Mad2 prolongs DNA damage checkpoint arrest caused by a double-strand break via a centromere-dependent mechanism

Farokh Dotiwala, **Jacob C. Harrison**, **Suvi Jain**, **Neal Sugawara**, and **James E. Haber**[1]
Department of Biology and Rosenstiel Basic Medical Sciences Research Center Brandeis University, Waltham, MA 02454-9110

Abstract

Eukaryotic cells employ a suite of replication and mitotic checkpoints to ensure the accurate transmission of their DNA. In budding yeast, both the DNA damage checkpoint and the spindle assembly checkpoint (SAC) block cells prior to anaphase [1-5]. The presence of a single unrepaired double-strand break (DSB) activates ATR and ATM protein kinase homologs, Mec1 and Tel1, which then activate downstream effectors to trigger G2/M arrest and also phosphorylate histone H2A (creating γ-H2AX) in chromatin surrounding the DSB [6-8]. The SAC monitors proper attachment of spindle microtubules to the kinetochore formed at each centromere and the biorientation of sister centromeres toward opposite spindle pole bodies. Although these two checkpoints sense quite different perturbations, recent evidence has demonstrated both synergistic interactions and cross-talk between them [9-11]. Here we report that Mad2 and other SAC proteins play an unexpected role in prolonging G2/M arrest after induction of a single DSB. This function of the SAC depends not only on Mec1 and other components of the DNA damage checkpoint but also on the presence of the centromere located ≥ 90kb from the DNA damage. DNA damage induces epigenetic changes at the centromere, including the γ-H2AX modification, that appear to alter kinetochore function, thus triggering the canonical spindle assembly checkpoint. Thus, a single DSB triggers a response by both checkpoints to prevent the segregation of a damaged chromosome.

Results and Discussion

Mad2 prolongs arrest before adaptation after DNA damage

Cells with an unrepaired DSB exit checkpoint arrest and resume cell cycle progression either after repairing the damage (recovery) or even when damage persists (adaptation) (reviewed in [2]). We induced a single DSB at the *MAT* locus on chromosome 3 (Chr3) in a strain deleted for the homologous donor loci, so that the DSB cannot be repaired by homologous recombination [6,12]. In this assay > 99% of cells remain arrested for about 15 h; this is equivalent to about 6 doubling times of isogenic cells lacking the HO endonuclease cut site (Fig. 1). Very similar arrest and adaptation is seen when a single DSB is induced on the left arm of Chr6 instead of at *MAT* [13]. In both cases the DSB is about 90-100 kb from its centromere. Cell cycle arrest is completely eliminated in cells lacking Mec1, but there is still a substantial delay in cells lacking Rad53 or Chk1 [13,14]. A slightly shorter, though

1 To whom correspondence should be addressed haber@brandeis.edu 781-736-2462.

statistically significantly different, arrest response is seen with a DSB on Chr11, 294 kb from its centromere (Fig. 1B).

Unexpectedly, deletion of *MAD2* markedly shortens the time that cells with a DSB remain arrested (Fig. 1). *mad2Δ* strains suffering a DSB on Chr3 or on Chr11 arrest only for about 10 h, 2/3 as long as in *MAD2* cells. The SAC mutants *mad1Δ* and *mad3Δ* had comparable effects though *bub1Δ* and *bub3Δ* did not (data not shown). These results suggest that arrest of cells at G2/M may consist of two stages, the first enforced by Mec1, Rad53 and to a lesser extent by Chk1 and the second regulated by Mad1, Mad2, and Mad3.

Epistasis analysis suggests that Mad2's role may be executed through a Rad53-dependent pathway. The long residual cell cycle delay in *chk1Δ* cells is partially suppressed by deletion of any of the 5 *mad* and *bub* mutants tested, even *bub1Δ* and *bub3Δ* that did not affect arrest as single mutants (Suppl. Fig. 1). Arrest in *mad2*Δ *chk1*Δ is markedly reduced, but only to the length seen in *rad53*Δ cells. In contrast, deleting the SAC genes did not further shorten the arrest in *rad53*Δ (Fig. 1A and Suppl. Fig. 1). These results suggest that Mad and Bub proteins act to prolong constraints on cell cycle progression that are imposed by Rad53. Deleting Mad or Bub genes in *rad53Δ chk1Δ* or in *rad9*Δ does not further shorten the small, but statistically significant residual delay compared to *mec1*Δ (data not shown). Thus, Mad and Bub proteins play a significant role in prolonging G2/M arrest and may define one pathway by which Rad53 halts the cell cycle.

MAD2 is required for the permanent arrest of most adaptation-defective mutations

Several classes of mutants block checkpoint adaptation including: (1) those with increased DNA end processing, (2) those with defects in aspects of homologous recombination, and (3) those unable to turn off the checkpoint signaling pathway. As seen in Fig. 2, *mad2Δ* at least partially suppresses the permanent arrest of all adaptation-defective mutants tested except *yku80*Δ, but even the *mad2*Δ-suppressed mutants still arrest for at least 10 h. Thus Mad2 and presumably other SAC proteins play a central role in maintaining the permanent arrest of cells initially arrested by the DNA damage checkpoint. The consequence of deleting Mad2 is not as great as deleting Rad9, which eliminates nearly all DSB-mediated arrest of these mutants (data not shown).

Deletion of Mad2 also shortens the delay of a DSB on Chr11, located 294 kb from its centromere (Fig. 1B). It is noteworthy that, when Mad2 is present, cell cycle delay with a DSB on Chr11 is significantly less ($p < 0.005$) than that seen on either Chr3 or Chr6, where the DSB is 90-100 kb from the centromere. Thus the distance of the DSB to the centromere may play a role in maintaining the DNA damage response.

Deletion of the centromere mimics *mad2*Δ in shortening checkpoint arrest

We tested whether centromere-derived signals can explain the role of Mad2 in the DSB response. A key question is whether Mad2's role reflects an alteration to the kinetochore in *cis* with the DSB. We have previously shown that 5′ to 3′ resection of the DSB continues for at least 24 hr at a rate of ~4 kb/h [15]; but the 90 kb distance of the DSB from *CEN3* is too far to be degraded within 10 h, the time at which Mad2 is required to maintain G2/M arrest. However, the effect on the centromere could reflect a change in the topological constraint of the now-broken chromosome or it could be caused by a long-distance chromatin modification such as γ-H2AX extending from the DSB [8,16]. To determine if the role of Mad2 depends on the presence of the centromere, we devised a way to delete *CEN3* at the same time that HO is induced to create a DSB at *MAT*. As shown in Fig. 3, we simultaneously induced an irreparable DSB at *MAT* and another DSB at a site less than 1 kb from *CEN3*. The DSB at *CEN3* is flanked by 2-kb identical DNA sequences, so 5′ to 3′ resection and repair of the DSB by single-strand

annealing (SSA) lead to a complete deletion of *CEN3*. Galactose-mediated induction of HO endonuclease cleavage is essentially 100% efficient, as shown in the southern blot in Fig. 3B. By 3 h, nearly all cells have deleted *CEN3*.

In the absence of a DSB at *MAT*, deleting *CEN3* has no effect on the time it takes for cells to progress into the next cell cycle and bud a second time (Fig. 3). This is consistent with previous results that repairing a short-lived DSB by SSA does not delay cell cycle progression [17]. When there is a DSB at *MAT*, deleting *CEN3* shortens the length of arrest caused by the remaining DSB at *MAT*, though not as profoundly as *mad2*Δ. Importantly, the *mad2*Δ *CEN3* double deletion was indistinguishable from *mad2*Δ alone. Moreover, deleting *CEN3* also significantly reduced the delay in a *chk1*Δ strain, as did *mad2*Δ. These data argue that deleting the centromere proximal to the DSB eliminates much of the Mad2-dependent response to the presence of a DSB.

If Mad2 responds to alterations at the kinetochore of the chromosome that suffered the DSB, then we would expect there to be no effect if we were to delete *CEN3* in a strain where the DSB is induced on Chr 6. Indeed this is the case (Fig. 3). This experiment provides clear evidence that a significant portion of DSB-induced G2/M arrest is attributable to an alteration at the kinetochore of the broken chromosome, rather than by an indirect effect by increasing the pool of Cdc20 that could promote mitosis.

We note that there is a greater effect of deleting *MAD2* than of deleting the *CEN3*. This difference in suppressing checkpoint-mediated arrest by deleting *CEN3* versus *mad2Δ* could be explained by the fact that *CEN3* deletion strain did – for a time – have two DSBs. Two unrepaired DSBs provoke permanent arrest, so that the checkpoint signal might have been initially stronger and possibly more difficult to turn off [12]. We therefore constructed a strain with an unrepairable DSB at *MAT* and a second DSB at the *URA3* locus on Chr5, where it could be repaired by a comparable SSA event that did not involve centromere deletion [18]. There was no alteration in the time it took for adaptation to occur, and the SSA event by itself did not cause any visible arrest (Fig. 3).

Eliminating γ-H2AX shortens the checkpoint delay

Shortly after a DSB is created, nearby histone H2A is phosphorylated (yielding γ-H2AX); this modification spreads prominently 40-50 kb on either side of the DSB with the strongest accumulation within ~20 kb of the DSB [16,19]. As resection proceeds γ-H2AX is locally displaced from chromatin, but additional γ-H2AX is modified by Mec1 kinase ahead of the resection [16]. To ask if γ-H2AX modification affects the persistence of adaptation, we analyzed an isogenic strain in which the two copies of the *H2A* gene encode the non-phosphorylatable S129A allele. As seen in Fig. 4A, eliminating γ-H2AX shortens the length of arrest about as much as does *mad2*Δ. Importantly, the double mutant lacking both *MAD2* and the phosphorylation site on H2A [*hta1-S129A hta2-S129A*] is no more defective than either defect alone. Cells carrying H2A-S129A also suppress adaptation-defective mutations, including *tid1*Δ, *yku70*Δ and *ckb2*Δ (data not shown). The H2A-S129A mutation markedly shortens the arrest seen in a *chk1*Δ mutant but, unlike *mad2*Δ, also reduces the duration of arrest in *rad53*Δ (Fig. 4). Just as Rad53 is likely to affect cell cycle arrest by several pathways, not all of which involve Mad2 [20], γ-H2AX may affect pathways other than those controlled by Rad53 (see below).

To establish more directly that γ-H2AX modification could alter centromere function, we asked if γ-H2AX modification does in fact reach the centromere after resection has proceeded for many hours. Using an antibody specific for the γ-H2AX modification, we monitored sites around *CEN3* as well as nearer the DSB at *MAT*. As shown in Fig. 4B, there is a 3-10 fold increase in γ-H2AX modification surrounding *CEN3* after 6 h.

These data suggest that the Mad and Bub proteins, acting in their normal role as monitors of spindle function and centromere integrity, can sense a DSB created ≥ 90 kb away. Consequently, the G2/M arrest triggered by a single DSB that first activates the DNA damage checkpoint is significantly prolonged. Either contemporaneous deletion of the adjacent centromere or elimination of γ-H2AX formation largely, but not completely, abrogates the Mad2-dependent phase of cell cycle arrest. We conclude that the major way in which Mad2 responds to the DSB likely involves an epigenetic modification of the centromere in *cis* to the DSB. Centromere function depends not only on the core nucleosome containing Cse4 but on surrounding centric chromatin [21]. It is also possible that the accumulation of γ-H2AX on the chromosome could cause a rearrangement of the normally high level of cohesin binding around the centromere, as γ-H2AX enables damage-dependent recruitment of cohesins, which may include sliding from the centromeric region [19,22]. An alternative model could be that the absence of Mad2 generally increases Cdc20 activity [23] that in turn could promote APC-mediated degradation of Pds1 and thus promote mitosis. However, this idea is hard to rationalize with the specific effect of completely deleting one centromere, which should in fact not perturb Mad2 monitoring of centromeres. However, release of Cdc20 could account for the larger effect of *mad2*Δ compared to centromere deletion (Fig. 1). We note that in a *mec1*Δ strain there is still Tel1-dependent γ-H2AX around the DSB site, but it fails to spread down the chromosome as resection proceeds because this requires Mec1 [16]; consequently the centromere region will not be modified and there is no Mad2-dependent delay of progression in a *mec1*Δ mutant.

Previous studies have also suggested that γ-H2AX plays an important role in Rad9-mediated steps in the DNA damage checkpoint response. Rad9 contains both BRCT domains that interact with γ-H2AX and a Tudor domain that binds to methylated histone H3-K79 [24,25]. It is therefore possible that removing phosphorylation of histone H2A would produce a partial *rad9* phenotype. This additional role can explain the shorter duration of arrest in H2A-S129A *rad53*Δ compared to *rad53*Δ. However, the fact that a *mad2*Δ H2A-S129A double mutant is not different from *mad2*Δ argues that the primary effect of eliminating γ-H2AX is acting through the Mad2 pathway.

To examine how partially limiting Rad9 response might affect DSB-induced arrest, we deleted the Dot1 methyltransferase that methylates H3-K79; this *dot1*Δ deletion has a more profound effect on DSB-induced arrest than H2A-S129A (Suppl. Fig. 2). Interestingly, a *dot1*Δ H2A-S129A double mutant shows significantly ($p < 0.05$) longer cell cycle delay than a *rad9*Δ mutation (Suppl. Fig. 2). This observation suggests there could be yet another histone modification to which Rad9 responds (we note that in Crb2 of *S. pombe* and in 53BP1 of mammals the methylated residue bound by the Tudor domain is H4-K20); moreover it appears that Rad9 may directly recognize single-stranded DNA [26].

To summarize, a persistent DSB establishes a spreading zone of ”chromosomal inflammation“, here marked by γ-H2AX. If this region is sufficiently close to a centromere the altered chromatin conformation can apparently trigger kinetochore dysfunction that is recognized by Mad2 and other components of the SAC. The SAC acts semi-redundantly with the DNA damage checkpoint to enforce long-term cell cycle arrest in the presence of chromosomal DSBs and thereby prevents the segregation of damaged chromosomes.

Experimental Procedures

Yeast strains, cell cycle arrest and adaptation assays, chromatin immunoprecipitation methods are presented in Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grant GM61766 to JEH. JCH was a postdoctoral fellow of the Leukemia and Lymphoma Society.

References

1. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science (New York, N.Y 1996;274:1664–1672.
2. Harrison JC, Haber JE. Surviving the breakup: the DNA damage checkpoint. Annual review of genetics 2006;40:209–235.
3. Longhese MP, Foiani M, Muzi-Falconi M, Lucchini G, Plevani P. DNA damage checkpoint in budding yeast. The EMBO journal 1998;17:5525–5528. [PubMed: 9755152]
4. Lew DJ, Burke DJ. The spindle assembly and spindle position checkpoints. Annual review of genetics 2003;37:251–282.
5. Lim HH, Zhang T, Surana U. Regulation of centrosome separation in yeast and vertebrates: common threads. Trends in cell biology 2009;19:325–333. [PubMed: 19576775]
6. Lee SE, Moore JK, Holmes A, Umezu K, Kolodner R, Haber JE. Saccharomyces Ku70, Mre11/Rad50 and RPA proteins regulate adaptation to G2/M arrest DNA damage. Cell 1998;94:399–409. [PubMed: 9708741]
7. Toczyski DP, Galgoczy DJ, Hartwell LH. CDC5 and CKII control adaptation to the yeast DNA damage checkpoint. Cell 1997;90:1097–1106. [PubMed: 9323137]
8. Shroff R, Arbel-Eden A, Pilch D, Ira G, Bonner WM, Petrini JH, Haber JE, Lichten M. Distribution and dynamics of chromatin modification induced by a defined DNA double-strand break. Curr Biol 2004;14:1703–1711. [PubMed: 15458641]
9. Garber PM, Rine J. Overlapping roles of the spindle assembly and DNA damage checkpoints in the cell-cycle response to altered chromosomes in Saccharomyces cerevisiae. Genetics 2002;161:521–534. [PubMed: 12072451]
10. Clemenson C, Marsolier-Kergoat MC. The spindle assembly checkpoint regulates the phosphorylation state of a subset of DNA checkpoint proteins in Saccharomyces cerevisiae. Mol Cell Biol 2006;26:9149–9161. [PubMed: 17060453]
11. Kim EM, Burke DJ. DNA damage activates the SAC in an ATM/ATR-dependent manner, independently of the kinetochore. PLoS Genet 2008;4:e1000015. [PubMed: 18454191]
12. Pellicioli A, Lee SE, Lucca C, Foiani M, Haber JE. Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. Mol Cell 2001;7:293–300. [PubMed: 11239458]
13. Dotiwala F, Haase J, Arbel-Eden A, Bloom K, Haber JE. The yeast DNA damage checkpoint proteins control a cytoplasmic response to DNA damage. Proc Natl Acad Sci U S A 2007;104:11358–11363. [PubMed: 17586685]
14. Gardner R, Putnam CW, Weinert T. RAD53, DUN1 and PDS1 define two parallel G2/M checkpoint pathways in budding yeast. Embo J 1999;18:3173–3185. [PubMed: 10357828]
15. Ira G, Pellicioli A, Balijja A, Wang X, Fiorani S, Carotenuto W, Liberi G, Bressan D, Wan L, Hollingsworth NM, et al. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. Nature 2004;431:1011–1017. [PubMed: 15496928]
16. Kim JA, Kruhlak M, Dotiwala F, Nussenzweig A, Haber JE. Heterochromatin is refractory to gamma-H2AX modification in yeast and mammals. J Cell Biol 2007;178:209–218. [PubMed: 17635934]
17. Vaze M, Pellicioli A, Lee S, Ira G, Liberi G, Arbel-Eden A, Foiani M, Haber J. Recovery from checkpoint-mediated arrest after repair of a double-strand break requires srs2 helicase. Molecular cell 2002;10:373. [PubMed: 12191482]

18. Sugawara N, Ira G, Haber JE. DNA length dependence of the single-strand annealing pathway and the role of Saccharomyces cerevisiae RAD59 in double-strand break repair. Mol Cell Biol 2000;20:5300–5309. [PubMed: 10866686]

19. Strom L, Lindroos HB, Shirahige K, Sjogren C. Postreplicative recruitment of cohesin to double-strand breaks is required for DNA repair. Mol Cell 2004;16:1003–1015. [PubMed: 15610742]

20. Liang F, Wang Y. DNA damage checkpoints inhibit mitotic exit by two different mechanisms. Mol Cell Biol 2007;27:5067–5078. [PubMed: 17485442]

21. Anderson DJ, Vargas JD, Hsiao JP, Hetzer MW. Recruitment of functionally distinct membrane proteins to chromatin mediates nuclear envelope formation in vivo. J Cell Biol 2009;186:183–191. [PubMed: 19620630]

22. Unal E, Arbel-Eden A, Sattler U, Shroff R, Lichten M, Haber JE, Koshland D. DNA damage response pathway uses histone modification to assemble a double-strand break-specific cohesin domain. Mol Cell 2004;16:991–1002. [PubMed: 15610741]

23. Yu H. Cdc20: a WD40 activator for a cell cycle degradation machine. Molecular cell 2007;27:3–16. [PubMed: 17612486]

24. Toh GW, O'Shaughnessy AM, Jimeno S, Dobbie IM, Grenon M, Maffini S, O'Rorke A, Lowndes NF. Histone H2A phosphorylation and H3 methylation are required for a novel Rad9 DSB repair function following checkpoint activation. DNA Repair (Amst) 2006;5:693–703. [PubMed: 16650810]

25. Wysocki R, Javaheri A, Allard S, Sha F, Cote J, Kron SJ. Role of Dot1-dependent histone H3 methylation in G1 and S phase DNA damage checkpoint functions of Rad9. Mol Cell Biol 2005;25:8430–8443. [PubMed: 16166626]

26. Lancelot N, Charier G, Couprie J, Duband-Goulet I, Alpha-Bazin B, Quemeneur E, Ma E, Marsolier-Kergoat MC, Ropars V, Charbonnier JB, et al. The checkpoint Saccharomyces cerevisiae Rad9 protein contains a tandem tudor domain that recognizes DNA. Nucleic acids research 2007;35:5898–5912. [PubMed: 17726056]

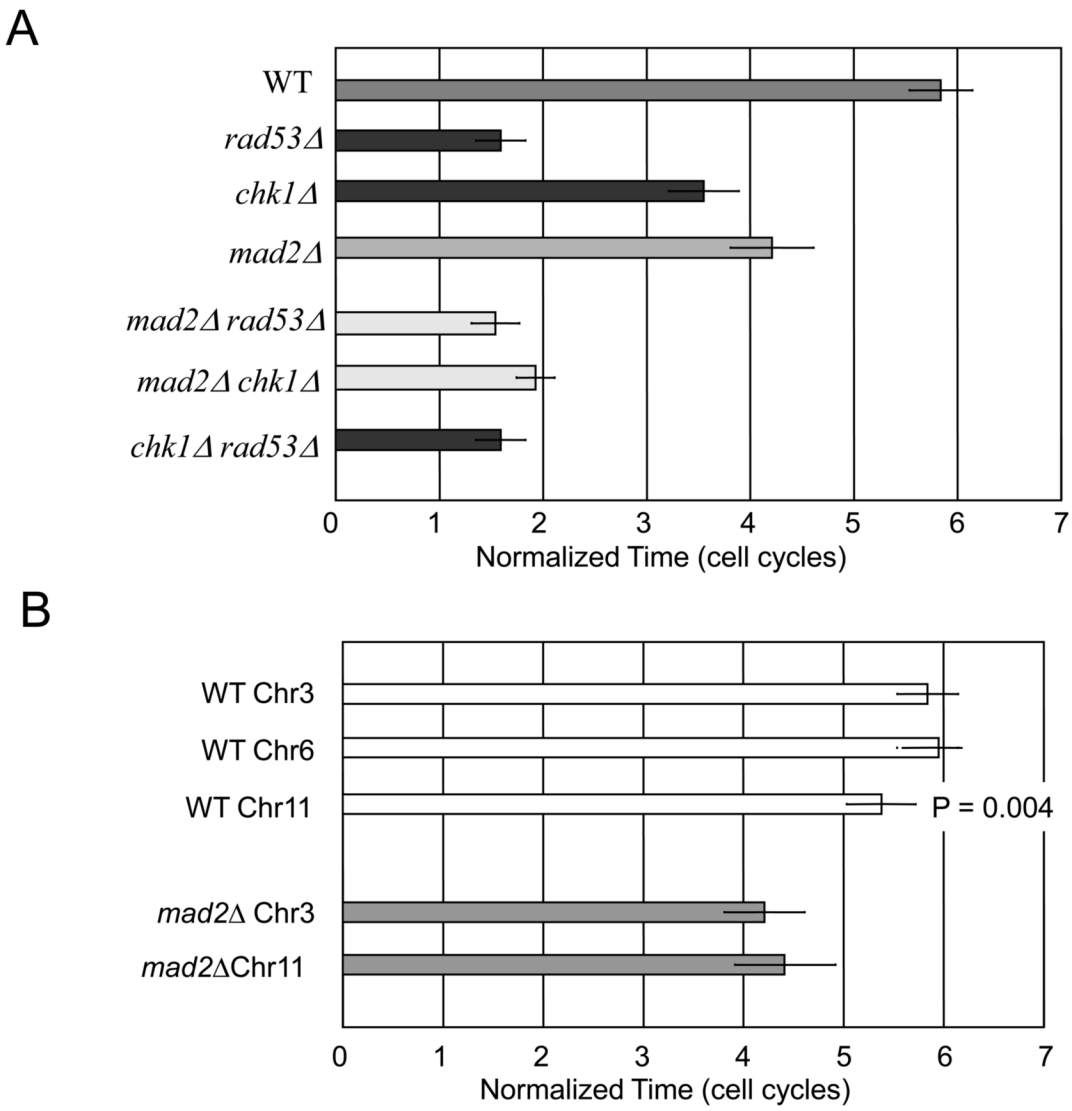

Figure 1.

*mad2*Δ shortens checkpoint-mediated cell cycle delay after induction of an unrepaired DSB. A) Induction of an irreparable DSB causes a delay in the cell cycle equivalent to 6 doubling times in wild type cells, which is fully eliminated by *mec1*Δ. *mad2*Δ reduces arrest in *chk1Δ* and is epistatic to *rad53*Δ. B) Response to a DSB in Chr6 is similar to that on Chr3, while a break on Chr11, where the DSB is 200 kb further from its centromere, provokes a significantly shorter Mad2-dependent arrest.

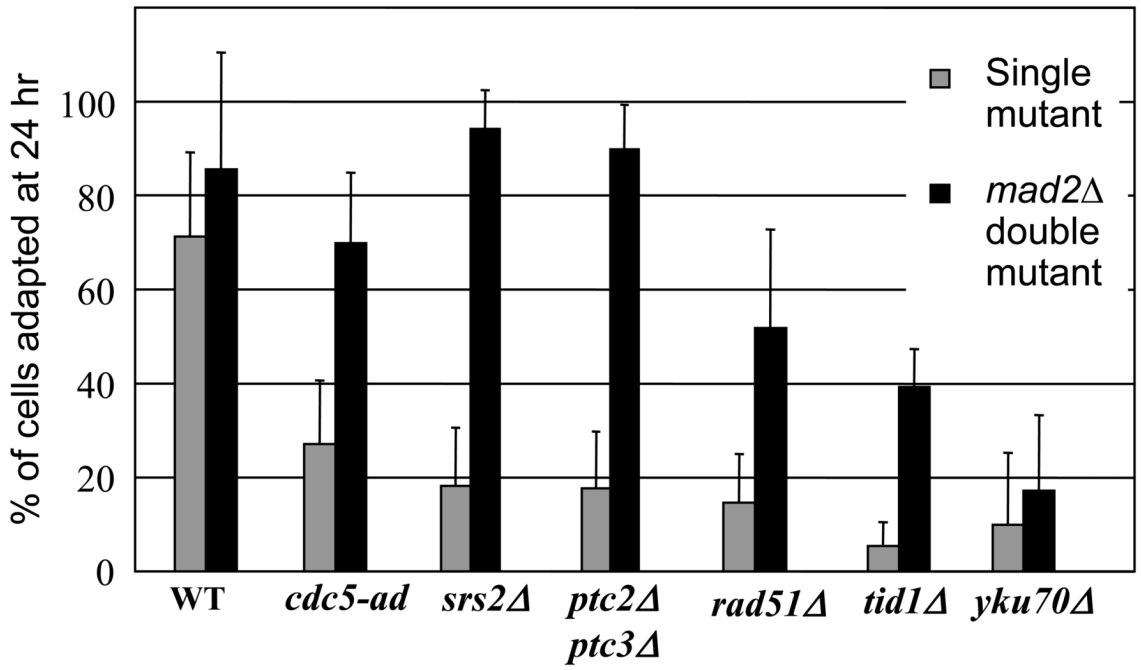

Figure 2.
Deletion of *MAD2* suppresses the permanent arrest phenotype of most adaptation-defective mutations. Unbudded (G1) cells were plated on YEP-Galactose and monitored at the times indicated.

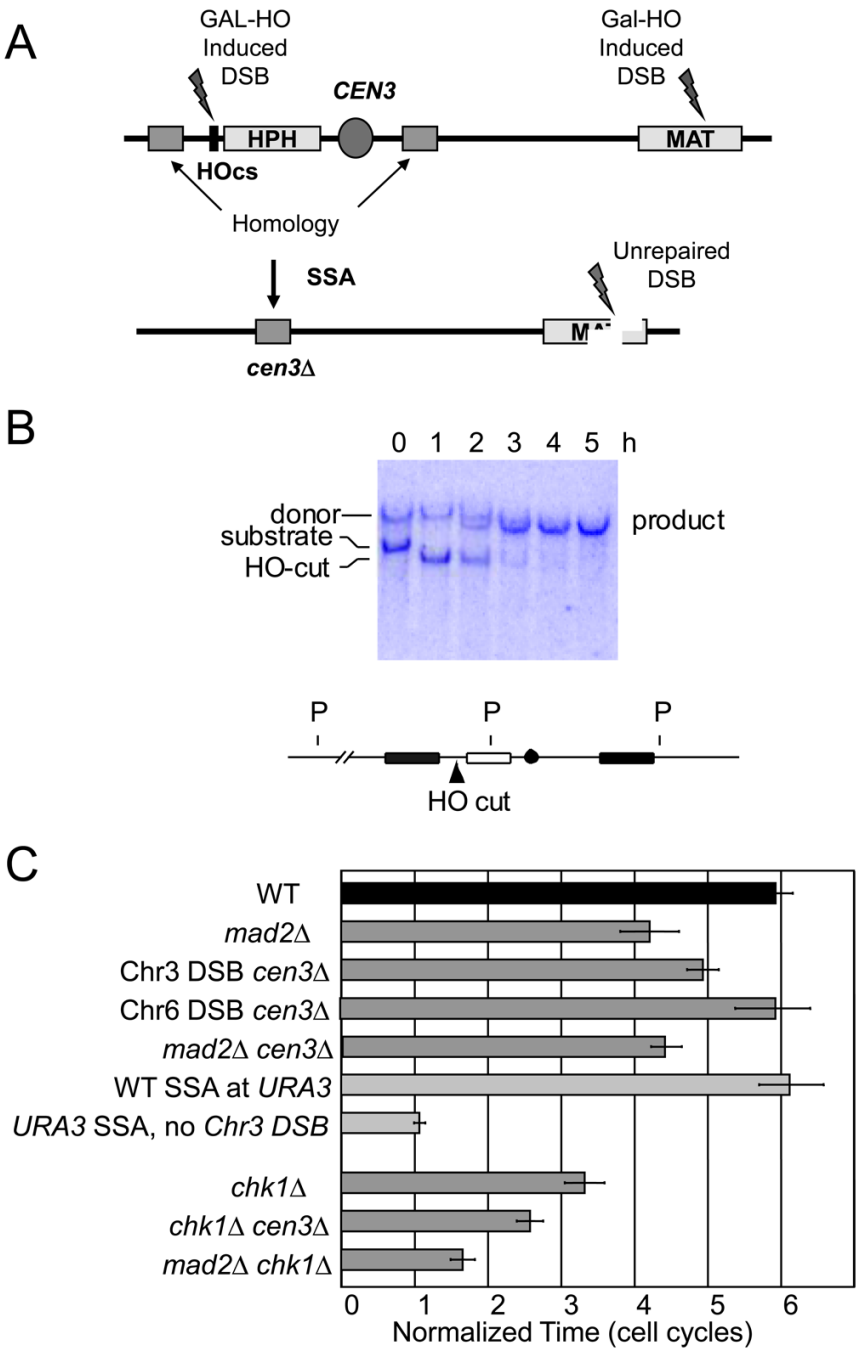

Figure 3.

Deleting the *CEN3* locus decreases cell cycle delay caused by the DNA DSB at *MAT*. A) An HO cut site was inserted to the left of *CEN3*. A 2 kb fragment from the left of the HO cutsite was inserted to the right of *CEN3*. Gal-HO induction creates DSBs at *MAT* and near *CEN3*. *CEN3* is deleted as the nearby DSB is repaired by SSA between flanking homologous DNA segments. B) CEN3 is deleted within 3 h of HO induction, as seen in a southern blot probed with the sequences (black) flanking CEN3. C) Deleting *CEN3* shortens the DNA DSB-dependent cell cycle arrest in WT cells but not in *mad2Δ* cells. An equivalent SSA event occurring at the *URA3* locus does not affect cell cycle arrest. Deleting *CEN3* does not affect

arrest when the DSB is made on another chromosome. Deleting *CEN3* shortens the checkpoint in a *chk1Δ* strain albeit not as much as deleting *MAD2*.

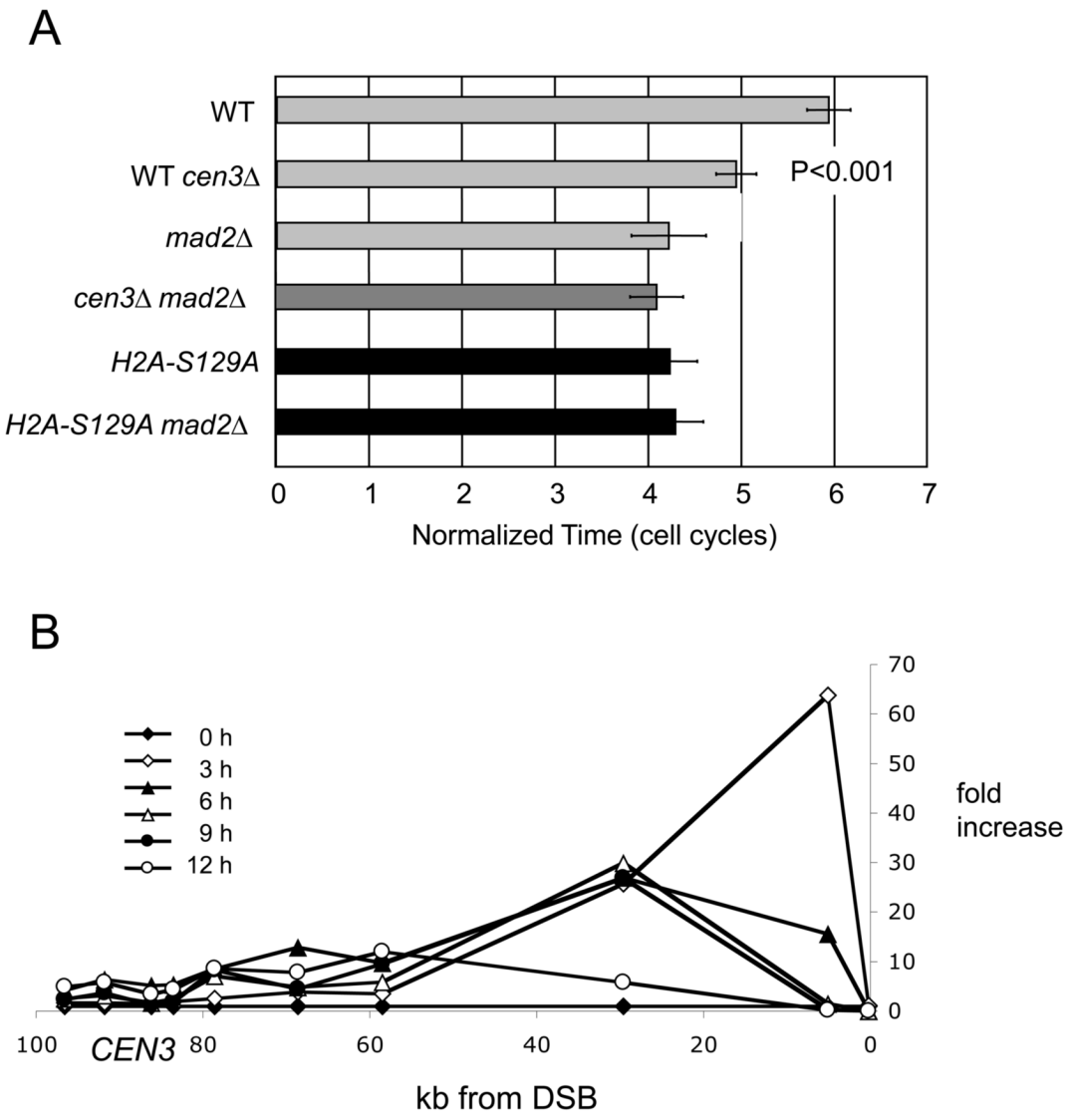

Figure 4.

γ-H2AX modification influences the persistence of the DNA damage checkpoint in a Mad2-dependent fashion. A) Mutating H2A-S129 shortens G2/M delay of a DSB at *MAT*. B) Spread of γ-H2AX leftward towards *CEN3* from a DSB at *MAT*. ChIP using an anti-γ-H2AX antibody shows the spreading of γ-H2AX down the chromosome and its progressive loss from regions near the DSB. Peak accumulation at *CEN3* of 10-fold was seen at 6 h, prior to the time cells adapt in *mad2*Δ.