Arrest, Adaptation, and Recovery following a Chromosome Double-strand Break in *Saccharomyces cerevisiae*

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DNA damage such as a double-strand break (DSB) of a chromosome causes eukaryotic cells to arrest cell cycle progression. Arrest provides a greater opportunity for cells to repair DNA damage prior to mitosis, which might cause cells to inherit chromosomes in which DNA replication was not complete or in which broken chromosome segments, lacking a centromere, would be lost (Hartwell and Weinert 1989; Elledge 1996; Weinert 1998). DNA-damage-induced arrest is enforced by a network of checkpoint proteins that detect the damage and signal both the inhibition of mitosis and the induction of damage-inducible genes through a cascade of protein kinases, including Mec1p (a homolog of the mammalian ATM/ATR checkpoint kinases), Rad53p (whose human homolog is Cds1p), and Chk1p (homologous to human Chk1p) (Sanchez et al. 1996; Longhese et al. 1998; Weinert 1998; Lowndes and Murguia 2000). How these kinases are activated in response to DNA damage is not yet well understood.

One putative DNA damage sensor involves both Rad24p and a complex of Rad17p, Mec3p, and Ddc1p (Kondo et al. 1999; Green et al. 2000). Another protein, Rad9p, is also required, independent of these other proteins (de la Torre-Ruiz et al. 1998). The ultimate targets of the damage signal cascade include Dun1p (Huang et al. 1998), a protein kinase that controls the induction of many damage-inducible genes; the Polo-like kinase Cdc5p (Shirayama et al. 1998) that regulates Cdc28p-cyclin B kinase controlling both entry and exit from mitosis; and Pds1p, an inhibitor of progression from metaphase to anaphase during mitosis (Cohen-Fix and Koshland 1999). Although much of the network of interacting proteins is conserved from yeast to mammals, damage-induced arrest in budding yeast appears to be different from damage-induced arrest in both mammalian cells and fission yeast, which block cell cycle progression at the transition from G_2 to M, primarily through regulating Cdk1–cyclin B kinase (Sanchez et al. 1999; Tinker-Kulberg and Morgan 1999).

Many studies of DNA-damage-induced checkpoint responses have used genotoxic, DNA-modifying agents including UV lights and the alkylating chemical, methylmethanesulfonate (MMS), or nucleotide depletion by hydroxyurea (HU). These treatments elicit a complicated cellular response, as they can create both single- or double-stranded DNA breaks as well as interfere with the completion of DNA replication and even RNA transcription (S. Elledge, pers. comm.). To study a defined type of DNA damage, we and other investigators have made use of the site-specific HO endonuclease to create one or more DSBs at defined chromosomal locations. Using a galactose-inducible *HO* gene, it is possible to induce a DSB and follow the response of the cell to a single DNA lesion and to follow the kinetics of DNA damage response either when the DSB can be repaired or when the break is irreparable. Studying the response to one or a few DSBs is important, as cells frequently experience DSBs arising during replication (Haber 1999).

HO endonuclease evolved to cleave the *MAT* locus and promote switching of *MAT***a** to *MAT*α, or vice versa, by homologous recombination with the *HML*α or *HMR***a** donor loci (Haber 1998). However, when these donors are deleted, or when the *RAD52* recombination gene is deleted, the cell can only repair the DSB by nonhomologous end-joining (NHEJ) (Kramer et al. 1993; Moore and Haber 1996; Lee et al. 1999). If a galactose-inducible *HO* gene is turned on for an hour and then cells are returned to glucose, about 30% of cells can accurately rejoin the 4-bp 3′-overhanging ends of the DSB, a process that depends on yKu70p, yKu80p, Lig4p, Lif1p (Xrcc4p), and the Mre11p-Rad50p-Xrs2p complex (Lee et al. 1999). If, however, *HO* is continually expressed, nearly all cells fail to form colonies, although about 0.2% create small deletions or insertions of the HO cut site, again requiring the Ku proteins and DNA ligase 4 (Lee et al. 1999). Thus, continuous HO expression creates an unrepairable DSB in nearly all cells in the population.

DSB-INDUCED CELL CYCLE ARREST: HOW IS DAMAGE DETECTED?

When G_1 cells carrying a *GAL::HO* gene are plated on galactose-containing plates, nearly all cells arrest as characteristic dumbbell-shaped cells with a single nucleus (Fig. 1A,B). A parallel fluorescence-activated cell sorting (FACS) analysis of liquid-grown cells shows that by 6 hours, essentially all cells accumulate with a 2 N DNA content (Fig. 1C). This arrest is abolished in

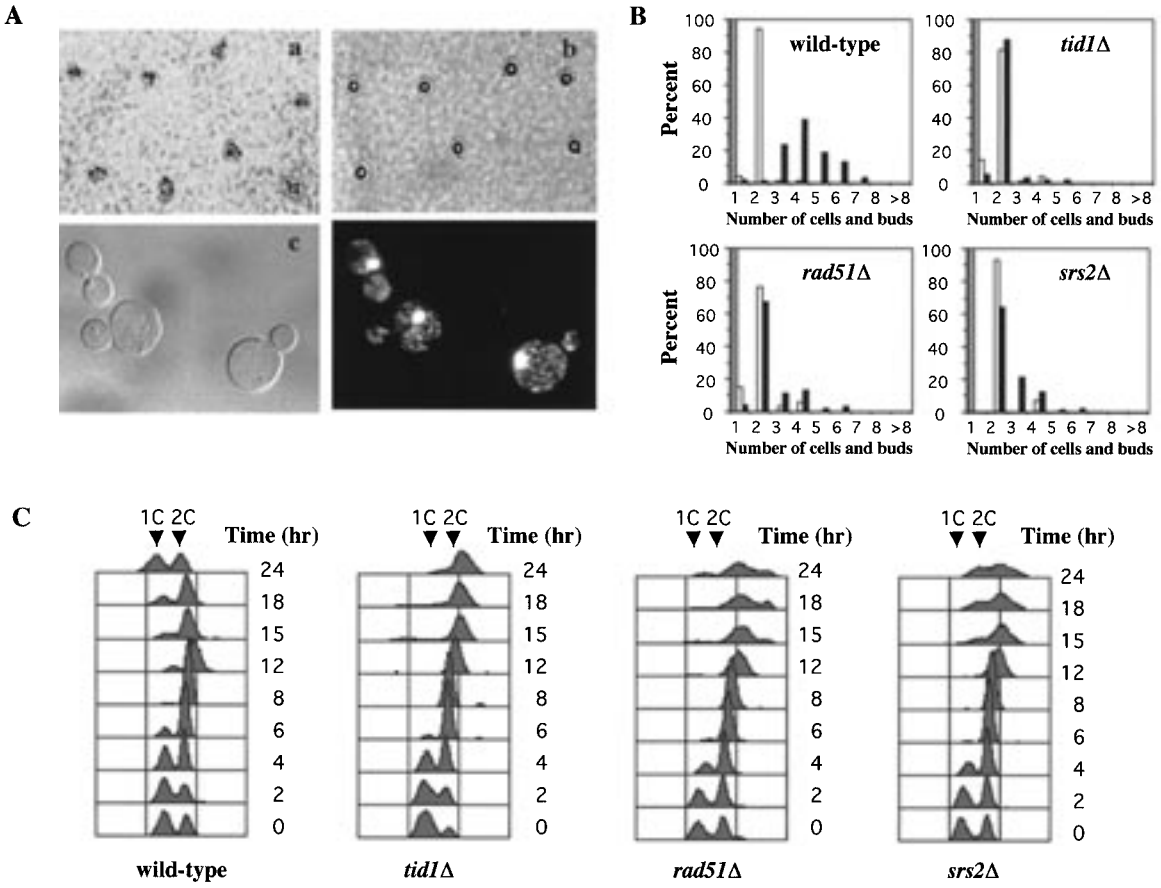

Figure 1. Arrest of cell cycle progression by a single unrepaired DSB in wild-type and mutant cells. (*A*) After 24 hr, wild-type cells initially plated as G_1 unbudded cells on galactose-containing medium to induce HO endonuclease have mostly adapted and resumed cell division, whereas an adaptation-defective *tid1*Δ strain remains arrested prior to anaphase, as shown also by DAPI staining of representative cells in the lower panel. (*B*) Arrest and adaptation of G_1 cells at 8 and 24 hr. (*C*) FACS analysis of cells induced for HO expression at $T = 0$.

strains deleted for *RAD9* or *RAD17,* although compared to isogenic cells lacking the *GAL::HO* gene, *rad9*Δ or *rad17*Δ cells still pause in the first cell cycle (Lee et al. 1998).

A molecular assessment of checkpoint response can be made by examining the phosphorylation and kinase activity of the Rad53p kinase, as described by Pellicioli et al. (1999). Figure 2C shows the kinetics of Rad53p phosphorylation and kinase activity in wild-type cells induced by a single, unrepaired DSB. An increase in Rad53p accumulation, phosphorylation, and kinase activation is first detected 1.5 hours after inducing HO expression in these logarithmically growing cells. In contrast, virtually complete HO cleavage is seen after 30 minutes (Lee et al. 1998; Holmes and Haber 1999). The kinetics of response to a lethal DSB are significantly slower than when cells are treated with a sublethal dose of 0.02% MMS (Pellicioli et al. 1999; and data not shown), where the checkpoint may be triggered by the recognition and repair of alkylated DNA, by a block in progression of replication forks, or by the creation of DSBs. These results suggest that a single DSB does not itself trigger a checkpoint response; rather, the checkpoint is activated only after the ends of the DSB have been resected by 5′ to 3′ exonucleases, to create long single-stranded DNA tails. In support of this idea, we note that when HO is induced for 1 hour in normal cells, where the DSB at *MAT* is repaired in about 1–2 hours by homologous recombination with the *HML* or *HMR* donor loci, there is no visible Rad53p kinase response (Fig. 2A), even though the Rad53p kinase is activated if HO expression in these cells is maintained, so that the *MAT* locus is continually cleaved (Fig. 2B). The failure to see a checkpoint kinase response during *MAT* switching can be explained if the ends of the DNA become involved in recombination intermediates. The fact that HO-induced DSBs on two different chromosomes can form translocations by Ku-dependent DNA end-joining argues that the DNA ends are not protected by the HO endonuclease from recognition by other DNA end-binding proteins (S.E. Lee and J.E. Haber, unpubl.).

A further indication that the checkpoint response to a single DSB is slow is that, whereas unbudded cells in G_1 uniformly arrest at G_2/M following HO induction, cells in S or G_2 may escape and only arrest in the next cell cycle, accounting for the long length of time before complete arrest is accomplished. The fact that a much shorter MMS

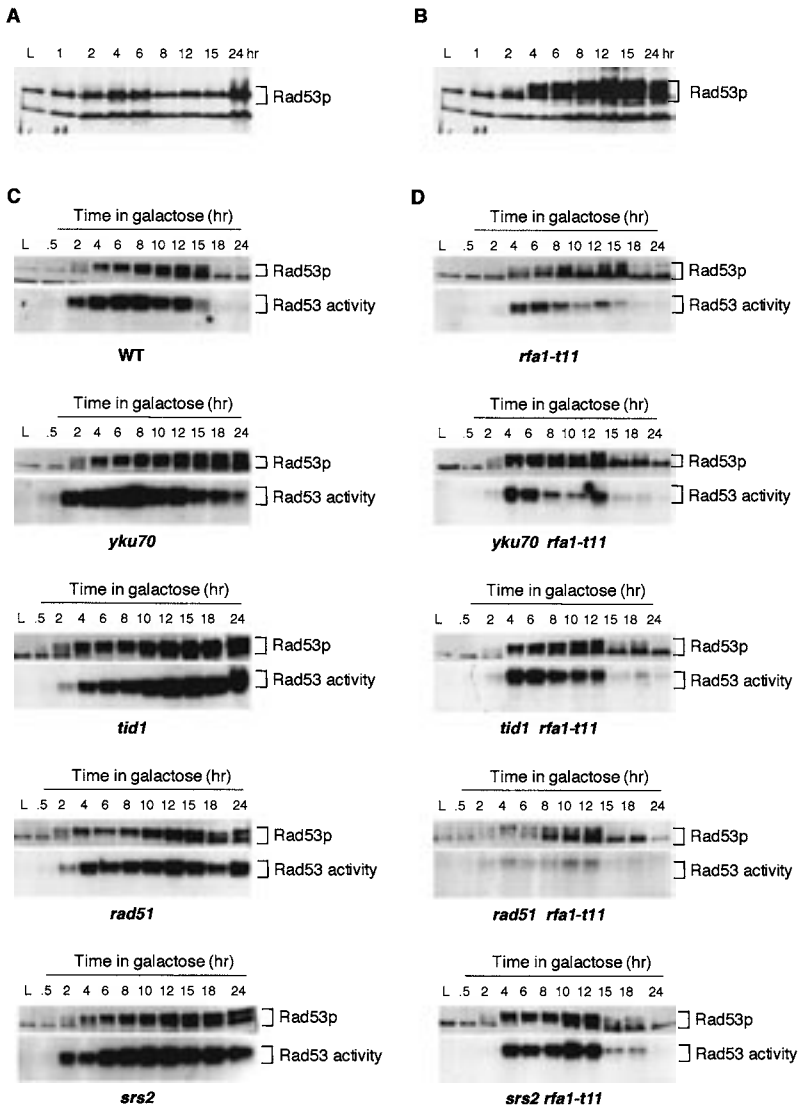

Figure 2. Phosphorylation and kinase activity of Rad53p. Western blot analysis to show slower-migrating phosphorylated forms of Rad53p are shown. (*A*) Absence of phosphorylation of Rad53p during an HO-induced *MAT* switch, which takes 1–1.5 hr to complete. Here, HO was induced for 1 hr, and recombination was allowed to proceed. (*B*) Hyperphosphorylation of Rad53p when HO is continually expressed and the *MAT* locus can recombine, but is continually cleaved. (*C*) Phosphorylation (*top*) and kinase activity (*bottom*) in response in logarithmically growing wild-type and adaptation-defective mutant cells to a single HO-induced DSB that cannot be repaired because *HML* and *HMR* donors have been deleted. (*D*) Suppression of kinase activity in *rfa1-t11* derivatives of wild-type and adaptation-defective mutant cells.

treatment causes a robust checkpoint activation is explained if the intra-S DNA replication checkpoint is much more sensitive to replication stalling than the DSB damage response is to the DSB itself.

DSB-INDUCED ARREST IS NUCLEAR AUTONOMOUS

Classic cell fusion experiments in mammalian cells suggested that the regulation of mitosis following the completion of DNA replication is dependent on signals transmitted through the cytoplasm (Rao and Johnson 1970). We have asked if DSB-induced checkpoint signaling is similarly controlled. We constructed a heterokaryon zygote by mating two cells, one that expresses both HO endonuclease and a LacI::GFP fusion protein but has no site where HO can cleave, and one that carries an array of *lacO* sites where LacI::GFP can bind and an HO-cleavable *MAT*α locus (but no donors with which the DSB can be repaired). One parent also carries the *kar1-*Δ15 mutation to prevent nuclear fusion (Fig. 3A). When HO is expressed, and the DSB cannot be repaired, in more than 93% of zygotes, the green fluorescent protein (GFP)-marked nucleus arrests with the characteristic short spindle (shown by red antitubulin staining) of pre-anaphase arrest, but the undamaged nucleus completes

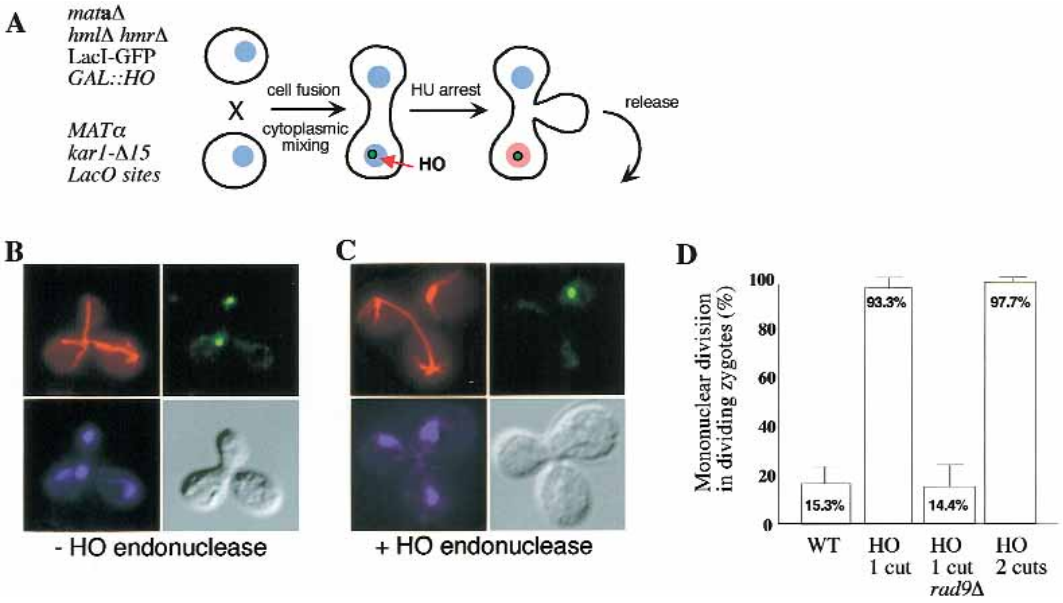

Figure 3. The DNA damage signal for mitotic arrest is nuclear-limited. (*A*) A *kar1*Δ/KAR heterokaryon zygote was created by mating cells in which one nucleus carries the *GAL::HO* gene but no HO cleavage site. The second nucleus carries *MAT*α and will be cleaved, and not repaired, once HO enters the nucleus. The damaged nucleus is marked by a green fluorescent dot by binding of LacI::GFP to a LacO array. (*B*) Both nuclei divide simultaneously when HO is not expressed. Microtubule arrays are visualized by antitubulin staining (*red*) and DNA was stained with DAPI (*purple*). (*C*) Only the undamaged nucleus divides when HO is expressed. (*D*) Proportion of simultaneous and mononuclear divisions. *rad9*Δ suppresses the effect of expressing HO. Also shown are heterokaryons in which the DNA damage signal is intensified either by deleting *YKU70* or by inducing two DSBs.

mitosis (Fig. 3C). In control zygotes, where there is no *HO* gene (Fig. 3B), or in zygotes homozygous for *rad9*Δ, both nuclei divide at the same time in approximately 85% of the cases (Fig. 3D) (Demeter et al. 2000). Because haploid cells with one DSB can adapt and resume cell cycle progression, but cells with two DSBs cannot (see below), we also repeated this experiment by inducing two DSBs. Even under these conditions, DNA damage to one nucleus failed to inhibit the undamaged nucleus in the same cytoplasm.

The zygote thus responds to the single, unrepaired DSB by causing nuclear-autonomous checkpoint-mediated arrest of mitosis. We note that this is apparently different from what has been seen in laser-damaged mammalian heterokaryons (Rieder and Cole 1998). This difference may reflect the fact that mammalian cells arrest at G_2/M, controlled predominantly through Cdk1-cyclin-mediated events, whereas *Saccharomyces* arrests in mitosis, prior to anaphase (Cohen-Fix and Koshland 1999; Sanchez et al. 1999; Tinker-Kulberg and Morgan 1999). It may also be explained by the fact that the laser damage inflicted on the mammalian cell may have damaged more than DNA or that yeast mitosis takes place without nuclear envelope breakdown. The nuclear-autonomous nature of *S. cerevisiae* arrest could be explained if the checkpoint kinases and their targets do not diffuse from the nucleus. Alternatively, the damaged nucleus may actively export a key regulator of cell cycle progression from the nucleus, as has been suggested for the DNA damage response in fission yeast (Yang et al. 1998; Lopez-Girona et al. 1999). The appearance of this regulator in the cytoplasm would prevent activation of mitosis in that nucleus, but not affect the undamaged nucleus.

DSB-INDUCED GENES DETECTED BY MICROARRAY ANALYSIS

It is well established that DNA damage elicits the induction and repression of a number of genes. Whether this induction is necessary for DSB repair is unclear. Indeed, the fact that a normal HO-induced gene conversion at *MAT* failed to elicit an activation of Rad53p may indicate that efficient repair per se is not dependent on elevated transcription of damage-inducible genes. Nevertheless, it is interesting to determine how many genes are induced in response to a single, unrepaired DSB, in comparison, for example, with genes induced by UV or by alkylating agents. Of course, many genes change their level of expression as a function of the cell cycle, and damaged cells arrest at one point in this cycle. To avoid studying this potentially large family of genes, we first arrested yeast cells at G_2/M by treatment with the microtubule inhibitor nocodazole and only then induced expression of the *HO* gene, to create a single unrepaired DSB (Fig. 4). Many of the genes affected are related to carbon metabolism, likely a direct effect of adding galactose to the medium to induce *HO*. An interesting subset of genes whose transcripts decrease in abundance provides a useful demonstration of the sensitivity of the method. Over several hours, the mRNA of genes surrounding the

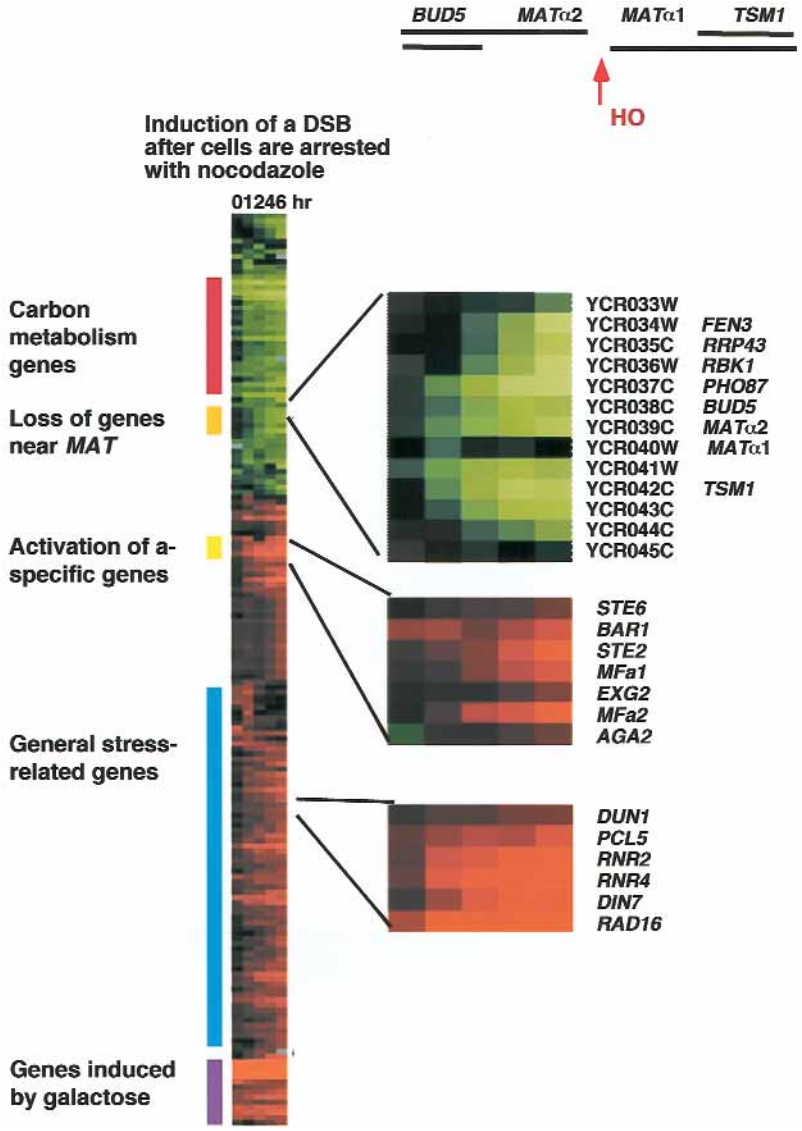

Figure 4. Microarray analysis of genes whose mRNA abundance decreases (*green*) or increases (*red*) in response to the creation of a single unrepaired DSB. Here, cells were first arrested with nocodazole before *GAL::HO* induction by the addition of galactose to the medium so that genes whose expression changes as cells accumulate at one point in the cell cycle are not observed. Creation of a DSB at *MAT* results in the progressive loss of mRNAs of genes near *MAT* as the ends of the DSB are resected at about 4 kb/hr. Consequently, a set of **a**-specific genes, normally repressed by Matα2p, are induced. Only a small number of genes involved in DNA metabolism or repair are induced.

site of HO cleavage at *MAT* (beginning with *MATα1*, *MATα2*, *BUD5*, and *TSM1*) all decrease, as the ends of the DSB are resected by 5′ to 3′ exonuclease and these genes can no longer be transcribed. Moreover, because Matα2p is no longer transcribed, the **a**-specific genes normally repressed by Matα2p-Mcm1p now become expressed, including *STE3*, *STE6*, and *MF***a**.

Among the induced genes are surprisingly few known to be involved in DNA repair. None of the *RAD* genes involved in recombination are induced even threefold. However, when these data were compared to genomic expression programs triggered by other stresses (A.P. Gasch et al., in prep.), a set of genes specifically induced by HO endonuclease cleavage and related to DNA damage responses emerged (Fig. 4). Among these genes are the DNA-damage-inducible ribonucleotide reductase subunits (*RNR2* and *RNR4*), the DNA-damage-inducible gene *DIN7*, the *PCL5* cyclin, a gene affecting plasmid segregation (*PKM2*), and the *DUN1* checkpoint kinase. In addition to these genes that may be directly related to DNA damage repair, a single DSB generated by HO endonuclease also triggers expression changes in hundreds of genes implicated in a general response to various environmental stresses (A.P. Gasch et al., in prep.). The complete data set may be seen at http://www-genome.stanford.edu/ho.

When the *HO* gene expression data were compared to a previous study of the global expression response to

MMS treatment (Jelinsky and Samson 1999), there was some overlap in the gene expression patterns, as well as differences between the genomic expression responses to HO endonuclease induction versus MMS treatment. Among the similarities was the induction of a few genes implicated in DNA damage responses, discussed above, as well as genes implicated in a general stress response. Much of the difference between the genomic expression responses can be rationalized by the fact that MMS initiates S-phase arrest in response to DNA damage, as opposed to G_2/M arrest triggered by HO endonuclease expression; furthermore, MMS likely inflicts pleiotropic damage in the cell, through protein methylation and oxidative stress. Moreover, the MMS study was performed on asynchronous cells, as opposed to the G_2/M-arrested cells in this study, adding to the differences between the experiments.

The ability to create a single DSB will now allow us to ask a number of important questions. For example, would prior treatment of cells with a DNA-damaging agent (or a different, unrepaired single DSB) cause a change in the kinetics, efficiency, or outcome of a subsequent HO-induced event that can be repaired?

ADAPTATION IS AN ADVANTAGEOUS STRATEGY

A key discovery concerning the DNA damage checkpoint was that damage-induced cell cycle arrest is not permanent: Cells can *adapt* even though a broken chromosome is still present (Sandell and Zakian 1993; Toczyski et al. 1997; Lee et al. 1998). For example, when cells suffer a single HO-induced DSB that cannot be repaired by homologous recombination (and where nonhomologous end-joining rescues fewer than 1%), they remain arrested for 8–12 hours; but by 24 hours, nearly all cells have resumed cell division (Lee et al. 1998). There is no obvious delay at the next mitosis (Sandell and Zakian 1993; Lee et al. 1998).

It is important to note that adaptation is not simply the futile flailing of a cell that will inevitably die as essential genes are lost, as the broken chromosomal DNA is degraded. For example, if an HO-induced DSB is made on one chromosome in a disomic haploid strain deleted for *RAD52*, the broken chromosome is eventually lost, but the cell is never at risk of death (Sandell and Zakian 1993). Two mutations that prevent adaptation, *yku70*Δ and *cdec5-ad*, have the same effect on both haploid and disomic cells experiencing a single unrepaired DSB (Toczyski et al. 1997; Lee et al. 1998; also see below).

For *Saccharomyces*, adaptation is in fact a valuable strategy to improve survival even in haploid cells suffering a DSB in G_1. First, although an acentric broken chromosome arm will not segregate properly in mitosis (which takes place without nuclear envelope breakdown), one of the two daughter nuclei is likely to inherit both replicated copies of the acentric piece, along with the centromere-attached broken chromosome arm. Hence, some repair events such as NHEJ or break-induced replication might be possible one or more generations later (Kramer et al. 1993; Malkova et al. 1996). Second, some of these repair events may occur more efficiently in the G_1 or S phase of the cell cycle than in mitotically arrested cells.

An example of this advantage is shown by studying the survival of a *rad52*Δ strain containing a single DSB at *leu2* on chromosome III. In *rad52*Δ cells that arrest but then adapt, about 2% survive, apparently by a combination of NHEJ events and *RAD52*-independent single-strand annealing. However, in an isogenic *rad52*Δ *cdc5-ad* strain, which is prevented from undergoing adaptation, cell survival drops to 0.3%. Thus, the ability to adapt and proliferate provides cells with an improved chance of survival.

ADAPTATION IS VERY SENSITIVE TO THE EXTENT OF SINGLE-STRANDED DNA

A key discovery concerning adaptation is that it is very sensitive to the extent of DNA damage. A cell with a single HO-induced DSB will adapt, but a cell that suffers two DSBs, on different chromosomes, remains permanently arrested (Lee et al. 1998). However, the cell is apparently not responding to the number of DSBs but to the total amount of single-stranded DNA produced by 5′ to 3′ resection of DSB ends. This was shown by analyzing *yku70*Δ cells with a single DSB, in which 5′ to 3′ resection is twice as fast as in wild-type cells. These mutant cells become permanently arrested with only a single DSB (Lee et al. 1998). Moreover, the permanent arrest of wild-type cells with two DSBs and *yku70*Δ cells with one DSB is suppressed by *rad50*Δ or *mre11*Δ mutations that reduce the rate of resection (Lee et al. 1998). Permanent arrest is also suppressed by deletions of either the *RAD9* or *RAD17* checkpoint genes (Toczyski et al. 1997; Lee et al. 1998).

The idea that the cell assesses the extent of DNA damage by monitoring single-stranded DNA (ssDNA) produced by resection of the DSBs was given strong support by our finding that a recombination-defective *rfa1-t11* (Rfa1-L45E) mutation in the largest subunit of yeast single-strand binding protein complex, RPA, can suppress the permanent arrest phenotype of both wild-type and *yku70*Δ cells (Lee et al. 1998). *rfa1-t11* cells still show a significant mitotic arrest in response to a single DSB in otherwise wild-type cells, but adaptation is more rapid than in $Rfa1^+$ cells.

THREE NEW MUTATIONS THAT IMPAIR OR PREVENT ADAPTATION

The idea that cells monitor the extent of ssDNA to decide whether to adapt is attractive, but in its simplest form, it fails to address an important concern. If 5′ to 3′ resection proceeds at a constant rate and the 3′-ended ssDNA tails are much more slowly degraded over many hours, there should be a continuously increasing amount of ssDNA. Hence, the assessment of how much damage the cell had suffered might need to change with time. However, RPA is not the only protein that binds to ssDNA. The DNA strand-exchange protein Rad51p forms filaments on ssDNA, and it is believed that such filament

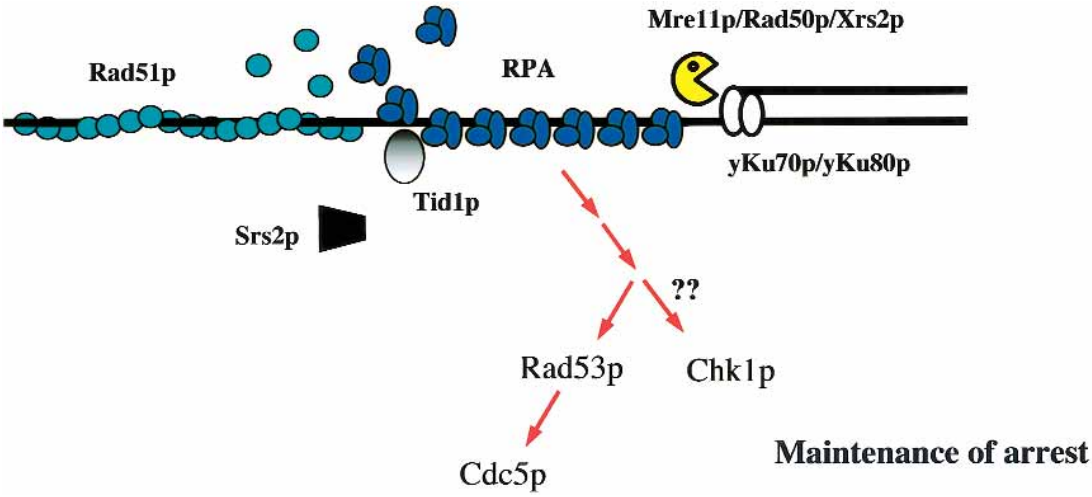

Figure 5. Model for DNA-damage-induced arrest and adaptation. A single DSB by itself does not signal checkpoint-mediated arrest, rather, after 5′ to 3′ exonuclease creates ssDNA. RPA binds to ssDNA but is later displaced by Rad51p and possibly other proteins (not shown). Checkpoint proteins may also contact ssDNA or RPA in the establishment of pre-anaphase arrest. Establishment and maintenance may be mechanistically different. The amount of RPA bound serves as a measure of the extent of DNA damage, such that two such regions (or one region resected twice as fast) doubles the amount of RPA bound. The Srs2p helicase and Tid1p (a Swi2/Snf2 homolog) both have key roles in this process, possibly in mediating the destabilization of RPA binding or in facilitating Rad51p binding, which may not be identical to the fashion in which Rad51p binds when it forms filaments to promote recombination.

formation must occur in order to facilitate a search for homologous sequences to engage in recombinational repair of the DSB (Ogawa et al. 1993; Sung and Robberson 1995; Petukhova et al. 1998; Zaitseva et al. 1999). Moreover, studies of Rad51p (or its bacterial counterpart RecA) loading onto DNA suggest that these strand-exchange proteins displace RPA from ssDNA (New et al. 1998; Shinohara and Ogawa 1998; Kowalczykowski 2000; Song and Sung 2000). Thus, much of the ssDNA might be coated with Rad51p and perhaps only a relatively small segment, which newly generated close to the exonuclease, might be covered with RPA. This could provide a constant "window" where RPA binding to newly generated ssDNA could reflect the rate of resection or the number of regions being resected (Fig. 5).

If Rad51p were absent, we would expect more RPA to be bound to the resected DNA and thus the cell would perceive apparently more damage. We therefore tested a *rad51*Δ derivative and found adaptation was impaired in 70–80% of the cells, although not as profoundly as *yku70*Δ (Fig. 6A). Both in vitro biochemical studies (Hays et al. 1998) and immunocytological studies in meiotic yeast cells (Gasior et al. 1998) have supported the idea that a set of other recombination proteins, including Rad52p, Rad54p, Rad55p, and Rad57p, all interact physically with Rad51p and facilitate the loading of Rad51p onto DNA. In homologous recombination assays in vivo, Rad52p is at least as necessary as Rad51p (Song and Sung 2000). Surprisingly, a *rad52*Δ strain adapted indistinguishably from a wild-type strain. There is a weak effect in the absence of Rad54p, but no effect in the absence of Rad55p. A *rad59*Δ mutation that appears to eliminate a *RAD51*-independent, but *RAD52*-dependent, recombination pathway (Bai et al. 1999; Sugawara et al. 2000) also has no effect.

Rad51p also interacts weakly with a homolog of Rad54p, termed Tid1p (Rdh54p), although this protein interacts more strongly with the meiosis-specific Rad51p homolog, Dmc1p (Dresser et al. 1997; Shinohara et al. 1997). Surprisingly, a *tid1*Δ deletion, which has only a minor role in mitotic recombination (Klein 1997; Shinohara et al. 1997; Arbel et al. 1999), has a dramatic effect on adaptation. A *tid1*Δ strain is as profoundly impaired as *yku70*Δ (Fig. 6A).

Rad54p, Tid1p, and the helicase Srs2p have apparently overlapping functions. A *srs2*Δ *tid1*Δ double mutant is synthetically lethal in diploids (Klein 1997), possibly because yet another redundant gene is turned off by heterozygous mating-type alleles. We therefore tested *srs2*Δ for adaptation after a single DSB. We found that *srs2*Δ was very similar to *rad51*Δ in causing 75% of the population to remain permanently arrested at the first mitosis and the remaining cells to remain arrested for 24 hours at the second division. In contrast, deleting another helicase involved in some recombination events, *SGS1*, had no effect on adaptation. A *rad51*Δ *srs2*Δ double mutant was no more defective than either single mutant (data not shown). Both *tid1*Δ *rad51*Δ and *tid1*Δ *srs2*Δ had the more severe defect seen with *tid1*Δ. *tid1*Δ *yku70*Δ was indistinguishable from either single mutant (data not shown). Thus, three proteins involved in homologous recombination are also involved in the adaptation response.

The permanent arrest phenotype of these mutants is only seen when the DNA damage checkpoint is active, because *rad9*Δ derivatives of these mutants, as with *yku70*Δ, prevent arrest and the double mutants proliferate similarly to wild-type cells (Fig. 6C). However, the set of proteins implicated in adaptation are very different from what would be expected if they were participating in a normal homologous recombination process. In our

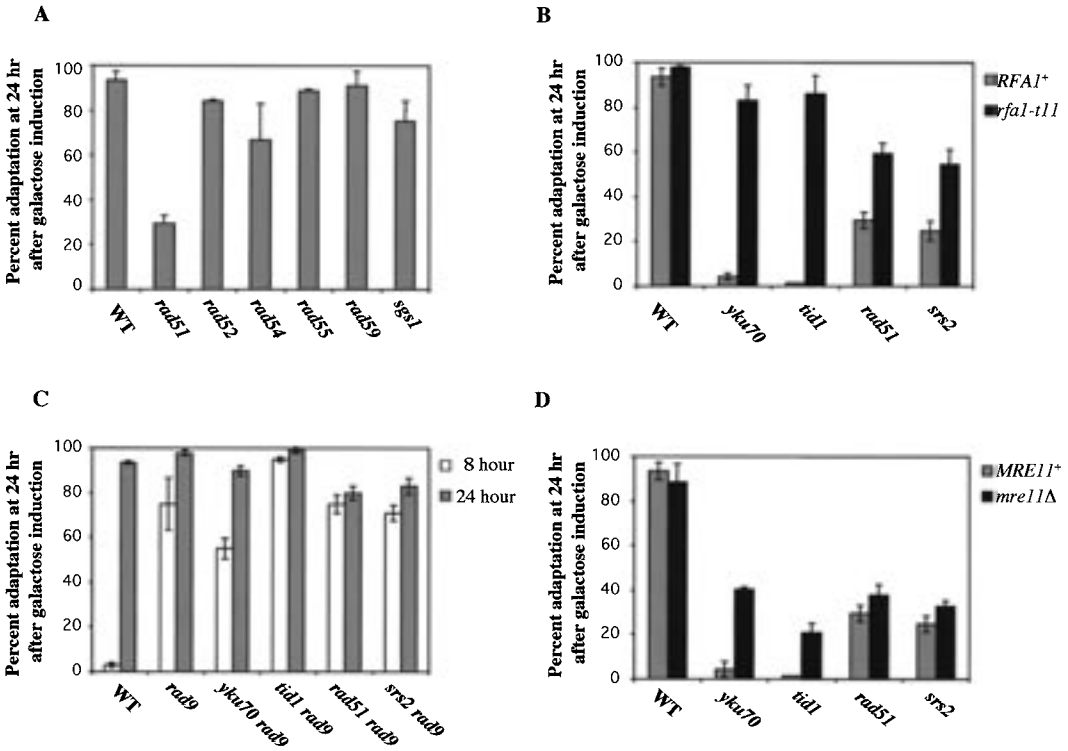

Figure 6. Adaptation of wild-type and mutant cells. (*A*) Cells progressing beyond the two cell + bud stage at 24 hr after HO-induction are scored as having adapted. (*B*) Effect of *rfa1-t11* on the adaptation of wild-type and mutant cells at 24 hr. (*C*) Effect of *rad9*Δ on the arrest and adaptation of wild-type and mutant cells at 8 hr and 24 hr. (*D*) Effect of *mre11*Δ on adaptation on wild-type and mutant cells at 24 hr.

strains, HO-induced gene conversions between homologous chromosomes show at best minor perturbations when Srs2p and Tid1p, Sgs1p, or Rad59p are deleted (L. Signon, A. Malkova, M. Naylor, and J.E. Haber, in prep.). In this same system, *rad51*Δ, *rad54*Δ, *rad55*Δ, and *rad57*Δ each cannot complete gene conversion but still carry out break-induced replication (White and Haber 1990; Ray et al. 1991; Firmenich et al. 1995; Johnson and Symington 1995; Sugawara et al. 1995; Malkova et al. 1996). *rad52*Δ eliminates essentially all recombination. Recombination is also not defective in *yku70*Δ strains (Milne et al. 1996).

EFFECTS OF ADAPTATION-DEFECTIVE MUTANTS ON THE 5′ TO 3′ RESECTION OF DNA

We have previously shown that a *yku70*Δ mutation causes a twofold increase in the rate of 5′ to 3′ resection of DNA ends, possibly by failing to compete with the exonucleases for the junction between single- and double-stranded DNAs (Lee et al. 1998). We confirmed that observation here, but found that none of the new adaptation-defective mutations have any effect on 5′ to 3′ resection (data not shown). This argues that their role is distinctly different from the rather indirect effect of Ku in creating more ssDNA from a single DSB.

Slowing down the rate of resection of a *yku70*Δ strain by a *mre11*Δ mutation partially reversed the permanent arrest phenotype of *yku70*Δ (Lee et al. 1998). We also found that *mre11*Δ partially suppressed the *tid1*Δ mutant but did not affect either *rad51*Δ or *srs2*Δ (Fig. 6D).

ADAPTATION APPEARS TO INVOLVE TURNING OFF THE CHECKPOINT KINASE CASCADE

In an adaptation-competent cell, Rad53p kinase activity and Rad53p phosphorylation decrease between 8 and 12 hours, when most cells have resumed cell cycle progression (see Fig. 2C). This suggests that the checkpoint kinase cascade is turned off at the time of adaptation, consistent with a suggestion by Sanchez et al. (1999). In contrast, all of the adaptation-defective mutations have a profound effect on the phosphorylation and kinase activity of Rad53p (Fig. 2C). In each case, Rad53p remains activated for at least 24 hours, although in *yku70*Δ, kinase activity declines over time. The persistent activation of the checkpoint in these several mutants further argues that the stimulus of the checkpoint kinase—broken and resected DNA—persists in the cell much longer than 8–12 hours. We note that at a measured rate of resection of 4 kb/hr, only about 100 kb would be removed from each end of the DSB in 24 hours, so that there are still broken

fragments of even a relatively small 315-kb chromosome III after this time. Southern blots have confirmed that sequences near the ends of the broken chromosome are still present as double-stranded DNA at 24 hours in a *rad52*Δ strain (Lee et al. 1998).

RFA1-t11 SUPPRESSES THE PERMANENT ARREST PHENOTYPES OF *TID1*Δ, *RAD51*Δ, AND *SRS2*Δ

We have suggested that RPA plays a central part in monitoring the extent of ssDNA. As noted above, *rfa1-t11* suppresses the permanent arrest phenotype of the *yku70*Δ strain with a single DSB (Lee et al. 1998). Here, we show that this mutation also suppresses the permanent arrest of *tid1*Δ, *rad51*Δ, and *srs2*Δ mutations (Fig. 6B). In each case, the presence of the *rfa1-t11* mutation causes a significant reduction in the intensity of Rad53p kinase phosphorylation and kinase activity (Fig. 2D).

WHAT IS THE RELATION OF ADAPTATION TO RECOVERY?

We now turn to the question of how cells resume cell cycle progression after sitting for a long time in an arrested state, i.e., what is the relationship between *adaptation*, when the DSB is not repaired, and *recovery*, when repair is completed? The adaptation-defective *cdc5-ad* mutation is an altered function mutation in an essential Polo-like kinase that controls several aspects of mitosis (Toczyski et al. 1997). We wished to explore whether this mutant is defective in monitoring the extent of DNA damage or is somehow unable to resume mitosis even after the checkpoint kinase cascade is turned off. As noted before, we could not simply use cells in which HO induces a normal switching of the *MAT* locus, as this occurs without activating Rad53p checkpoint kinase. We therefore devised a system in which a single DSB would persist for 6 hours, long enough for cells to become arrested in mitosis by the checkpoint proteins, but then be efficiently repaired. The kinetics of single-strand annealing can be regulated by moving one of the recombining sequences flanking the DSB further away from the site of the DSB (Fig. 7). On the basis of a resection rate of 4 kb/hr, we reasoned that if a 1-kb segment of DNA were placed 25 kb from the DSB, it should take 6 hours for repair to begin. Indeed this is the case (Fig. 7); 85% of the cells survive (again arguing that the 3′ end near the DSB has remained undegraded for 6 hours).

We then asked if a *cdc5-ad* derivative of this strain would be able to recover. We first confirmed that the *cdc5-ad* mutation does indeed prevent adaptation in a *rad52*Δ derivative of our strain, when there is no repair of the DSB and cells normally adapt (data not shown). However, *cdc5-ad* does not prevent recovery in the 6-hour annealing assay described above. Thus, the process of recovery and the process of adaptation from a single DSB are clearly separate.

CONCLUSIONS

The ability to create a single controlled DSB with an inducible endonuclease provides a powerful approach to the analysis of checkpoint responses to DNA damage. One fundamentally important finding of our work is that a single DSB does not rapidly induce a checkpoint response. Whereas MMS treatment induces a very rapid activation of checkpoint kinase, the presence of one unrepaired DSB only causes Rad53p activation after more than 1 hour. Moreover, it appears that the kinetics of ssDNA formation also correlates well with the kinetics, but not the magnitude, of Rad53p kinase activation. Thus, the kinetics of appearance both of the phosphorylated form of Rad53p and kinase activity is more rapid in a *yku70*Δ

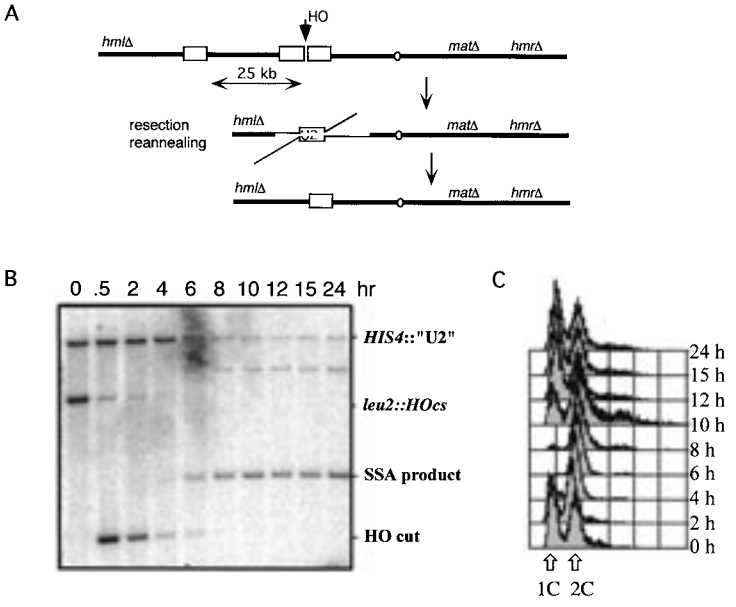

Figure 7. Analysis of recovery from a DSB after cells have been arrested by the DNA damage checkpoint. (*A*) A 6-hr single-strand annealing event produces a deletion. This process is initiated by HO cleavage and is limited by the slow rate of 5′ to 3′ exonuclease activity. (*B*) Repair of the DSB by the formation of a viable deletion begins at 6 hr, at which time cells have arrested with a 2C DNA content (*C*).

strain with one DSB than in the wild-type strain, although the magnitude of the response is not changed (Fig. 2). It will be interesting to see if activation of Rad53p is even slower in *mre11*Δ or *rad50*Δ mutants that slow down 5′ to 3′ resection of DSB ends.

The failure to induce Rad53p during the process of a programmed recombinational DSB repair event such as *MAT* switching could be explained if the DNA ends become engaged in recombination intermediates very rapidly and are sequestered from the signaling apparatus; but in fact, there is strong evidence that one of the two ends of the cleaved *MAT* DNA, containing the Y**a** or Yα sequences that are replaced by opposite mating-type information, is not involved in strand invasion (White and Haber 1990; Holmes and Haber 1999). Moreover, these nonhomologous regions are not excised until the very end of the switching process (White and Haber 1990).

The rapid response of cells to MMS or UV treatment (Pellicioli et al. 1999) seems understandable: Cells need to know very quickly if one or more replication forks have been impeded. But it may not be so critical for a yeast cell to respond immediately to an unrepaired DSB. First, there is increasing evidence that most of the DSBs encountered by eukaryotic cells are created during the process of replication (Haber 1999) and that virtually all of these are very efficiently repaired by recombination with a sister chromatid that is very close by (Arbel et al. 1999). Cells apparently have sufficient time to repair these breaks without ever activating the checkpoint proteins. Thus, Rad53p is not activated during normal replication (Pellicioli et al. 1999; Sanchez et al. 1999).

What a cell *does* need to know is when a DSB is unlikely to be repaired in a timely fashion. Thus, the cell does not respond to the DSB per se but only after a substantial amount of ssDNA has been generated. We note also that even if a cell were to complete mitosis before DSB repair, all is not lost. Yeast tolerate substantial aneuploidy and the loss of a chromosome in a diploid is generally not fatal. For a haploid, survival depends on inheriting all essential genes. But this does not mean that cells inevitably die if mitosis takes place prior to DSB repair. One of two daughter cells is likely to retain perhaps both replicated copies of an acentric chromosome fragment that could still be re-joined to the centromere-containing end. Moreover, some DNA repair processes are apparently considerably more efficient in G_1, S, or G_2 than when broken chromosomes are found in condensed chromosomes prior to anaphase. This could be the case for break-induced replication that can repair chromosomes whose telomeres have been shortened or lost, and it appears also to be the case for single-strand annealing and/or NHEJ (Malkova et al. 1996; Moore and Haber 1996; Diede and Gottschling 1999). It is also possible that when the checkpoint kinase cascade is turned off during adaptation, the cell may use different mechanisms of DNA repair than when the checkpoint is engaged. We further note that whereas *chk1*Δ cells fail to arrest after DNA damage, there is little decrease in viability compared to *rad53*Δ cells (Sanchez et al. 1999). It is also possible that when the entire checkpoint kinase cascade is turned off, the cell may use different mechanisms of DNA repair than when the checkpoint is engaged. Adaptation is therefore a well-evolved strategy of first giving the cell enough time to repair a DSB by means available in mitotically arrested conditions but then permitting additional pathways of repair in later cell cycles.

Yeast are exquisitely sensitive to the extent of DNA damage. With one DSB, they adapt, but with two DSBs—or one DSB resected twice as fast—they do not (Lee et al. 1998). At this point, we can provide no rationale for why "too much" damage discourages adaptation, nor do we really know how adaptation is triggered. One model would be that there is a key protein that is required to maintain arrest and that this protein is synthesized at a lower rate (or is turned over at a higher rate) in mitotically arrested cells, such that when it falls below a certain level, cells are released from their arrest. In this conception, there must also be a continuing signal to prevent reactivation of the checkpoint as cells progress through the next cell cycle.

We believe RPA is the key monitor of how much DNA damage there is, by binding to newly forming ssDNA. However, the single-stranded binding protein will be displaced by other DNA-binding proteins, most notably the *RAD51* strand-exchange protein, which forms a filament on ssDNA (Ogawa et al. 1993; Sung and Robberson 1995). This will create a constant "window" of RPA-bound ssDNA that will serve as a measure of how much damage the cell has experienced (see Fig. 5). It has been suggested that replacement of yeast RPA by Rad51p depends on a number of auxiliary proteins, but as of yet, we do not know what proteins such as Rad52p, Rad54p, Rad55p, and Rad57p actually do, or if they are in fact all needed prior to Rad51p filament formation in vivo.

In support of this idea, we find that the absence of Rad51p prevents a large fraction of cells from adapting to a single DSB. This would increase the extent of RPA bound to ssDNA and create a signal of greater DNA damage. Srs2p is a 3′ to 5′ helicase that apparently helps stabilize early steps in strand invasion (Pâques and Haber 1997), but beyond that we do not know how it works. One possibility is that the helicase is one of several proteins involved in the replacement of RPA by Rad51p. As we noted above, there is *RAD51*-independent, but *RAD52*-dependent, homologous recombination processes (break-induced replication) (Malkova et al. 1996) that may very well involve another ssDNA-binding protein. The fact that *rad51*Δ and *srs2*Δ show a slower onset of permanent arrest (i.e., some cells progress through one more cell cycle) than *yku70*Δ or *tid1*Δ may mean that there are still other ssDNA-binding proteins that could displace RPA.

The proteins involved in adaptation are an unexpected subset of those involved in homologous recombination. Most striking is the important role of Tid1p. Tid1p has a very important role in meiotic recombination, where it interacts with a meiosis-specific Rad51p homolog, Dmc1p (Dresser et al. 1997; Klein 1997; Shinohara et al. 1997; Arbel et al. 1999). In mitotic recombination, *tid1*Δ has a relatively minor phenotype, with its greatest defects only when Rad54p is missing (Klein 1997; Shinohara et al. 1997; Ar-

bel et al. 1999). Yet in DSB damage-sensing, *tid1*Δ is the most severe of all the adaptation mutants we have tested. Tid1p, like Rad54p, is a member of the Swi2p/Snf2p family of DNA helicase-like, DNA-binding proteins whose avatar is involved in chromatin remodeling (Peterson 1998). Thus, Tid1p may also be involved in the displacement and binding of RPA, Rad51p, or other proteins onto DNA. The important role of Tid1p in DNA damage monitoring helps explain the observation that mRNA levels for *TID1* are much higher in mitotic cells than in meiotic cells (Klein 1997), despite its obviously key role in meiotic recombination in conjunction with Dmc1p.

Perhaps the most important unanswered questions are: With what does RPA interact to provide information about the extent of ssDNA and how is this information transmitted to turn off the checkpoint? RPA is known to interact with Rad52p, but this does not seem to be the interaction important for adaptation, as *rad52*Δ does not suppress *yku70*Δ or *tid1*Δ mutations. The ability to combine precise DNA damage with genetic and biochemical approaches should help to answer these questions.

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