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Limiting amounts of budding yeast Rad53 S-phase checkpoint activity results in increased resistance to DNA alkylation damage

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ABSTRACT

The *Saccharomyces cerevisiae* protein kinase Rad53 plays a key role in maintaining genomic integrity after DNA damage and is an essential component of the 'intra-S-phase checkpoint'. In budding yeast, alkylating chemicals, such as methyl methanesulfonate (MMS), or depletion of nucleotides by hydroxyurea (HU) stall DNA replication forks and thus activate Rad53 during S-phase. This stabilizes stalled DNA replication forks and prevents the activation of later origins of DNA replication. Here, we report that a reduction in the level of Rad53 kinase causes cells to behave very differently in response to DNA alkylation or to nucleotide depletion. While cells lacking Rad53 are unable to activate the checkpoint response to HU or MMS, so that they rapidly lose viability, a reduction in Rad53 enhances cell survival only after DNA alkylation. This reduction in the level of Rad53 allows S-phase cells to maintain the stability of DNA replication forks upon MMS treatment, but does not prevent the collapse of forks in HU. Our results may have important implications for cancer therapies, as they suggest that partial impairment of the S-phase checkpoint Rad53/Chk2 kinase provides cells with a growth advantage in the presence of drugs that damage DNA.

INTRODUCTION

Eukaryotic cells have evolved efficient surveillance mechanisms that sense different kinds of DNA damage. These mechanisms delay or arrest cell cycle progression and induce repair processes that ensure genome integrity (1–3). It is believed that the delay or arrest provides additional time for cells to repair the damage efficiently prior to resuming the cell division cycle. These surveillance mechanims are signal transduction cascades called checkpoints that, when activated, regulate repair responses including transcription of the DNA damage response genes, activation of DNA repair processes and recruitment of proteins to sites of DNA damage to form, in some cases, foci at lesions (4–10). All major components of checkpoint pathways are remarkably well conserved in eukaryotes (5–8,10,11), in fact some of them are implicated in embryonic development while others have been found mutated in human cancer or in rare syndromes that predispose to cancer (12–14). In the budding yeast *Saccharomyces cerevisiae* the ATR-homologue Mec1 plays a key role in the signalling cascade by phosphorylating downstream effector kinases in response to lesions in DNA and to defects at DNA replication forks (15). In particular Mec1–Ddc2 complexes are recruited to sites of damage or stalled DNA replication forks (16–19) to, then, transduce the checkpoint signal by phosphorylating effector kinases Rad53 or Chk1 (15,20).

Recently it has been shown that the essential role of the Mec1-Rad53 cascade is to maintain DNA replication forks stability when cells face replication fork blocks or DNA damage (21,22). In fact, both checkpoint proteins are involved in a process of stabilization of DNA replication forks that prevents them from collapsing spontaneously in S-phase or after chemical exposure (21–26). *mec1* and *rad53* mutant budding yeast cells are unable to recover from any kind of stress that stalls progression of DNA replication forks (22,27), indicating that the essential function of the intra S-phase checkpoint is the recovery itself. The intra S-phase checkpoint response also activates a Mec1/Rad53-dependent mechanism that represses the firing of late and dormant replication origins (28–30). The absence of this inhibitory mechanism explains the fast rate of DNA synthesis in *rad53* and *mec1* mutant cells when DNA is damaged (21,31).

In this study, which focuses on the characterization of *S.cerevisiae* mutant cells with low levels of the Rad53 checkpoint kinase, we find that, as expected with a limited capacity for checkpoint response, the reduction in Rad53 levels results

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in DNA replication fork collapse and checkpoint impairment in response to the depletion of nucleotides by hydroxyurea (HU). However, we present clear evidence indicating that this partial reduction of the Rad53 effector kinase strikingly allows S-phase cells to maintain DNA replication fork stability in response to methyl methanesulfonate (MMS)-mediated DNA alkylation and that cells become resistant to the DNA damaging agent.

MATERIALS AND METHODS

General methods of Molecular and Cellular Biology were used as described by Sanchez *et al*. (32).

Strains, cell cycle control and checkpoint induction

The *S.cerevisiae* strains used in this work were 15Dau and W303 backgrounds (as indicated). Yeast strains were grown in rich YPA medium (1% yeast extract, 2% peptone) containing 2% glucose. For block-and-release experiments, cells were grown in YPA with 2% glucose (except where indicated) at 28°C and synchronised with α-factor pheromone in G1 by adding 40 ng/ml (final concentration, 2.5 h) for the 15Dau strains or 7.5 μg/ml (2 h) + 2.5 μg/ml (one additional hour) for the W303 strains. Cells were then collected by centrifugation and released in the presence of MMS or HU as indicated. Overexpression experiments with cells grown in YPA medium with 2% raffinose at 28°C were conducted by adding 0.3% galactose (to induce) or 2% glucose (to repress) to the medium and further incubating with/without MMS or HU as indicated. Proteolysis in degron strains was induced from asynchronous cultures of cells grown at 24°C in YP medium containing 2% Raffinose as the carbon source and 0.1 mM $CuSO_4$. To inactivate Rad53 degron fusion protein, expression of *UBR1* was induced by addition of 2.5% Galactose (final concentration) to the medium (still containing 0.1 mM $CuSO_4$) for 30 min at 24°C before changing to YPGal medium prewarmed at 37°C, and continuing incubation for 120 min. Experiments in Petri dishes with degron strains were performed at 24 or 37°C in YPD (containing 0.1 mM $CuSO_4$), YPRaf (containing 0.1 mM $CuSO_4$), or YPGal as required. FACS analysis of DNA content was performed as described (32).

Rad53-Ha, Rad53 degron fusion strains and *RAD53* deletion strains

To construct the *rad53Ha* allele (in all genetic backgrounds) the single step PCR-based gene modification strategy by Longtine *et al*. (33) was used. The oligonucleotides used were 5′-AAGGTTAAAAGGGCAAAATTGGACCAAACCTCAAAAGGCCCCGAGAATTTCAATTTTCGCGGATCCCCGGGTTATTAA-3′ and 5′-GGTATCTACCATCTTCTCTCTTAAAAAGGGGCAGCATTTTCTATGGGTATTTGTCCTTGGGAATTCGAGCTCGTTAAAC-3′. The selection marker used was *KANMX6*, which allows selection with Geneticin, or *TRP1*. The resulting genomic constructions were confirmed by PCR and sequencing. A similar strategy was employed to construct *GAL1,10: rad53Ha* by gene targeting of *rad53Ha* strains. The oligonucleotides used in this case were 5′-AAAGGACGGTAGAGATTATTGGAAGACAAACTAATTTTGTATATGCATTCGATTTGAATTCGAGCTCGTTTAAAC-3′ and 5′-AAAACCTTTGAGTAGCCTGCGTGGATTGCTGTGTGGGTTGTGTAATATTTTCCATCATTTTGAGATCCGGGTTTT-3′. *SML1* deletions were generated similarly. The oligonucleotides used were 5′-GATCTTACGGTCTCACTAACCTCTCTTCAACTGCTCAATAATTTCCCGCTCGGATCCCCGGGTTAATTAA-3′ and 5′-CAGAACTAGTGGGAAATGGAAAGAGAAAAGAAAAGAGTATGAAAGGAACTGAATTCGAGCTCGTTTAAAC-3′ for the 15Dau strain, and for the W303 strain the oligonucleotides used were 5′-ATCTGCTCCTTTGTGATCTTACGGTCTCACTAACCTCTCTTCAACTGCTCCGGCATCAGAGCAGATTGTAC-3′ and 5′-AAGGAACTTTAGAAGTCCATTTCCTCGACCTTACCCTGGTTGAACATAGAGTATTTCACACCGCATATGATC-3′. In *sml1* deletions the selectable marker used was *HIS3*.

To construct the *rad53-tpd* heat-inducible degron strain the same single step-PCR-based gene modification was used (33), in a yeast strain in which the only copy of the *UBR1* gene was regulated by the galactose-inducible *GAL1, 10* promoter (34). The oligonucleotides used were 5′-AGGACGGTAGAGATTATTGGAAGACAAACTAATTTTGTATATGCATTCGAATTAAGGCGCGCCAGATCTG-3′ and 5′-CTTTGAGTAGCCTGCGTGGATTGCTGTGTGGGTTGTGTAATATTTTCCATGGCACCCGCTCCAGCGCCTG-3′. Again, the selection marker used was *KANMX6*, which allows selection with Geneticin.

In all cases correct integrations were confirmed by PCRs using combinations of oligonucleotides corresponding to sequences either side of the integration site or within the inserted cassette.

Western blot and *in situ* autophosphorylation assays

Cells extracts were prepared by standard yeast protein protocols (35). Immunoblotting was carried out with anti-Rad53 (gift of Dr J. F. X. Diffley) or with anti-Rad53 (#sc-6749) from Santa Cruz Biotechnology, Inc., used as indicated by the supplier. *In situ* autophosphorylation assays (ISA) were carried out as described (35,36).

Two-dimensional DNA gels (2D-gel analysis)

DNA samples for neutral-neutral two-dimensional gel electrophoresis were prepared and analyzed as described previously (22,37,38). DNA was cut with the NcoI restriction enzyme and hybridized to probes spanning the *ARS306* and *ARS501* origins of DNA replication.

RESULTS

Carboxy Ha-tagging of Rad53 enhances cell survival following DNA alkylation

MMS modifies both guanines and adenines to methyl derivatives causing DNA base mispairing, hence inducing DNA damage and slowing down progression through DNA replication (21,25,31,39). *RAD53* has a central role in the checkpoint response to this alkylating chemical. Exposure to MMS leads to Rad53 phosphorylation and checkpoint activation (25,31,40,41). Here we characterised a *RAD53* allele tagged with three Ha epitopes (*rad53Ha*) and found major differences in the cellular responses to different

genotoxic agents. When untreated, *rad53Ha* cell viability was comparable to the wild-type control and tagged cells progressed through a normal S-phase without losing viability (Figure 1), suggesting that the *rad53Ha* allele was fully functional. However, we found that cells carrying the Ha-tagged allele were hyposensitive to low concentrations of MMS. This resistance to the effect of the DNA-alkylating agent was independent of the strain background used for the analysis (Figure 1A). In contrast, a 10-fold dilution assay in plates with 50 mM HU, a ribonuclease reductase inhibitor (42), showed that *rad53Ha* cells were hypersensitive to the drug (Figure 1A). The level of response was better than in a strain deleted for *RAD53*, indicating that *rad53Ha* mutant cells were partially active in the checkpoint response to blocks in DNA replication. Interestingly, we have also found that *rad53Ha* cells were partially resistant to cisplatin. However, they were not sensitive to ultraviolet, camptothecin (CPT) or bleomycin treatments (Supplementary Data). We conclude that *rad53Ha* is a mutant allele of *RAD53*.

When *S.cerevisiae* cells are treated with drugs that interfere with S-phase progression, such as MMS or HU, the Mec1 and Rad53 checkpoint kinases are sequentially activated to respond to the DNA stress (15,43). We then analysed checkpoint activation and found that *rad53Ha* cells had low levels of the Rad53 kinase. The phosphorylation of Rad53 changes its electrophoretical mobility in denaturing PAGE-gels and has been related to its activation (35). When cells were exposed to HU, MMS or bleomycin, Rad53 and Rad53Ha proteins became phosphorylated as judged by the shift in their mobility; indicating that the wild-type and the mutant-form proteins were active (Figure 1B). In block and release experiments with α-factor-synchronised cells, we found no differences between Ha-tagged or Rad53 wild-type controls in the temporal pattern of activation of the checkpoint kinase. However, *rad35Ha* cells accumulated lower levels of the kinase than wild-type cells.

We next compared the activity of the Rad53 kinase in wild-type and *rad53Ha* strains. In keeping with the differences observed in the level of the checkpoint protein, an ISA (35) revealed that *rad53Ha* cells contained an active Rad53Ha protein kinase that was able to phosphorylate itself and that accumulated low amounts of the checkpoint kinase compared to the wild-type (Figure 1B).

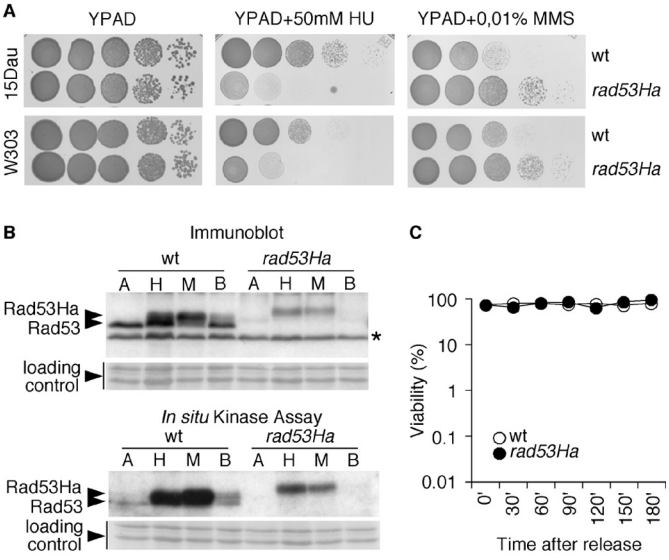


Figure 1. The Ha carboxy-terminal tagging of Rad53 reduces protein levels and renders cells sensitive to HU and resistant to MMS. (**A**) 10-fold serial dilutions of cultures of wild-type and *rad53Ha* mutant cells growing in YPAD and spotted onto YPAD plates with or without HU (50 mM) or MMS (0.01%) as indicated. Note that the assay was performed in the 15Dau and W303 *S.cerevisiae* strain backgrounds. (**B**) Immunoblot analysis and *In situ* Kinase Assay of Rad53 in asynchronous cultures of wild-type and *rad53Ha* strains (A), or in cells treated for 90 min with 200 mM HU (H), or with 0.033% MMS (M), or with 10 mU/ml of Bleomycin (B) (Note: a longer exposure of the western and ISA were needed to detect Rad53Ha in bleomycin samples). (**C**) Cultures of wild-type and *rad53Ha* strains growing exponentially in YPAD medium were synchronised in G1 with alpha-factor pheromone at 28°C and then released into fresh YPAD medium at the same temperature to allow cells to initiate S-phase synchronously. Samples were taken at indicated intervals and processed for viability assays and percentage of viables was plotted.

DNA damage and replication stress induced transcription in *rad53Ha* mutant cells

In *S.cerevisiae* activation of the checkpoint kinase cascade (Mec1/Rad53/Dun1) in response to DNA replication stress or DNA damage during S-phase rapidly induces the transcription of *RNR* genes, including *RNR1*, *RNR2*, *RNR3* and *RNR4* (44–47). To understand the consequences of reducing the cellular level of Rad53 checkpoint kinase in the transcriptional induction response we next analysed the expression of the *RNR2* gene after MMS or HU treatments. The *RNR2* gene codes for one of the two essential small subunits of the ribonucleotide reductase complex in *S.cerevisiae*. *RNR2* mRNA level is highly induced in response to the stress of DNA damage or nucleotide depletion (45,48). Northern analysis showed that *RNR2* gene expression was similarly downregulated upon induction of checkpoint response to both MMS and HU treatments in *rad53Ha* mutant cells compared to wild-type controls (Figure 2). Comparable results were obtained when *HUG1* expression was analysed (data not shown). *HUG1* is another component of the checkpoint response in budding yeast (49). These data indicate that a reduction in Rad53 levels limits the transcriptional response of the checkpoint cascade to replication blocks and DNA alkylation.

Defects of *rad53Ha* are not bypassed by deletion of *SML1*

In the budding yeast *S.cerevisiae*, *MEC1* and *RAD53* are essential for cell growth and checkpoint response. The cell growth defect is the consequence of the Mec1 and Rad53-protein kinases control of dNTP production. Consistently, cell viability is restored in cells lacking *MEC1* or *RAD53* by deletion of *SML1*, a physiological inhibitor of the ribonucleotide reductase complex (42,50–52). We have shown above that *rad53Ha* cells are resistant to MMS and sensitive to HU (Figure 1). One possibility is that the observed effects of HU and MMS in *rad53Ha* cells could simply be explained by the effect of the low levels of expression of *RNR2* gene in Ha-tagged cells when exposed to the drugs (Figure 2). In such case, deletion of *SML1* would rescue *rad53Ha*-associated HU sensitivity and MMS resistance. To test this possibility we deleted *SML1* in *rad53Ha* cells and tested double mutants

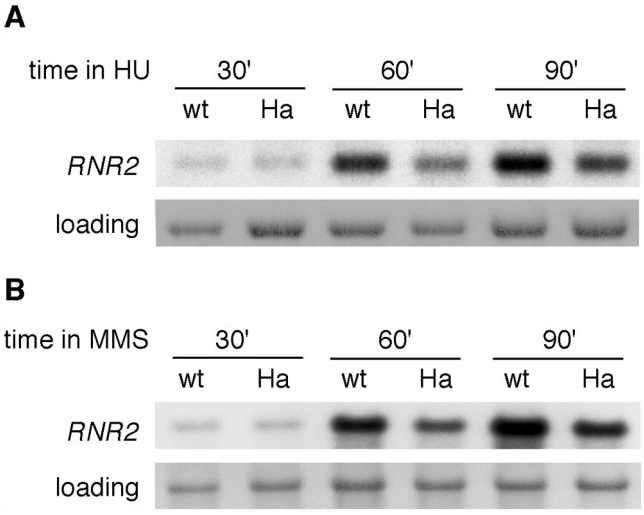


Figure 2. Limited *RNR2* expression in *rad53Ha* mutant cells in response to blocks in DNA replication and DNA damage. Northern blot analyses of RNA isolated from wild-type and *rad53Ha* cells treated with HU (200 mM) (**A**) or MMS (0.033%) (**B**). Wild-type and Rad53Ha-tagged cells were synchronised with α-factor and then released in the presence of HU or MMS. RNA samples were isolated at the times indicated and then electrophoresed, blotted to nylon membranes and the membranes were probed for *RNR2* expression. The loading control is the 18S rRNA stained with methylene blue.

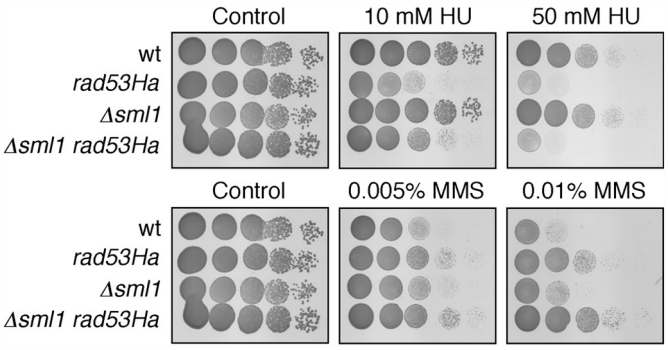


Figure 3. Sensitivity assay to HU and MMS-mediated DNA damage in wild-type, *rad53Ha*, Δ*sml1* and *rad53Ha* Δ*sml1* mutants. 10-fold serial dilution assays of cultures of the indicated strains exposed to sublethal concentrations of DNA replication inhibitor HU or DNA alkylating chemical MMS.

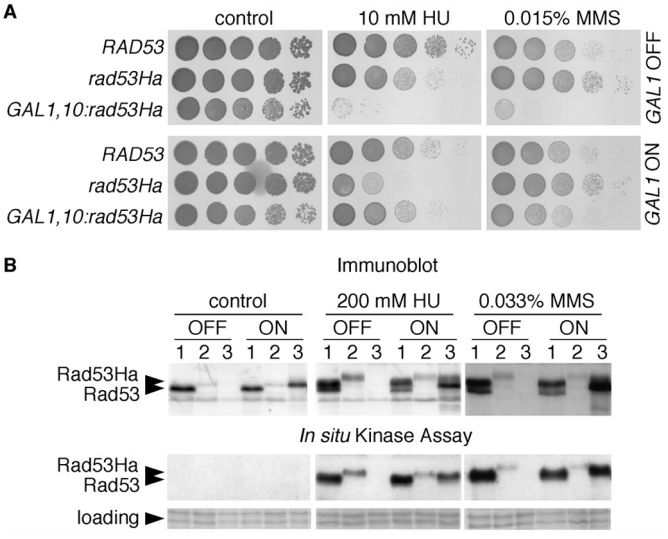


Figure 4. Suppression of MMS-induced DNA damage and HU-replicative stress sensitivity defects by increased expression of the *rad53Ha* allele. (**A**) 10-fold serial dilution assay of indicated strains. Cells growing in YPAD medium were spotted onto YPAD (2% glucose, labeled as *GAL1 OFF*) or YPARG (0.3% galactose, 1% raffinose, labeled as *GAL1 ON*). Petri dishes with no drug (control), with 10 mM HU or with 0.015% MMS (as indicated), were incubated for 48 h at 28°C and then photographed. (**B**) Analysis of Rad53 protein levels (Immunoblot) and Rad53 activity (ISA) in asynchronous (control) HU- and MMS-treated cultures of wild-type (1), *rad53Ha* (2) and *GAL1,10:rad53Ha sml1*Δ (3) strains. When treated, yeast cultures were exposed to HU or MMS (as indicated) for 90 min.

on plates with HU or MMS (Figure 3). Compared to controls, we found that deletion of the ribonucleotide reductase *SML1*-encoding gene had minor effects on *rad53Ha*-associated phenotypes, Δ*sml1 rad53Ha* cells were resistant to MMS and hypersensitive to HU as found for *rad53Ha* cells.

An increase in Rad53Ha protein levels restores the wild-type response to MMS

If low levels of the Rad53 checkpoint protein lead to all defects observed in *rad53Ha S.cerevisiae* cells, it could be predicted that higher levels of Rad53Ha protein should rescue the defective responses to MMS and HU. We therefore constructed a *GAL1,10:rad53Ha* strain (in a Δ*sml1* background to ensure cell viability) that only expressed high levels of Rad53Ha when induced. As expected of a strain conditionally deleted for *RAD53*, *GAL1,10:rad53Ha*, cells were hypersensitive to HU and MMS (Figure 4A, *GAL1* OFF plates), comparable to a *rad53* deletion mutant strain (15,43,53,54). We then tested the levels and the kinase activity of Rad53Ha protein with the *GAL1,10:rad53Ha* strain (Figure 4B). We found that indeed they were fairly similar to those in wild-type cells (Figure 4B, lower panels). Accordingly, the resistance to MMS associated with *rad53Ha* cells was rescued by Rad53Ha overexpression and likewise the hypersensitivity of these cells to HU was suppressed (Figure 4A), indicating that the defects observed in Rad53Ha-tagged strains were directly related to insufficient protein levels.

rad53-tpd: a degron allele of *RAD53* with low levels of the checkpoint kinase

To further support our hypothesis that resistance to MMS-mediated DNA damage and sensitivity to HU-induced replication blocks was a simple consequence of reducing Rad53 levels we looked for alternative situations in which we could limit the amount of the Rad53-checkpoint kinase. We reasoned that depletion of the mRNA by transcriptional repression (*tet02* or *GAL1* systems) would not be an efficient mechanism to limit Rad53 levels because the checkpoint protein is fairly stable (S. Ufano and A. Bueno, unpublished data). A method called 'heat-inducible-degron' has been described that allows any essential protein to be depleted rapidly and conditionally by targeting the protein for degradation at 37°C (34,55). We fused a 'heat-inducible-degron' to the amino terminus of Rad53 and, in addition, we screened for (Rad53-) degron strains that were viable at 37°C and selected those where cell size was markedly heterogeneous (suggesting insufficient inactivation of the protein). We

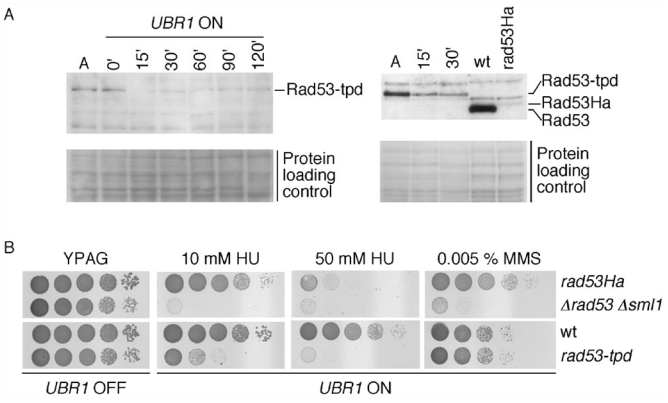


Figure 5. The partial inactivation of Rad53 by fusion to a heat-inducible degron renders cells hypersensitive to HU and wild-type-like to MMS. (**A**), left panels, Western blot analysis of a Rad53-tpd protein in mutant strains asynchronously growing (A) or after partial inactivation of the degron fusion protein at indicated intervals. (A), right panels, comparison of protein levels of Rad53-tpd with appropriate controls (Rad53Ha and wt Rad53). The protein loading controls are portions of respective gels stained with Ponceau. (**B**) Serial dilutions were made of a Rad53 degron strain (*rad53-tpd*) together with *rad35Ha*, Δ*rad53* Δ*sml1* and wild-type controls.

isolated five strains with this phenotype. We expected selected strains to express levels of Rad53 sufficient to support cell viability but insufficient to respond properly to genotoxic insults. One of the strains was choosed for further work (Figure 5), we named it *rad53-tpd* (where *tpd* denotes a temperature-sensitive partial degron, to follow previous nomenclature). As expected *rad53-tpd* induced a partial degradation of Rad53 at 37°C (Figure 5A). At this temperature the level of Rad53-tpd protein was higher than in *rad53Ha* cells but lower than in wild-type cells. Importantly, when assayed in the presence of drugs we noticed that cells were clearly sensitive to HU (not to the same extent as Δ*rad53* Δ*sml1* cells) and almost as resistant as wild-type cells to MMS (Figure 5B). We also noticed that *rad53Ha* cells were more resistant to the action of the alkylating agent than *rad53-tpd*. *rad53Ha* and *rad53-tpd* behave dissimilarly in HU and MMS most likely because of differences in levels of the Rad53Ha and Rad53-tpd proteins (Figure 5A). However, these results support our observation that reducing the level of the Rad53-effector kinase causes cells to behave very differently in response to MMS-mediated DNA alkylation or to HU-induced nucleotide depletion.

DNA replication fork stability upon MMS- or HU-induced stalling in *rad53Ha* mutant cells

In response to MMS wild-type *S.cerevisiae* cells slowed down progression through S-phase. In contrast, *rad53* deletants passed through S-phase as fast as untreated cells, losing their viability (21,29). These phenotypes are interpreted as the consequences of inappropriate activation of late and dormant origins of DNA replication and the collapse of DNA replication forks (21,29). Accordingly, we analysed S-phase progression in MMS-treated *rad53Ha* mutant cells (Figure 6) and found that α-factor-presynchronised Rad53Ha-tagged cells rapidly doubled their DNA content, as measured by FACS analysis (Figure 6B). This was in contrast to wild-type controls, which slowed progression through DNA replication, as previously reported (29,31). Consistently, the late origin *ARS501* was not inhibited in *rad53Ha* mutant cells (Figure 6A; see arrow in 30 min *rad53Ha* sample). However, 2D-gel analysis showed no indication of DNA replication fork collapse in replicating *ARS306* or *ARS501* genomic sequences, while western blot and *ISA* confirmed low levels of the checkpoint kinase in mutant cells (Figure 6C). This is in sharp contrast to collapsing structures observed in replicating *ARS306* in cells deleted for *RAD53* when treated with MMS (Figure 6D and F), as previously described by Lopes *et al*. (26).

We then analysed the replication intermediates in *rad53Ha* cells released from a G1 block in the presence of HU and found that, in agreement with their hypersensitivity to the drug (Figure 1), the DNA replication forks collapsed (Figure 7). These unusual replication intermediates were similar to abnormal DNA structures previously observed in HU-treated *rad53*-kinase dead mutants (22).

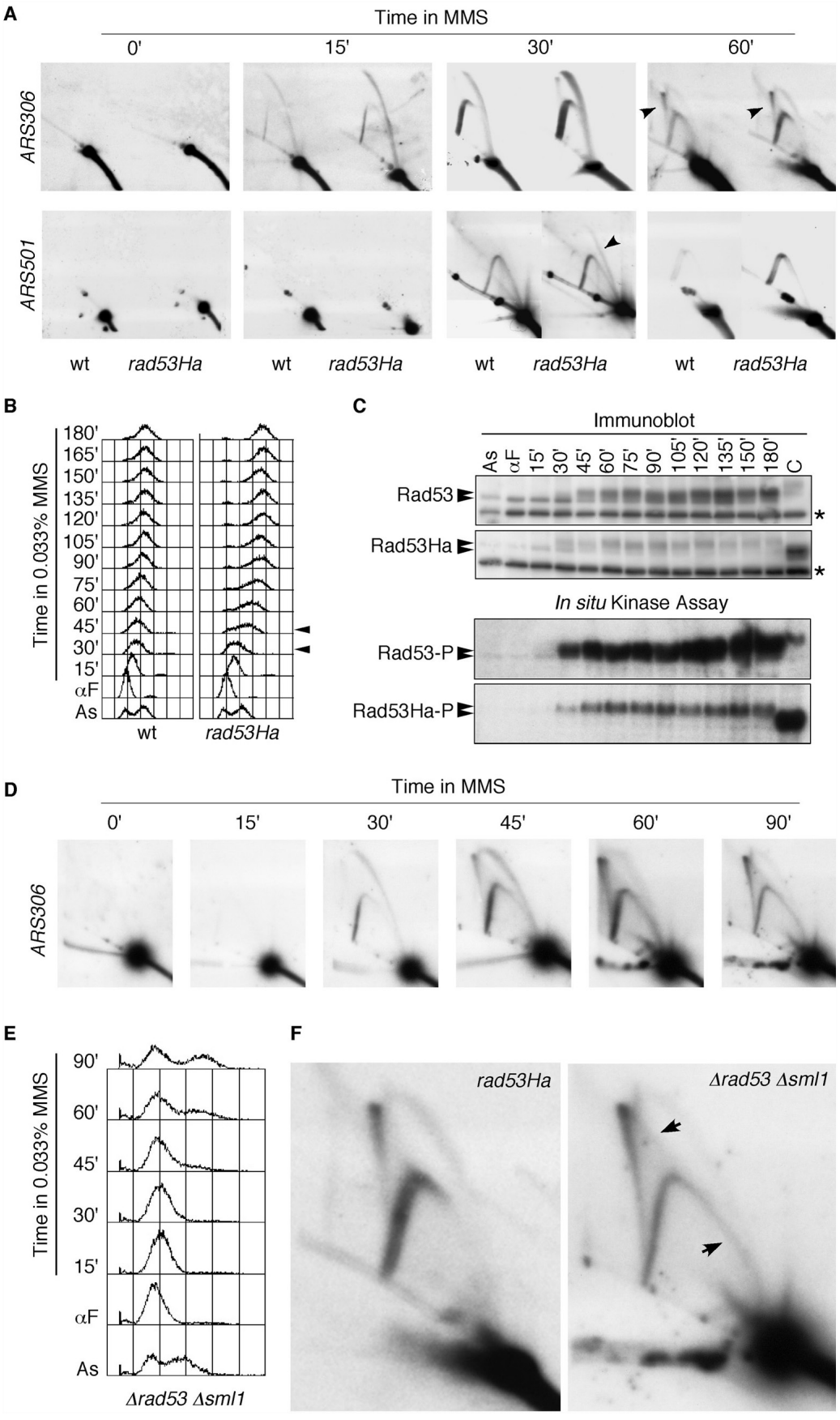
Our results show that *rad53Ha* cells have enough checkpoint effector kinase molecules to deal with fork stability but not enough to prevent DNA replication late origins from firing in the presence of the DNA-alkylating agent, contrasting with their own defects regarding fork collapse in HU.

Premature checkpoint inactivation in cells with low levels of Rad53 kinase

We next became interested in the analysis of *rad53Ha* resistance to MMS. Recovery from a DNA damaging insult requires down-regulation of the Rad53 checkpoint kinase (35). Rad53 down-regulation allows cells to reset cell cycle progression (41). Although *rad53Ha* cells harboured low levels of the checkpoint kinase, they inactivated it when recovering from MMS or HU treatments as in wild-type cells, as judged by their pattern of dephosphorylation (Figure 8). This indicates that Rad53Ha checkpoint kinase is properly dephosphorylated as cells recover from drug-induced DNA replication blocks or DNA damage. However, consistent with DNA replication fork collapse in HU (Figure 7), we noticed that Ha-tagged cells maintained Rad53Ha-associated kinase activity longer than wild-type controls (Figure 8A). This indicates that cells dealing with fork collapse sense the damage and maintain an active checkpoint.

To understand the DNA damage resistance of *rad53Ha* mutant cells observed on MMS plates, we also compared the activation of Rad53 checkpoint kinase in wild-type and *rad53Ha* cells when exposed to MMS for long periods of time. Therefore, we analysed the relative concentration and activity of Rad53 in cells exposed to different doses of the DNA alkylating drug (Figure 9). Rad53 remained active throughout all MMS treatments in wild-type controls. However, Rad53Ha in tagged cells rapidly became down-regulated after an initial activation upon drug exposure, even at the higher doses used, implying that *rad53Ha* cells had prematurely down-regulated Rad53-associated kinase activity in continuous exposure to the alkylating DNA-damaging agent.

Because this premature down-regulation of Rad53Ha kinase could enhance the mutation rate and genomic


A
Time in MMS
0'
15'
30'
60'
ARS306
ARS501
wt
rad53Ha
wt
rad53Ha
wt
rad53Ha
wt
rad53Ha
B
Time in 0.033% MMS
180'
165'
150'
135'
120'
105'
90'
75'
60'
45'
30'
15'
αF
As
wt
rad53Ha
C
Immunoblot
As
αF
15'
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In situ Kinase Assay
Rad53-P
Rad53Ha-P
D
Time in MMS
0'
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60'
90'
ARS306
E
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F
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Δrad53 Δsml1

instability, we then monitored the forward mutation rate to canavanine resistance (56) on *rad53Ha*-tagged mutant cells exposed to MMS and found that it was almost 2-fold higher than in wild-type cells (Figure 10), suggesting that a reduction in cellular levels of the kinase decreases replication fidelity in the presence of the alkylating agent and consequently increases genetic instability.

Since Rad53Ha is prematurely dephosphorylated in the continuous presence of MMS and this down-regulation results in a short cell cycle arrest, we were interested in understanding whether the PI3-kinases Mec1 and Tel1 were continuously required for *rad53Ha* resistance to the DNA alkylating drug. For this purpose, we studied the effect of caffeine (1,3,7-trimethylxanthine) in *rad53Ha* resistance to MMS. In budding yeast caffeine has mutagenic effects that appear to be mediated through the inhibition of PI3-related kinases Tel1 and Mec1 (57). Viability was checked in *rad53Ha* cells and wild-type controls first treated with MMS and then with or without caffeine (in the presence of MMS). We found that *rad53Ha* cells treated with caffeine were less viable than controls lacking the drug (Figure 11). This result clearly suggests that *rad53Ha* resistance to MMS-mediated DNA damage depends on functional Mec1 and/or Tel1 kinases. Importantly, this evidence, in the context of the premature dephosphorylation of Rad53Ha, also supports the view that a minimal concentration of Rad53 protein may be required for the maintenance of the hyperphosphorylated state of the checkpoint kinase, more likely by the autophosphorylation activity of Rad53 kinase.

DISCUSSION

We have explored the consequences of reducing Rad53 protein levels in *S.cerevisiae* cells when dealing with blocks to DNA replication or DNA damage induced by an alkylating chemical. The results of our studies indicate that the limitation of Rad53 protein leads to HU sensitivity and checkpoint impairment in response to depletion of nucleotides, however, unexpectedly, it also leads to a dramatic increase in cellular resistance to MMS-induced DNA damage.

By tagging the carboxy-terminus of the Rad53 protein with the Haemmaglutinin epitope we have generated a yeast strain with a low level of the checkpoint protein. How does the C-terminal tagging of Rad53 cause cells to accumulate low amounts of the checkpoint protein? We have studied the stability of Rad53Ha and found that the tagged protein is unstable (S. Ufano and A. Bueno, unpublished data). However, yeast cells carrying the *rad53Ha* allele are viable and show no obvious defects in the growth and cell cycle parameters analysed. In fact, *rad53Ha* cells are viable even without the deletion of *SML1* gene (encoding a ribonucleotide reductase inhibitor), as is required not only for *rad53* but also for *mec1* null mutants (42,50,51). Thus, the checkpoint kinase level in *rad53Ha* cells appears to be sufficient to induce the degradation of Sml1 protein every S-phase. Consistently, S-phase progression is normal as observed by FACS analysis of DNA content (data not shown). The Ha-tagged Rad53 checkpoint kinase is properly and promptly activated in response to HU and MMS, as shown by western and *ISA*. However, *rad53Ha* strains are sensitive to HU but resistant to MMS. These defects in *rad53Ha* cells are directly attributable to the reduction in the level of the checkpoint kinase, because a *GAL1,10*-regulated increase of the Ha-tagged protein restores wild-type like responses to HU and MMS. These experiments demonstrate that the Rad53Ha protein kinase is fully functional because the checkpoint response is restored when normal levels of the checkpoint protein are reached. Further support for our hypothesis comes from the analysis of the *rad53-tpd* degron strain that we have generated. Despite the fact that *rad53-tpd* cells are not resistant to MMS (at least to the same extent as *rad53Ha* cells) they are as resistant as wild-type cells to the alkylating agent, contrasting to their hypersensitivity to HU. Thus, our study identifies two different mutants with low Rad53 levels, *rad53Ha* and *rad53-tpd*, that behave similarly in dealing with HU-induced DNA replication blocks or DNA alkylation damage.

When treated with the alkylating agent MMS, *rad53Ha* cells progress through S-phase as fast as fully defective mutants (21,29). Accordingly, the *ARS501* late DNA replication origin is activated and *rad53Ha* cells had a low level of the checkpoint kinase. Our data again suggest a defect associated with an insufficient number of molecules of the checkpoint protein to prevent the activation of late origins, as in *rad53*Δ *sml1*Δ mutants (21,28,29). However, *rad53Ha* strains are resistant to MMS and, remarkably, DNA replication forks remain stable in the presence of the DNA alkylating agent, contrasting with fork instability in *rad53* null mutants (21,25,26). Our data indicate that *rad53Ha* cells have enough checkpoint effector kinase molecules to deal with fork stabilisation but not enough to prevent late DNA replication origins from firing in the presence of the alkylating agent. These findings are consistent with earlier evidence from the analysis of *mec1-100* mutants, suggesting that the essential function of the checkpoint response to DNA damage is to maintain DNA replication fork stability (25), and, importantly, indicate a hierarchy of Rad53 functions in checkpoint response to MMS.

Figure 6. MMS-induced S-phase DNA damage checkpoint in *rad53Ha* mutant cells. Cultures of wild-type and *rad53Ha* mutant cells presynchronised in G1 with α-factor were released into fresh YPAD medium containing 0.033% MMS to induce the intra S-phase DNA damage checkpoint response. Samples were taken at the indicated intervals and processed for FACS analysis, 2D-gel analysis of *ARS306* and *ARS501*, Western blotting and ISA assays of Rad53. (**A**) Genomic DNA samples were prepared from aliquots at the indicated intervals and cut with NcoI, restriction fragments were electrophoresed in 2D-gels, transferred to nylon membranes and hybridized to probes spanning the *ARS306* and *ARS501* origins of DNA replication. (**B**) FACS analysis of the DNA content of *rad53Ha* and wild-type control cells in response to MMS treatment. Note that *rad53Ha* mutants passed through S-phase faster than wt controls (samples indicated by arrows). (**C**) Protein extracts from aliquots from the same samples were analysed by Western blot assays with anti-Rad53 antibody or *In Situ* Authophosphorylation assays (ISA), as indicated, to test the activation of Rad53. A cross-referenced sample (labelled C) was used in both the Western and ISA assays that corresponded to the 120 min sample of *rad53Ha* or wild-type experiments respectively. (**D**) Genomic DNA samples from Δ*rad53* Δ*sml1* cells treated with MMS were prepared at indicated intervals as in section A and hybridized to the ARS306 probe. Note that replication in these Δ*rad53* Δ*sml1* cells starts 15 min later than in wild-type and *rad53Ha* cells in A, and also that small Ys and cone-shaped signals are evident from 45 min (to the end of the experiment) indicating genuine DNA replication fork collapse. (**E**) FACS analysis of Δ*rad53* Δ*sml1* cells in D treated with MMS. (**F**) Comparison of replication intermediates in *rad53HA* and Δ*rad53* Δ*sml1* cells (cone-shaped signals and small Ys are indicated by arrows in the Δ*rad53* Δ*sml1* mutant).

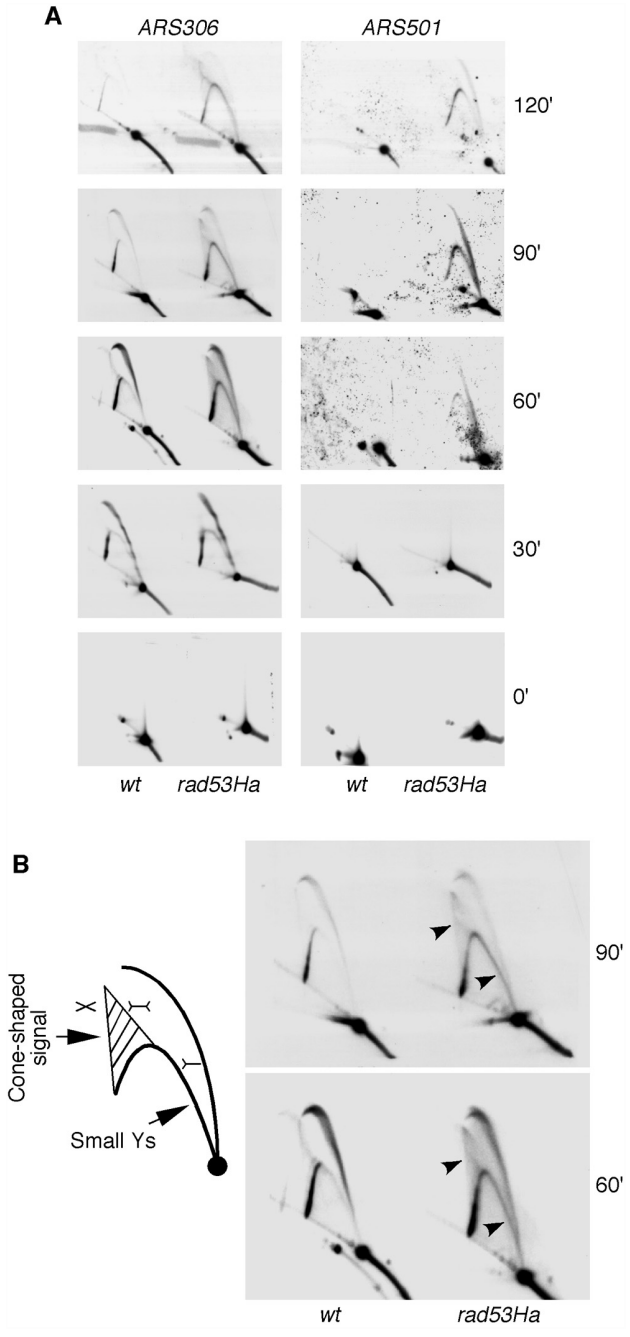

Figure 7. Effect of HU treatment on initiation from an early (*ARS306*) origin of DNA replication in *rad53Ha* cells. (**A**) wild-type and *rad53Ha* mutant cells were grown in YPAD medium to exponential phase, synchronised with α-factor in G1 and then released into fresh YPAD medium containing 0.2 M HU. Genomic DNA was prepared from cells at indicated intervals (from the release) and cut with NcoI. Restriction fragments were electrophoresed in N:N 2D-gels, transferred to nylon membranes and hybridized to a probe spanning the *ARS306* early origin of replication. (**B**) *rad53Ha* mutant cells acumulate abnormal DNA replication structures (cone-shaped signals and small Ys as indicated by arrows). A drawing of the abnormal intermediates related to DNA replication fork collapse, according to Lopes *et al*. (2001), is also shown.

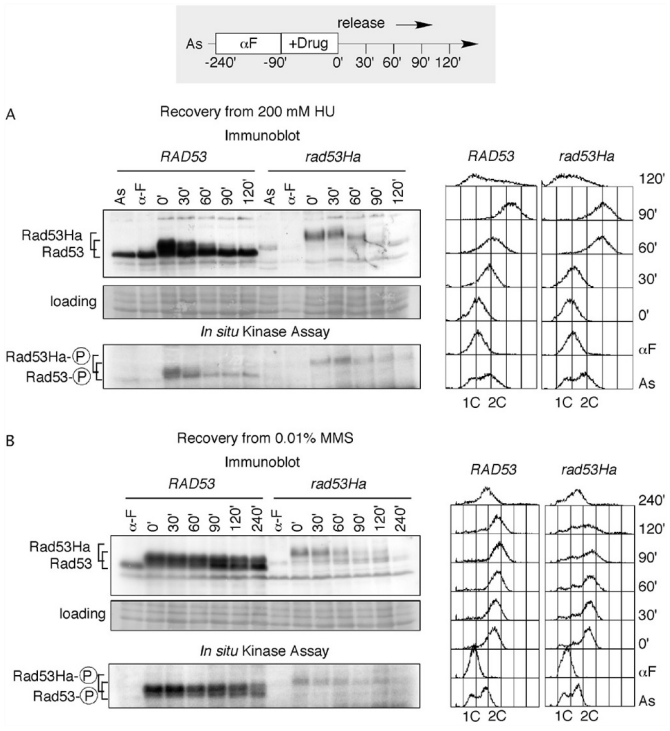

Figure 8. Recovery from replication arrest (HU) and DNA damage (MMS) in *rad53Ha* cells. Wild-type and *rad53Ha* mutant cells growing exponentially at 28°C were first synchronised in G1 with α-factor and then released into S-phase in fresh YPAD medium in the presence of HU (**A**) or MMS (**B**) and incubated for a further 90 min. Cultures were then released from drug exposure into fresh YPAD medium and further incubated at 28°C (recovery). Protein extracts were prepared at the indicated intervals and analysed by western blotting using an anti-Rad53 polyclonal antibody and *in situ* kinase assay (ISA).

Interestingly *rad53Ha* strains are sensitive to HU and show every phenotype previously described for *rad53* fully defective or kinase dead mutants in the response to this ribonucleotide reductase inhibitor drug (22,28–30), indicating that a low number of molecules of active Rad53 per cell is not enough to properly respond to a block in DNA replication caused by HU. Accordingly, we have observed a significant degree of DNA replication fork collapse. Thus, our study identifies important differences in stalled DNA replication fork stability in MMS and HU and strongly suggests that replication fork stabilization in HU or MMS are genetically separable functions. Future studies will test this hypothesis.

The inactivation of Rad53Ha when cells are recovering cell cycle progression after a MMS block and release mimics the pattern of the Rad53 wild-type protein that it is indeed comparable to previous reports on the Rad53 checkpoint effector kinase (22,35). We have observed that a lower level of the checkpoint kinase in *rad53Ha* cells implies a earlier exit from the block (upon release from drug treatment). Accordingly, the *rad53Ha* mutation increases genetic instability. In experiments of continuous exposure to the DNA damaging agent, we have shown that the premature inactivation of Rad53Ha precedes cell cycle resetting and so correlates with the resistance to the DNA alkylating agent. However, wild-type levels of Rad53 maintain checkpoint activation in response to MMS exposure [(21,25) and this work]. Thus, a reduction in the number of Rad53 molecules per cell results in premature checkpoint down-regulation, allowing cells to reset the cell cycle and proliferate. Our findings suggest that the premature inactivation of

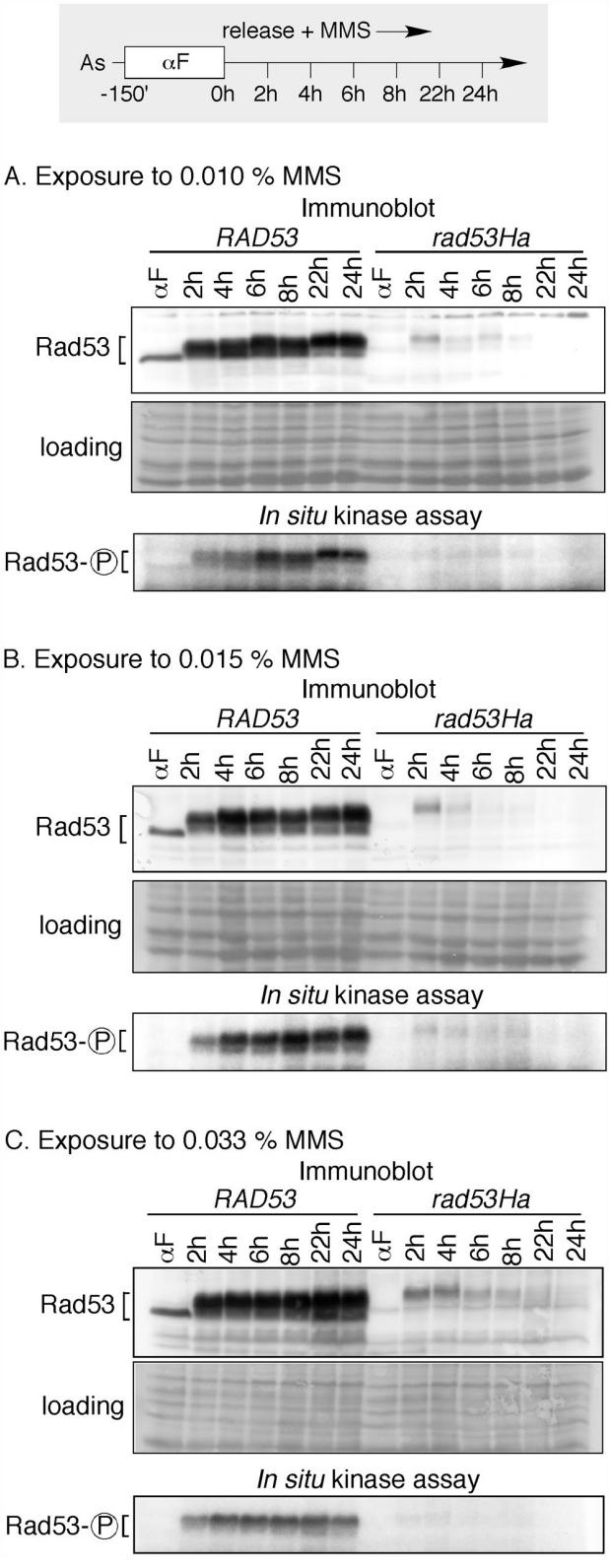

Figure 9. A reduction in the levels of the Rad53 checkpoint kinase causes cells to inactivate the checkpoint kinase in response to continuous MMS-induced DNA damage. (**A**–**C**) Cultures of wild-type and *rad53Ha* cells synchronised in G1 with α-factor were released into S-phase in fresh YPAD medium in the presence of the indicated MMS concentrations. Samples were taken at the indicated intervals and prepared for immunoblot and *in situ* kinase assays (ISA).

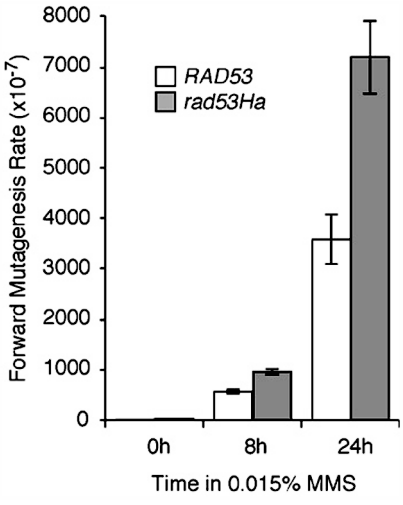

Figure 10. Forward mutation analysis in wild-type and *rad53Ha* strains. Canavanine resistance was assayed in *rad53Ha* and wild-type control cells treated with 0.015% MMS at 28°C for 0, 8 and 24 h. A plot of the resulting forward mutation rate is shown.

Rad53Ha kinase leads to resistance to the DNA alkylating agent. We have also found that *rad53Ha* cells are partially resistant to cisplatin (*cis*-diamminedichloroplatinum: a platinum compound widely used in cancer chemotherapy). It is of interest to study whether or not the resistance to cisplatin is, as expected, the consequence of a similar checkpoint defect. The mechanism of *rad53Ha* resistance to cisplatin will be the subject of future studies.

It is important to emphasise that *rad53Ha* defects are not equivalent to the defects described for *orc2-1* in *S.cerevisiae*. *orc2-1* thermosensitive mutant cells compromise the activation of Rad53 in S-phase by means of replication stress or DNA damage, resulting in cells that are sensitive to both HU and MMS (58). In contrast, *rad53Ha* cells have a genuine defect in kinase levels but the kinase is promptly activated in response to replication blocks (HU) or DNA damage (MMS) (Figures 1B and 6C), resulting in cells sensitive to HU but resistant to MMS-induced DNA damage. It is also of interest to compare *rad53Ha* with *mec1-100* strains. *mec1-100* mutants are deficient in the timing of checkpoint activation, resulting in cells sensitive to blocks in DNA replication but wild-type-like to MMS-induced DNA damage (40). Consistently, Rad53 checkpoint kinase levels reach wild-type levels in *mec1-100* cells (40).

Finally, our findings in yeast may have important implications for cancer-therapy and they suggest an explanation for the role of mutations that reduce checkpoint activity in human lung cancer (59,60). These mutations were shown to confer resistance to radiation-induced DNA damage. In particular, it has been shown that a mutation in *CHK2* related with human lung cancer (59,61) encodes an unstable protein that is expressed only at a significantly reduced level of the wild-type (20% of the wt level) (59). The authors suggested that reduced expression of Chk2 may be an important inactivating mechanism of the DNA damage checkpoint pathway, contributing to the development of this fatal adult lung cancer. Our work suggests that such change may down-regulate the checkpoint response and thus favour tumour cell

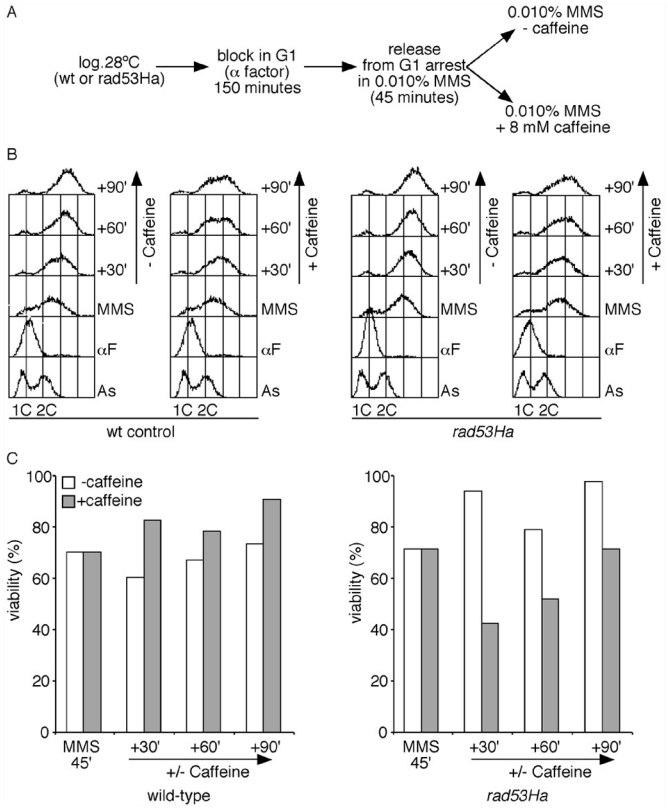

Figure 11. Analysis of caffeine effect in *rad53Ha* resistance to MMS. (**A**) *rad53Ha* and wild-type cells were grown at 28°C, blocked in G1 at the same temperature with α-factor for 150 min and released in the presence of 0.010% MMS. Cultures were then divided in two (always in the presence of MMS) either with or without caffeine. (**B**) The DNA content of cells was determined by flow cytometry at indicated intervals of the experiment described in (A). (**C**) Cell viability during the course of the experiment.

proliferation upon DNA damaging therapy. Accordingly, previous work on p53 mutants indicated that checkpoint defects make cells resistant to irradiation or treatment with chemotherapeutic compounds (62) and established an experimental correlation between p53 mutations and poor prognosis in cancer development (63,64). Our work suggests that in the case of cells that are partially defective in Rad53/Chk2 checkpoint pathways, it may be more effective to use drugs that strongly stall the progression of DNA replication forks.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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