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Activation of the Budding Yeast Securin Pds1 but Not Rad53 Correlates with Double-Strand Break-Associated G_2/M Cell Cycle Arrest in a mec1 Hypomorphic Mutant

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Abstract

Budding yeast Mec1, encoded by the yeast ATR/ATM homolog, negatively regulates cell cycle progression by activating Rad53 (Chk2) and Chk1, two parallel downstream checkpoint pathways. Chk1 phosphorylates Pds1 (securin), which prevents Pds1 degradation. We determined whether activation of both downstream pathways is required to establish G_2 arrest in response to double-strand breaks (DSBs). In a hypomorphic *mec1* mutant, Rad53 activation was not required to establish G_2 arrest triggered by a single HO endonuclease-generated DSB. However, Pds1 phosphorylation did correlate with G_2 arrest and *mec1–21 pds1* cells did not arrest in G_2 after exposure to ionizing radiation. The G_2 checkpoint genes, *CHK1* and *PDS1*, did confer radiation resistance in *mec1–21*, indicating that *CHK1*-mediated pathway is functional in the *mec1* hypomorph. Thus, phosphorylation of Pds1 but not Rad53 correlates with G_2 arrest in response to DSBs in the *mec1* hypomorphic mutant.

Keywords

Saccharomyces cerevisiae ; G_2 checkpoint; MEC1; RAD53; PDS1

INTRODUCTION

Eukaryotic cells activate checkpoint pathways to arrest cell cycle progression in response to DNA damage. Cell cycle arrest allows time for DNA repair to occur before DNA replication or before sister chromatid separation. Defective checkpoint pathways enhance genome instability, such as chromosomal rearrangements, which are associated with cancer in higher eukaryotes.[1,2]

Observations that the G_2 checkpoint suppresses chromosomal rearrangements underscore the importance of understanding the contribution of individual pathways in triggering G_2 arrest.[3,4] In *Saccharomyces cerevisiae* (budding yeast), DNA damage initiates checkpoint

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pathways that block entry into anaphase by preventing the degradation of cohesins and sister chromatid separation. Sister chromatid cohesion is achieved by stabilizing Pds1 (securin), which is normally degraded by the anaphase promoting complex (APC).[5] Chk1-mediated phosphorylation of Pds1 prevents Pds1 degradation by APC^{Cdc20};[6,7] which is important in the DNA damage-mediated checkpoint.[8,9] Alternatively, Rad53 (Chk2) inhibits Cdc20-Pds1 interaction[7] and phosphorylates Dun1, which in turn, inhibits mitotic exit by activating the Bfa1-Bub2 complex.[10] However, *rad53* and *pds1* mutants exhibit a partial defect in DNA damage-induced G_2 arrest;[11] whereas, the *rad53 pds1* double[11] and *rad9* mutants[12] are completely deficient in DNA damage-induced G_2 arrest. These observations suggest that there is some redundancy in the Rad53 and Pds1-mediated G_2 checkpoint pathways.

MEC1, the yeast homolog of human ATR (ATM and *RAD3*-related)/ATM (ataxia-telangiectasia, mutated), is required for all DNA damage-associated checkpoint pathways.[13,14] *mec1* null mutants are completely deficient in DNA damage-associated G_2 arrest, since Mec1 is required to activate both Rad53 and Chk1 downstream pathways through the recruitment of Rad9 to sites of DNA damage.[15–17] Both Mec1-dependent pathways are required for establishing and maintaining G_2/M arrest following the accumulation of single-stranded DNA at the telomeres.[11] Mec1 also mediates mitotic exit by stabilizing Pds1 via phosphorylation of the mitotic inducer Cdc20.[18] Thus, Mec1 is central to the DNA damage checkpoint in budding yeast and controls multiple effector pathways.

Considering that G_2 arrest can be initiated after exposure to diverse DNA damaging agents, including UV, ionizing radiation,[12,19] and cross-linking agents,[20] it is unclear whether both Rad53 and Chk1-mediated pathways contribute equally in establishing and maintaining G_2 arrest initiated by diverse DNA lesions. *mec1* hypomorphic mutants, such as *mec1–21*,[21] that exhibit separation of function phenotypes are particularly valuable in determining the contribution of downstream checkpoint pathways in establishing G_2 arrest after exposure to specific DNA damaging agents. *mec1–21* results from a G to A substitution (G882S) at position 2644 outside of the kinase domain.[22] *mec1-21* retains essential function, is less sensitive to the replication inhibitor hydroxyurea (HU) and DNA damaging agents UV and methane methylsulfonate (MMS), compared with *mec1* null mutant, but is defective in S phase checkpoint function and Rad53 signaling.[6,21–23] We used *mec1–21* to determine how G_2 arrest is signaled following exposure to ionizing radiation. Considering that a single DSB is sufficient to trigger G_2 arrest,[24] we also determined whether a directed HO endouclease-generated DSB could trigger G_2 arrest and downstream checkpoint activation in *mec1–21*. We found that G_2/M arrest is triggered in *mec1–21* cells after exposure to DSBs generated by ionizing radiation or a single HO-induced DSB, and that this correlated with phosphorylation of Pds1 but not of Rad53.

MATERIALS AND METHODS

Yeast strains, plasmids and media.

The genotypes of yeast strains used in this study are listed in Table 1. The original *mec1–21* strain (Y620)[21] was backcrossed ten times into S288c strain background. Mutants containing *chk1::KanMX*, *pds1::KanMX* and *sml1::URA3 mec1*-Δ*::KanMX* were generated by one-step gene disruption[25] using PCR amplified gene fragments and selecting for

Kan^{R26} or Ura^{+}. Additional mutants were made by standard genetic crosses and tetrad dissections (Table 1). The primers used for amplifying these gene fragments are listed in the "Yeast Deletion Database" (www-deletion.stanford.edu). Plasmid pOC52 (HA-PDS1) was provided by Y. Sanchez. YP (yeast extract, peptone), YPD (YP, dextrose), SC (synthetic complete, dextrose), SC-LEU (SC lacking leucine), SC-TRP (SC lacking tryptophan), and SC-URA (SC lacking uracil), are describedby Burke et al.[27] YPL medium contains YP with 2% lactate (pH 5.8); YPGal medium contains YP medium with 2% ultra-pure galactose. YPD(Kan) is YPD supplemented with 200 μg/ml of kanamycin sulfate.

Exposing cells to ionizing radiation.

To measure cell cycle progression or checkpoint activation following radiation exposure, cells were grown to the appropriate cell density, washed and resuspended in sterile H_2O. Cells were exposed to X rays using a Faxitron X-ray irradiator (Wheeling, IL) at a dose rate of 120 rads/min.

To measure radiation sensitivities, cells were grown to log phase in YPD medium at 30°C or at 25°C. Cells were then plated onto YPD plates after appropriate dilution, and exposed to γ rays using a Nordion 1.8 kCi ^{137}Cs at 6.8 krad/h. Results obtained using the Nordion irradiator were more consistent for longer radiation exposures, compared to the X-ray irradiator. Colonies were counted after incubation for four to six days at appropriate temperature (30°C or 25°C). We used at least three independent cultures for each strain.

Flow cytometry.

Flow cytometry analysis was carried out as previously described by Gasch et al.[28] Briefly, 0.3 ml of freshly growing cells was added directly to 0.9 ml of ethanol and allowed to fix at 4°C overnight. Cells were rehydrated in PBS for at least 1 h, washed once and resuspended in 100 μl of FACS buffer (0.2 M Tris pH 7.5, 20 mM EDTA), and incubated with 1 mg/ml RNase A at 37°C for 4 h. Cells were then washed in PBS, treated with 5 μg/ml propidium iodide in a final volume of 0.5 ml of PBS, and analyzed for fluorescence content with the use of a BD LSR II Flow cytometer.

Targeting HO endonuclease-generated DSBs to the *TRP1* locus.

HO endonuclease generates DSBs at the *MAT* locus. We inserted the 117 bp *MAT*a fragment containing the HO endonuclease recognition sequence (HOcs) within a *his3* fragment.[3] This fragment, *his3-Δ3'::HOcs* was then inserted at the *trp1* locus on chromosome IV in a *MAT*a-inc strain, so that only the *trp1* locus is digested by HO endonuclease (Fig. 1). The strain construction used in this study was previously used to measure recombination stimulated by HO endonuclease-generated DSBs.[3] pGHOT-*GAL3* containing the HO gene under *GAL* control,[3] was introduced into *MEC1*, *mec1–21* and *mec1* null strains by selecting for Trp^{+} transformants. Stationary phase cells in SC-TRP were diluted 1:10 in YPLactate and cultures were grown to midlog phase (10^7 cells/ml). The cells were then synchronized in G_1 with α-factor (10 μg/ml). Cells were washed in sterile H_2O, and incubated in YPGal or YPGlu (YPD) for 3 h to either repress or induce the expression of HO endonuclease, respectively. HO endonuclease digestion at this site

generates chromosomal IV fragments,[29] and in this particular strain, DSB repair occurs by homologous recombination with a juxtaposed *his3* fragment, *his3*-Δ*5′*.

DAPI staining.

Cell cultures from above were then harvested and fixed with 75% ethanol. The fixed cells were rehydrated with PBS for 30 minutes, stained with 1μg/ml DAPI for 10 seconds, washed with distilled water five times. Then samples were processed for microscope examination. Images of G_2 arrested nuclei were captured using a Zeiss Axioskop 40 microscope.

Western blots.

Protein extracts from yeast cells were prepared as previously described by Foiani et al.,[30] separated either on 10% acrylamide/0.066% bis-acrylamide gels for Pds1 detection, or on 10% acrylamide/0.266% bis-acrylamide gels for Rad53 detection, and transferred to nitrocellulose membranes. Rad53 and HA-Pds1p were detected by Western blotting using goat anti-Rad53 (yC-19, Santa Cruz) and mouse anti-HA (16B12, Covance, Madison, WI) antibodies respectively. The secondary antibodies used were anti-goat IgG-HRP and anti-mouse IgG-HRP (Santa Cruz).

RESULTS

Because *MEC1* is required for G_2 arrest after exposure to DNA damaging agents, *mec1* hypomorphic mutants, such as *mec1-21*, that are partially defective in signaling pathways may elucidate which genes are required for cell cycle arrest following exposure to radiation. We first determined whether resistance to ionizing radiation in *mec1-21* correlated with G_2 checkpoint function. Considering that ionizing radiation causes both single and DSBs, we also determined whether a single HO-induced DSB would trigger G_2 arrest in *mec1-21* (Fig. 1). Finally, we determined whether Rad53 or Pds1 activation correlated with G_2 arrest following ionizing radiation exposure or induction of HO-endonuclease.

G_2 checkpoint genes confer radiation resistance in *mec1-21*.

We measured the γ-ray sensitivity of *mec1-21* and *mec1* null cells. We found that *mec1* null cells are significantly more sensitive than *mec1-21* cells to ionizing radiation, especially after exposure to higher radiation doses (Fig. 2A). For example, *mec1* null cells were over 10-fold (1.3%/14.3% survival) more sensitive than *mec1-21* after exposure to 18 krads. Thus, *mec1-21* cells retain DNA repair function(s) lacking in *mec1* null cells.

We then asked whether radiation resistance in *mec1-21* was conferred by the G_2 checkpoint genes, *RAD9*, *CHK1* and *PDS1*. We disrupted *RAD9*, *PDS1* and *CHK1* in *mec1-21* and measured the radiation sensitivity in the single and double mutants. Although the *chk1* mutant is as resistant as wild type cells to ionizing radiation, there is a synergistic increase in the radiation sensitivity of *mec1-21 chk1* compared to the single mutants, and the sensitivity of *mec1-21 chk1* is similar to that *mec1*-Δ (Fig. 2A). However, compared to *rad9*, there is only an additive increase in the radiation sensitivity of *mec1-21rad9*. These data indicate that the DNA repair deficiencies in *mec1-21* and *chk1* reside in different pathways, while DNA repair deficiencies in the *mec1-21* and *rad9* reside in over-lapping pathways.

Chk1 regulates the metaphase-to-anaphase transition in response to DNA damage by preventing Pds1 degradation. We measured radiation sensitivity in *mec1-21pds1* double mutant and the corresponding single mutants (Fig. 2B). Because *pds1* cells are temperature sensitive, all cells were irradiated and incubated at 25°C. After log phase cells were exposed to ionizing radiation, *mec1-21pds1* double mutant displayed an increase in sensitivity compared to the single mutants. The percent survival of *mec1-21 pds1* and *mec1-21 chk1* after radiation exposure and incubation at 25°C were similar (data not shown). These data suggest that some DNA repair functions of *PDS1* and *CHK1* still function in *mec1-21*. Because *PDS1* and *CHK1* function in DNA damage-induced G_2 arrest, we then determined whether G_2 arrest can be triggered in *mec1-21*.

Radiation-induced G_2 arrest in *mec1-21* requires *PDS1*.

We investigated whether ionizing radiation exposure triggered *PDS1*-dependent G_2 arrest in *mec1-21*. Wild type, *mec1-21*, and *mec1-21 pds1* log phase cells were exposed to 3 krads ionizing radiation, and then returned to rich (YPD) medium, and incubated at 25°C. Cell-cycle progression was determined by FACS analysis (Fig. 3). We expected that if radiation triggered G_2 arrest, the 2N peak, indicative of G_2 cells, would increase while the 1N peak, indicative of G_1 cells, would decrease. We observed an increase in the G_2 peak and a decrease in the G_1 peak for both *mec1-21* and wild-type cells 90–120 minutes after radiation exposure (Fig. 3A and B), while we observed no increase in the G_2 peak for *mec1-21 pds1 cells* (Fig. 3C). Thus, exposure to ionizing radiation triggers *PDS1*-dependent G_2 arrest in *mec1-21*.

A targeted DSB causes G_2 arrest in *mec1-21*.

Since one DSB is sufficient to trigger G_2 arrest in wild type cells,[24] we determined whether a single DSB would trigger G_2 arrest in *mec1-21*. We used strains containing a single HO cut site at the *trp1* locus; this cut site is present within a *his3* fragment, *his3-Δ3'* (Fig. 1). We introduced a galactose-inducible HO gene, present on pGHOT-*GAL3*, into wild type, *mec1-21* and *mec1* null cells. Mid-log phase *mec1-21*, *mec1* and *MEC1* cells were synchronized in G_1 with α-factor and HO endonuclease was induced for 3 h by addition of galactose. We visualized G_2-arrested cells by DAPI staining. As shown in Figure 4, both wild type (Fig. 4A) and *mec1-21* (Fig. 4B) cells exhibit a dumb-bell morphology with the nucleus at the neck of the bud, which is a typical G_2 arrest phenotype in budding yeast. In contrast, we observed diffuse DAPI staining in *mec1* null cells (Fig. 4C), suggestive of nuclear fragmentation, as described for a *mec1-ts* mutant growing at non-permissive temperature.[31] These data indicate that DNA damage-induced G_2 cell-cycle arrest occurs in *mec1-21* cells after generation of a single HO-induced DSB.

Pds1 but not Rad53 is activated in *mec1-21* after exposure to HO-induced DSBs.

DNA damage-associated G_2 arrest involves *MEC1*-dependent activation of Pds1 and Rad53.[6–9,11] Considering that DSBs trigger G_2 arrest in *mec1-21*, we determined by western blots whether Rad53 and Pds1 were activated in wild-type and *mec1-21* cells after radiation exposure or after HO induction. Pds1 phosphorylation was previously observed after exposure to 4 and 8 krads radiation.[8] Thirty minutes after 6 krads radiation exposure, we found that both Rad53 and Pds1 were activated in wild-type cells (Fig. 5A and B), and we

measured the ratio of activated protein to non-activated protein by scanning densitometry. The ratio of P-Rad53 to Rad53 was 0.67 and 0.38 for wild-type and *mec1-21* cells after X-ray exposure, respectively. We then determined whether Pds1 was phosphorylated after X-ray exposure, using a HA-tagged Pds1 protein expressed on pOC52. Pds1-HA appeared as a doublet, with a minor (phosphorylated) form migrating slower than the major form. After 6 krads X-ray exposure, Pds1 activation resulted in a shift from the major to the minor form. For wild type after X-ray exposure, the ratio of P-Pds1 to Pds1 was 5.4, while for *mec1-21*, the ratio of P-Pds1 to Pds1 was 2.1. No activation of either Rad53 or Pds1 was observed in *mec1*-Δ cells (data not shown).

Considering that HO-induced DSBs triggered G_2 arrest in *mec1-21*, we determined whether Rad53 and Pds1 activation also occurred in *mec1-21* cells following HO endonuclease induction. We extracted protein from wild-type and *mec1-21* cells, containing pGHOT-*GAL3* and pOC52, grown in YPL, YPD, and YPGal media. As also observed by Nakada et al.,[32] Rad53 phosphorylation occurred in wild-type cells after HO induction in YPGal; however, we did not observe Rad53 phosphorylation in *mec1-21* cells after HO induction (Fig. 5C). When cells were grown in YPL or YPD media (Fig. 5D), we observed the Pds1-HA doublet in both wild type and *mec1-21* cells (Fig. 5D, lanes 1, 2, 4 and 5). After HO endonuclease, induction we observed a shift from the major form of Pds1-HA to the minor (phosphorylated) form in both wild- type and *mec1-21* cells (Fig. 5D lane 3, 6 and E). Previous observations have indicated that there are also mobility changes in the non-phosphorylated form of Pds1-HA after exposure to DNA damage.[8] After HO endonuclease induction, there was a slight shift in the mobility of the non-phosphorylated form of Pds1-HA, as indicated by a shift in the peak dimensions on the scanning densitometer (Fig. 5E, lanes 3 and 6). Our results suggest that *mec1-21* cells retain a Pds1 signaling pathway that triggers G_2 arrest. Thus, Pds1 and not Rad53 activation correlated with G_2 arrest triggered by a single DSB in the *mec1-21* hypomorph.

DISCUSSION

MEC1, the yeast ATR/ATM homolog, is required for G_2 arrest in response to DNA damage and activates two parallel pathways, Chk1/Pds1 and Rad53 (Chk2).[6] G_2 arrest is then achieved by blocking the degradation of cohesins through the maintenance of Pds1 (securin), which is normally ubiquitinated by the anaphase promoting complex APC^{Cdc20} and targeted for degradation.[5] *RAD53* and *CHK1* are required to maintain and establish G_2 arrest in *cdc13* mutants,[11] which accumulate single-stranded DNA at the telomeres.[33] However, it is unknown whether activation of both Chk1/Pds1 and Rad53 are required to establish G_2 arrest in response to DSBs. Here, we used log phase *mec1-21* cells, which are deficient in the S phase checkpoint,[21] to determine which checkpoint genes are signaled to establish G_2 arrest and ionizing radiation resistance. Our major conclusion is that Pds1 phosphorylation correlates with G_2 arrest initiated by DSBs, while Rad53 phosphorylation is dispensable.

These studies indicate that *mec1-21* is a hypomorphic mutant that separates the cell cycle arrest from Rad53 signaling function that is signaled by DSBs. Previous studies indicated that *rad53* mutants were only partially defective in establishing and maintaining G_2 arrest.[11] Since *RAD53* is essential due to its regulation of dNTP,[34–35] Rad53 essential function

must be maintained in *mec1-21*. We cannot exclude that a non-phosphorylated Rad53 may still function in establishing G_2 arrest in *mec1-21*. Further experiments are necessary to determine whether G_2 arrest can be established in a *mec1-21 rad53* double mutant in response to double-strand breaks.

These data may seem to be incongruent with previous observations that *mec1-21* is deficient in maintaining G_2 arrest in nocodazole arrested cells exposed to UV.[36] However, these previous experiments addressed the maintenance but not the establishment of G_2 arrest, and are consistent with observations that Clb2 levels are decreased in *mec1-21*.[18] We used log phase cells and α-factor arrested cells that could enter S phase after exposure to DSBs. Further experiments are necessary to determine whether the G_2 arrest phenotype can be maintained for extended periods of time following ionizing radiation exposure in *mec1-21 rad52* cells. Thus, the previous experiments are different from those discussed in this study.

The *PDS1*-dependent G_2 arrest phenotype in *mec1-21* complements observations that the *mec1-21 chk1* mutant is synergistically more sensitive to radiation, compared to *mec1-21* and *chk1*. These data thus support the idea that when Rad53 signaling is functional, Chk1 is dispensable for radiation repair, while when Rad53 signaling is deficient, as in *mec1-21*, Chk1 is required for radiation repair. Thus, *rad9* mutations, which confer G_2 checkpoint and Chk1 and Rad53 signaling deficiencies,[16,17] do not confer a synergistic increase in radiation sensitivity in *mec1-21* cells.

Although *RAD53* signaling may be dispensable for repair of DSBs, these observations do not imply that all *RAD53* functions are dispensable for recombinational repair between sister chromatids. Indeed, we previously observed that *rad53* mutants were deficient for all types of DNA damage-associated SCE except UV-associated SCE.[37] *RAD53* is required for the phosphorylation of *RAD55* after exposure to DNA damage.[38] Thus, we speculate that some *RAD53* DNA repair function is maintained in *mec1-21*. Exactly which DNA repair function(s) confers radiation resistance in *mec1-21* is currently being investigated.

Other pathways for G_2 arrest have been observed in *mec1* null mutants. For example, Clerici et al.[39] observed a Tel1/MRX-dependent G_2 checkpoint which depends on completion of replication of the DNA lesion, an active Clb-CDK complex, and *PDS1*. They propose that *TEL1* may be directly involved in Pds1 phosphorylation, and that Mad2 (spindle checkpoint) may contribute to checkpoint arrest. Thus, it will be important to determine the role of *TEL1* and *MAD2* in checkpoint signaling in *mec1-21* after exposure to ionizing radiation. Further studies would be necessary to determine the kinase activity and substrate specificity of the mutant Mec1 protein.

In summary, we have shown that establishment of G_2 arrest does not require Rad53 activation in a *mec1* hypomorphic mutant. These results underscore previous studies that multiple pathways can establish G_2 arrest in yeast, and may further elucidate the many pathways involved in G_2 arrest in higher eukaryotes.

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ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
ATR	ATM and rad3 related
HU	hydroxyurea
MMS	methyl methanesulfonate
DSB	double-strand break
HO	mating-type switching endonuclease
SCE	sister chromatid exchange
DAPI	4,6-diamidino-2-phenylindole

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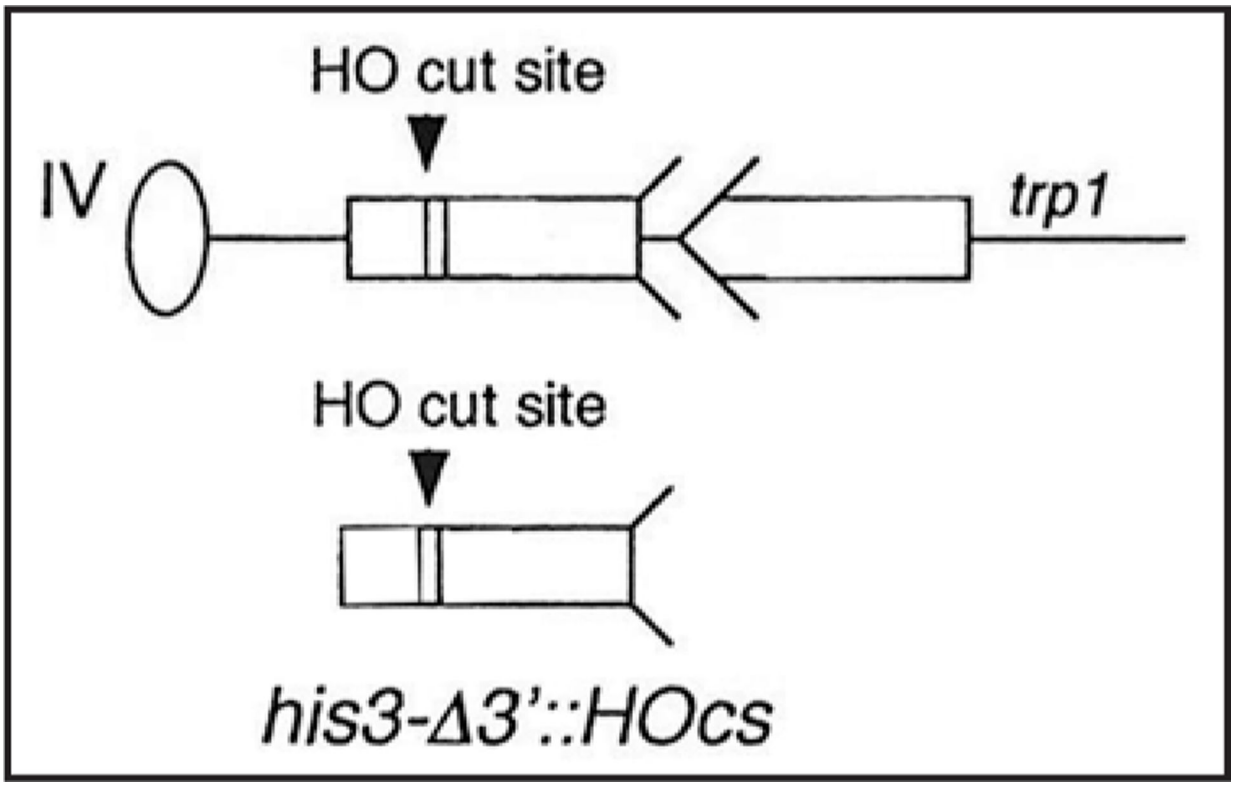

Figure 1.

The location of the 117 bp HO cut site (HOcs) at *trp1* on chromosome IV. The oval represents the centromere and the single line represents duplex DNA. For simplicity, the left arm of chromosome IV is not shown. The HOcs is located within one *his3* fragment, *his3*-Δ3', which is juxtaposed to the *his3* fragment, *his3*-Δ5'. The *his3*-Δ3' lacks the 3' sequences (arrow head), while the *his3*-Δ5' lacks to promoter sequences (feathers). Both *his3* fragments are located with the amino acid reading frames oriented to the centromere. The *his3* fragments share a total of 450 bp sequence homology. The boundaries of the HOcs are represented by two parallel vertical lines within *his3*-Δ3' Digestion at the HOcs triggers G_2 arrest and homologous recombination between the two *his3* fragments.

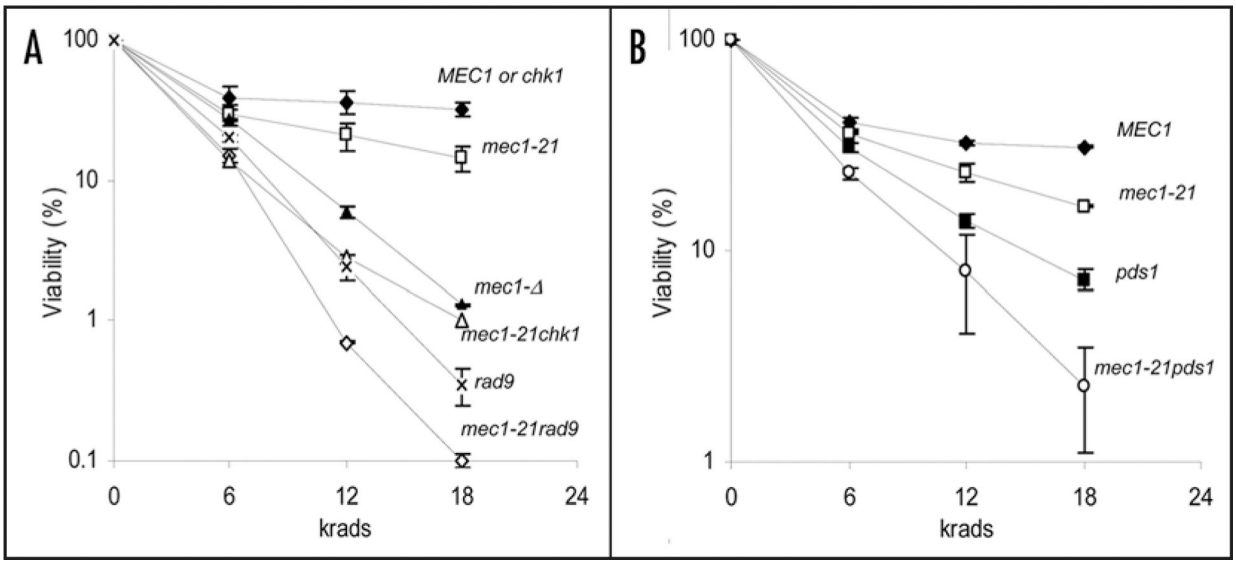

Figure 2.

Ionizing irradiation sensitivity of *mec1* and G_2 checkpoint mutants. (A) Radiation sensitivity of cells incubated at 30°C. (B) Radiation sensitivity of cells incubated at 25°C. Viability (percent survival) is plotted against radiation dose; 1 krad = 10 Gy. Genotypes are indicated adjacent to the appropriate survival curve. Wild-type (YB163, diamond), *mec1* null (YB339, solid triangle), *mec1-21* (YB368, square), *rad9* (YB147, cross), *mec1-21chk1* (YB337, open triangle), *mec1-21rad9* (YB331, open diamond), and *mec1-21pds1* (YB383, circle). Symbols may obscure standard deviations.

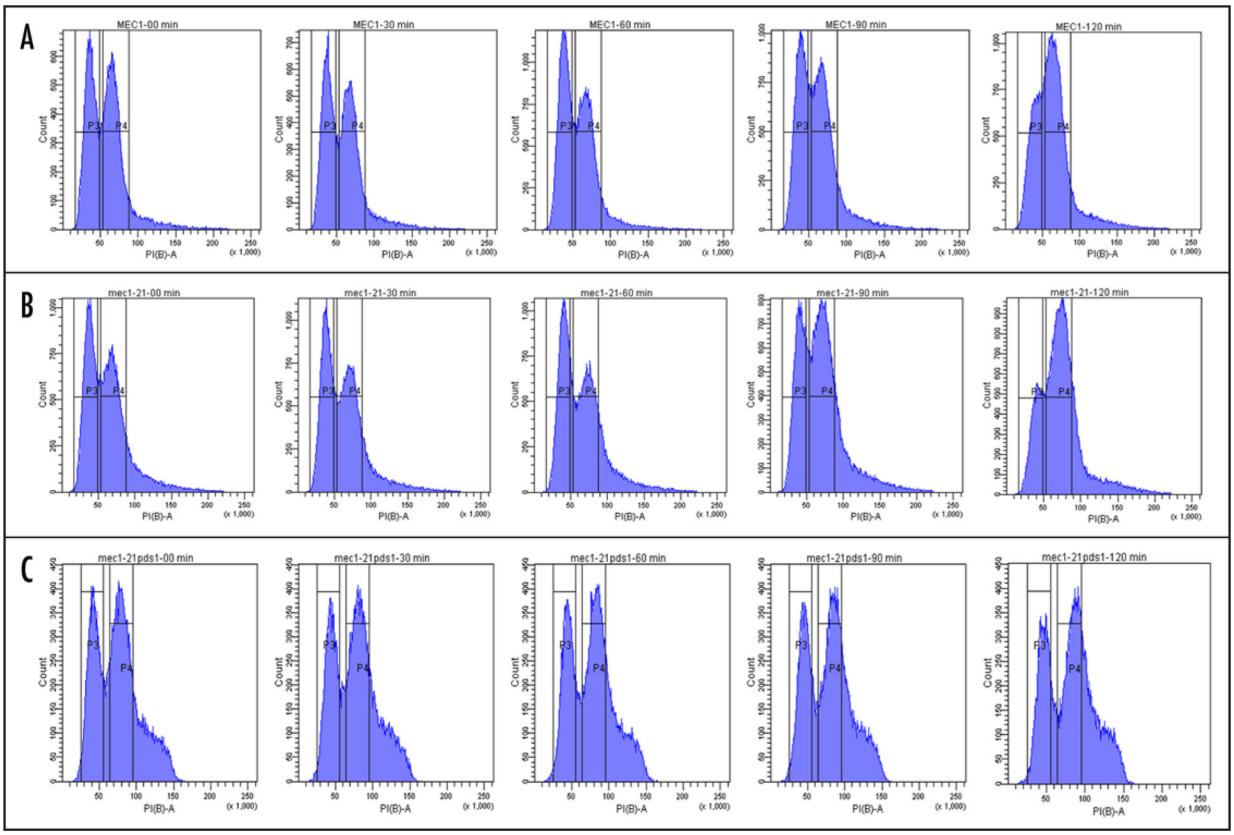

Figure 3.

Cell cycle progression in *mec1* mutants after exposure to ionizing radiation. Logarithmically growing cells were exposed to 3 krads X-ray, and returned to growth media (YPD). FACS analysis was performed on cells collected at each indicated time point after radiation exposure. The genotypes are listed above the panels. (A) FACS profiles for wild-type cells (YB163); (B) *mec1-21* cells (YB368); (C) *mec1-21pds1* cells (YB383).

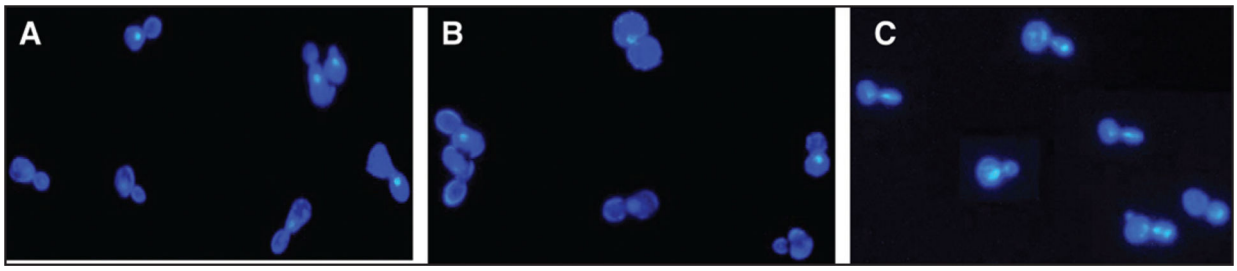

Figure 4.

DAPI staining of wild-type, *mec1-21*, and *mec1*-Δ cells 3 h after HO endonuclease induction. HO endonuclease was induced in α-factor arrested G_1 cells. Cells were then incubated for 3 h in growth medium (YPGal) and their nuclei morphology was determined by DAPI staining. The cells were photographed by fluorescence microscopy. (A) *MEC1* (YB163); (B) *mec1-21* (YB368); (C) *mec1*-Δ (YB339).

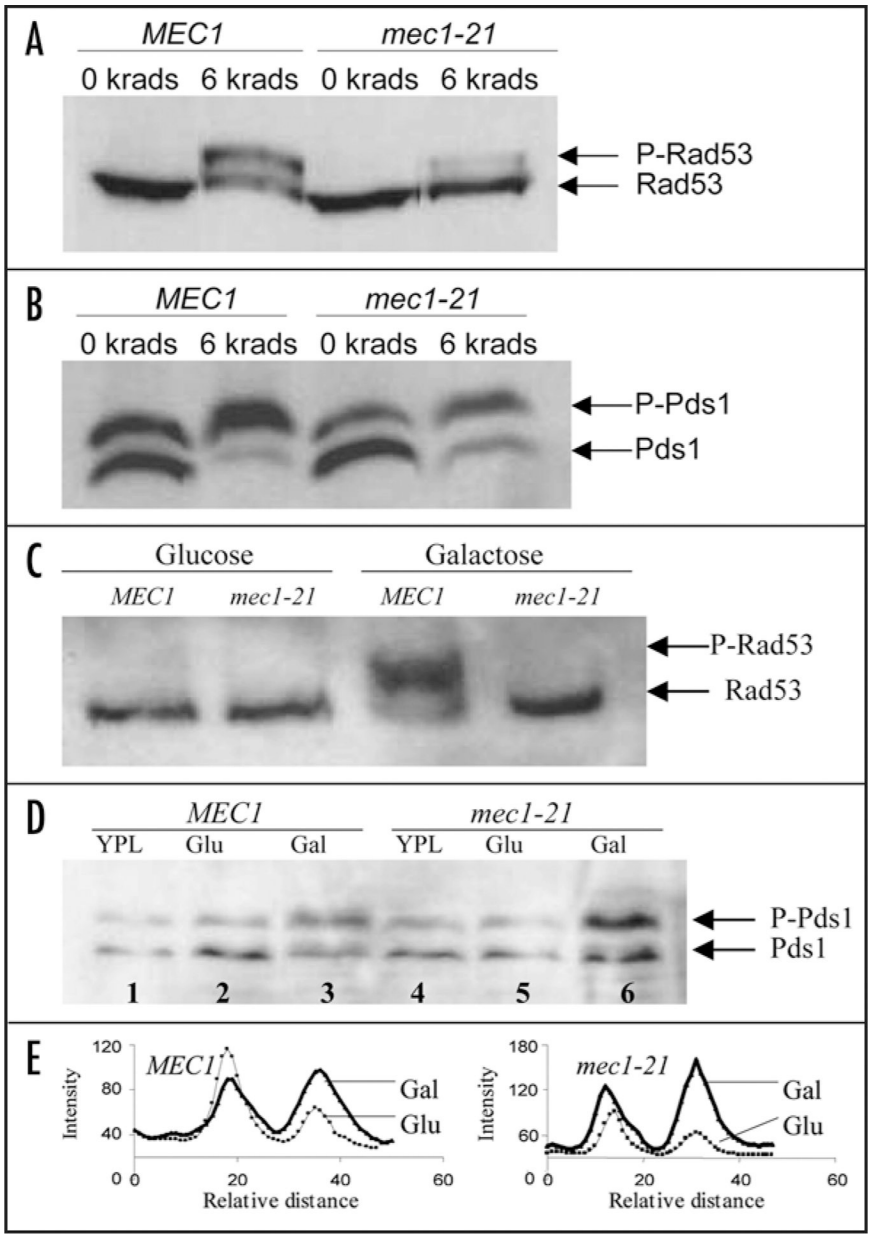

Figure 5.

Phosphorylation of Pds1 and Rad53 in wild-type and *mec1-21* cells after exposure to either 6 krads ionizing radiation or an HO endonuclease-induced DSB. HO endonuclease was induced or repressed after the addition of galactose (Gal) or glucose (Glu), respectively. HA-Pds1 and Rad53 were detected by Western blots using anti-HA and anti-Rad53 antibody, respectively. Arrows point to the position of phosphorylated and non-phosphorylated forms of Rad53 and Pds1. (A) Rad53 phosphorylation after exposure to ionizing radiation; (B) Pds1-HA phosphorylation after exposure to ionizing irradiation; (C) Rad53 phosphorylation after induction of HO endonuclease; (D) Pds1-HA phosphorylation after induction of HO endonuclease; (E) Densitometry scan of lane 2, 3 (*MEC1*) and 5, 6 (*mec1-21*) of (D). The

solid and dashed lines are of Pds1-HA from galactose and glucose treated respectively. Scans were performed from bottom to top

Table 1

Yeast strains

Strain	Genotype[a]	Source (Synonym)
YB163	*MAT*a-*inc ura3-52 his3-*Δ*200 ade2-101 lys-801 trp1-*Δ*1 gal3*⁻ *trp1::[his3-*Δ*3′::HOcs, his3-*Δ*5′]*	This laboratory
YB147	*MAT*a *rad9::URA3*	This laboratory
YB312	*MAT*α *mec1-21*	This laboratory
YB331	*MAT*a-*inc mec1-21 rad9::URA3*	This laboratory
YB335	*MAT*a-*inc chk1::KanMX*	*chk1::KanMX* disruption in YB163
YB337	MATα *mec1-21 chk1::KanMX*	From cross of YB312 with YB335
YB338	*MAT*a-*inc sml1::URA3*	*sml1::URA3* disruption in YB163
YB339	*MAT*a-*inc sml1::URA3 mec1-*Δ*::KanMX*	*mec1::KanMX* disruption in YB338
YB368	*MAT*a-*inc leu2-*Δ*1 mec1-21*	This laboratory
YB383	MATα *mec1-21 pds1::KanMX*	*pds1::KanMX* disruption in YB312

[a]All strains listed below YB163 have the same genotype as YB163 unless indicated. Mating type is added for clarity.