dna2-K1080E*, N-term. His_7 tag
pBL582	pGEX6P1-Amp	*dna2-(1-499)*, N-term. GST tag
pBL582-1	pGEX6P1-Amp	*dna2-(1-499),Y130A*, N-term. GST tag
pBL582-2	pGEX6P1-Amp	*dna2-(1-499)*, *W128A,* N-term. GST tag
pBL582-3	pGEX6P1-Amp	*dna2-(1-499)*, *W128A,Y130A,* N-term. GST tag
pBL582-4	pGEX6P1-Amp	*dna2-(1-499)*, *W459A,* N-term. GST tag
pBL582-5	pGEX6P1-Amp	*dna2-(1-499)*, *W459A, Y461A,* N-term. GST tag
pBL582-6	pGEX6P1-Amp	*dna2-(1-499),F173A*, N-term. GST tag
pBL589	pRS424-*TRP1 GAL-UAS*	*ddc1-(1-404),W352A--dna2-(41-243)*

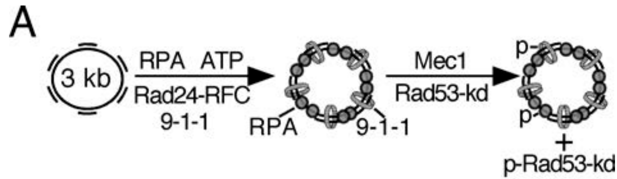
Supplemental Table 3. Yeast strains used in the study.

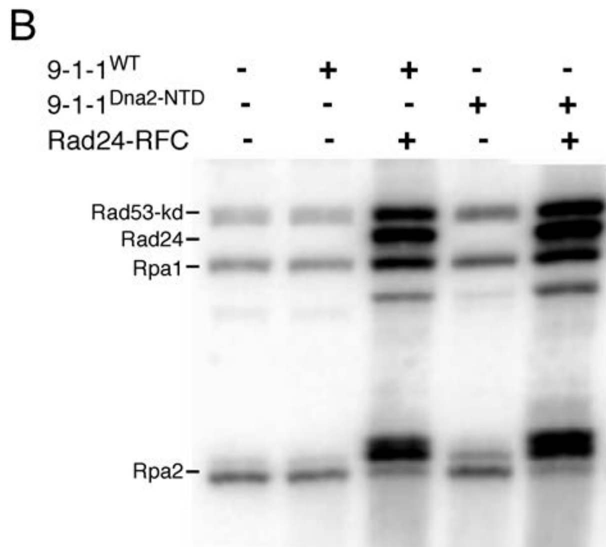
Strain	Genotype	Source
PY269	MAT*α can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 tel1Δ::NAT pBL583 (URA3 DNA2)*	this study
PY280	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 tel1Δ::NAT pBL583 (URA3 DNA2)*	this study
PY282	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 sml1Δ:: HygMX4 pBL583 (URA3 DNA2)*	this study
PY284	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 tel1Δ::NAT pBL583 (URA3 DNA2)*	this study
PY286	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 tel1Δ::NAT sml1Δ:: HygMX4 pBL583 (URA3 DNA2)*	this study
PY288	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 pBL583 (URA3 DNA2)*	this study
PY290	MAT*a can1 his3 leu2 trp1 ura3 dna2Δ::HIS3 tel1Δ::NAT pBL583 (URA3 DNA2)*	this study
PY292	MAT*a his3 leu2 trp1 ura3 dna2Δ::HIS3 sml1Δ:: HygMX4 pBL583 (URA3 DNA2)*	this study
PY294	MAT*a can1 his3 leu2 trp1 ura3 tel1Δ::NAT dna2Δ::HIS3 sml1Δ::HYG pBL583 (URA3 DNA2)*	this study
PY301	Diploid from a cross between PY269 and RSY1061	this study
PY302	MAT*a can1 his3 leu2 trp1 ura3 ddc1Δ::KanMX4 tel1Δ::NAT*	this study
PY305	Mat*α can1 his3 leu2 trp1 ura3 sml1Δ::HYG tel1Δ::NAT*	this study
PY310	MAT*a his3 trp1 ura3 dna2Δ::HIS3 ddc1Δ::KanMX4 sml1Δ::HygMX4 mec1Δ::KanMX6 pBL583 (URA3 DNA2)*	this study
YLL244	MAT*a ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1Δ::KanMX4*	M. Muzi-Falconi
YKH12	MAT*α ade1 his3 leu2 lys2 trp1 ura3 GAL+ dna2Δ::HIS3 pBL583 (URA3 DNA2)*	Y. S. Seo
RSY1061	MAT*a can1 his3-11 leu 2-3,112 lys2 trp1-1 ura3-1 bar1Δ::Leu2 sml1Δ::HygMX4*	J. Tyler
BDY110-1	MAT*α sml1Δ::HygMX4 tel1Δ::NAT mec1Δ::KanMX6*	GW Brown

Supplemental Figures

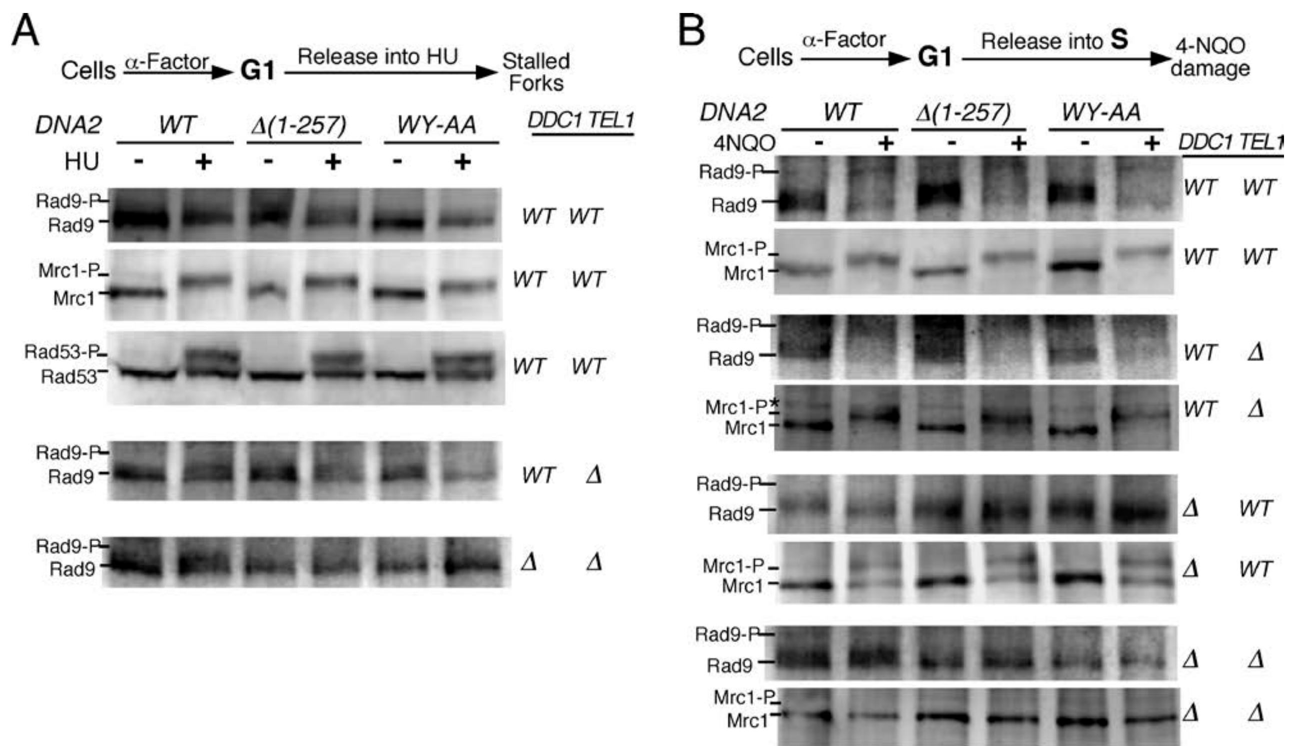


Supplemental Figure 1. Mec1 activation by Dna2. (A) Cartoon diagram of Dna2 with amino acids mutated indicated. **(B)** Basic scheme of the assay is shown (Top). Various proteins as shown (at ~100-200 nM) were tested for their ability to stimulate Mec1-dependent phosphorylation of Rad53-kd. **(C)** Mec1 kinase assay was carried out with and without 20 nM Dna2 and 2.5 nM deca-primed bluescript SK2 ssDNA, at indicated mM NaCl (left). Quantification of the data (right). **(D)** Indicated Dna2 proteins were resolved on a 8% SDS-PAGE gel. The gel was stained with colloidal Coomassie blue. **(E)** Mec1 stimulation assay by either wild type, nuclease-dead (D657A), or helicase-dead (K1080E) Dna2. **(F)** Mec1 stimulation assay with selected Dna2(1-499) mutants. The stimulatory activity of other mutants is shown in Fig. 1D.

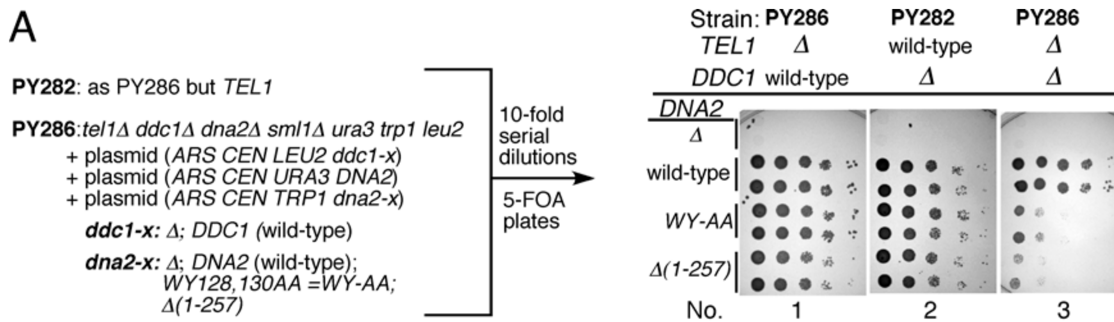


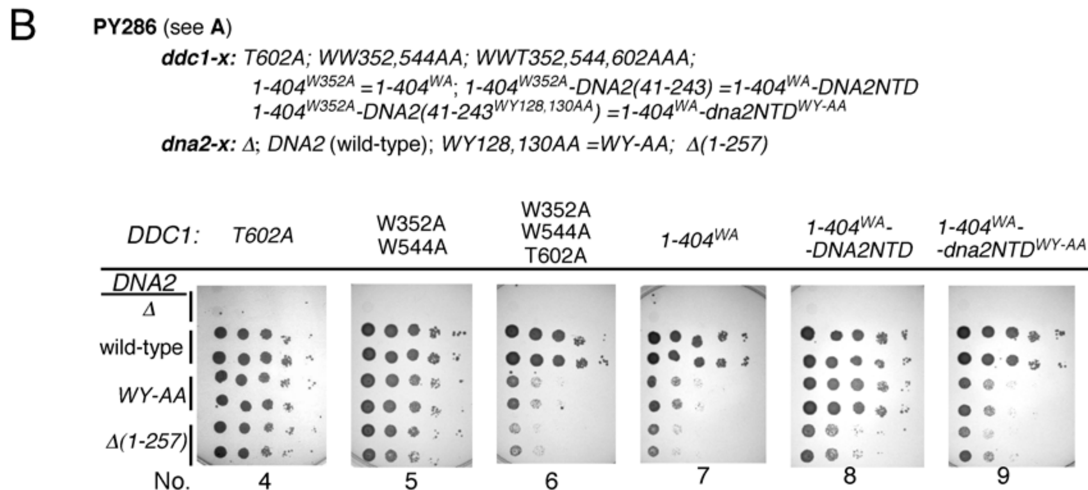


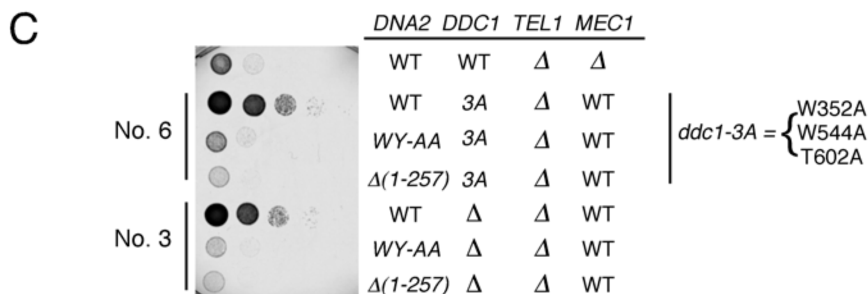
Supplemental Figure 2. The 9-1-1 clamp with Ddc1(1-404)^{W352}-Dna2(41-243) fusion subunit activates Mec1 *in vitro*. **(A)** Flow diagram showing the loading of 9-1-1-checkpoint clamp by the clamp loader (Rad24-RFC) onto decaprimed ssDNA, followed by activation of Mec1 by the loaded clamp. Previous studies have shown (i) that 9-1-1 loading onto DNA is required in order for it to function in Mec1 stimulation (Majka et al. 2006), (ii) that 9-1-1 with the Ddc1(1-404)^{W352} subunit is loaded onto DNA but defective for Mec1 stimulation (Navadgi-Patil and Burgers 2009). **(B)** Mec1 stimulation reaction was carried out in presence of 30nM of the indicated clamp with and without 30nM of clamp loader, for 10 min at 30 ˚C.



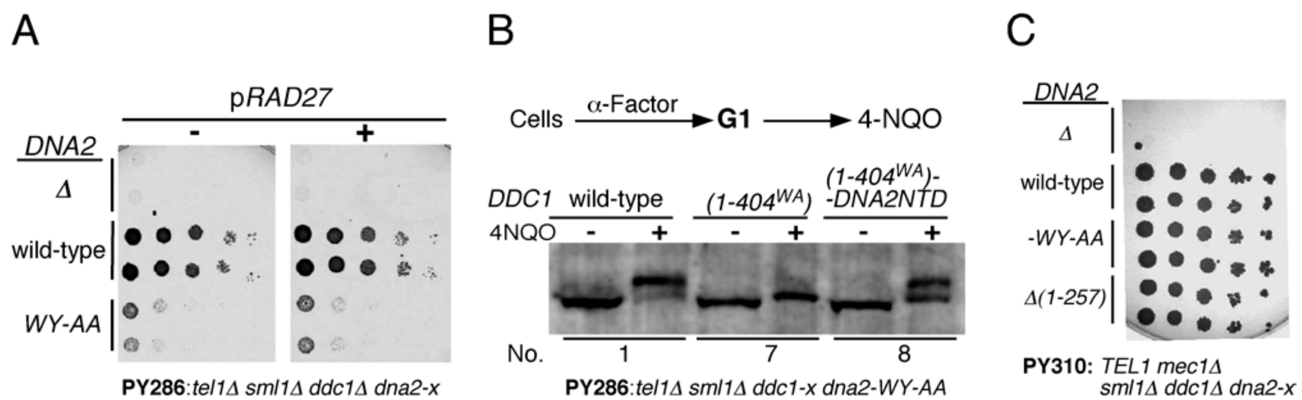
Supplemental Figure 3. Checkpoint activation in S phase as measured by phosphorylation of Rad9, Mrc1, and Rad53. **(A)** Yeast strains PY288 (panels 1-3), PY290 (panel 4) and PY286 (panel 5) having the indicated genotypes were synchronized in G1 by α-Factor and released into S phase in the presence of hydroxyurea. Activation of checkpoint was monitored by phosphorylation of Rad9 (panels 1,4 and 5), Mrc1 (panel 2) and Rad53 (panel 3) **(B)** Yeast strains PY288 (panels 1 and 2), PY290 (panels 3 and 4), PY282 (panels 5 and 6) and PY286 (panels 7 and 8) having the indicated genotypes were synchronized in G1 as described in (A), and released into fresh YPD supplemented with 2 μg/ml 4-NQO. Checkpoint activation was monitored by phosphorylation of Rad9 and Mrc1. * represents the appearance of a non-specific band.







Supplemental Figure 4. Growth phenotypes of *DNA2* mutants in checkpoint-deficient strains. Cut-outs of the full data shown here are also shown in Figure 4A and 4C of the paper. **(A)** Serial ten-fold dilutions of the indicated strains with the indicated *TEL1*, *DDC1* and *DNA2* mutations, were spotted onto 5-fluoroorotic acid plate to evict the resident complementing plasmid pBL583 (*DNA2 URA3*), and cells were allowed to grow at 23 ˚C for 3 days. **(B)** Scheme of the strains used in the experiment is shown on the top. Plating on 5-FOA plates was as in (A). In both A and B, all strains grew robustly on selective media before eviction of pBL583 containing wild-type *DNA2* (data not shown). Restreaking of strains from 5-FOA onto YPD plates reproduced the same phenotypes as shown, i.e. strains growing poorly on 5-FOA also grew poorly during subsequent propagation on YPD (e.g., see (C), and data not shown). **(C)** Serial ten-fold dilutions of a *mec1Δ tel1Δ* strain, together with strains No. 3 and No. 6 used in (A) and (B), respectively, were grown on YPD. A cut-out of this figure was used in Fig. 4C.



Supplemental Fig. 5. Growth and checkpoint activity of checkpoint-defective strains. (A) Poor growth of checkpoint defective strain cannot be suppressed by *RAD27* overexpression. Plasmid pBL176 (having *RAD27* gene under its native promoter; 2μ ori, right panel) or pRS306 (vector; left panel) was transformed into PY286 strain having the indicated *DNA2* alleles, or empty vector, and the strains were spotted onto 5-FOA plate to evict the resident complementing plasmid pBL583 (*DNA2 URA3*), and cells were allowed to grow at 23 ˚C for 3 days. **(B)** Western blot of PY286 strain having mutant *dna2 WY-AA* allele as a sole source of *DNA2*, is transformed with the indicated *DDC1* constructs. Cells were arrested in G1 and treated with 4-NQO and the numbers below the blot correspond to the plate numbers in Supplemental Fig. 4. Compare No. 7 and 8 with Supplemental Fig. 4B (No. 7 and 8), which shows that any strain that restores checkpoint activity (even through the artificial Ddc1-Dna2 fusion) also restores a robust growth phenotype. **(C)** Strains PY310, as PY282 but *mec1Δ*, containing the indicated allele of *DNA2* were plated on 5-FOA plates to evict pBL583 as in (A). This analysis shows that there is no growth phenotype associated with the *dna2-WY-AA* and *dna2-Δ(1-257)*, when *MEC1* is deleted but *TEL1* is wild-type, and secondly, that deletion of *MEC1* does not suppress the lethality of *dna2Δ*.

Supplemental References

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