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***RAD53* is limiting in double-strand break repair and in protection against toxicity associated with ribonucleotide reductase inhibition**

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Abstract

The yeast Chk2/Chk1 homolog Rad53 is a central component of the DNA damage checkpoint system. While it controls genotoxic stress responses such as cell cycle arrest, replication fork stabilization and increase in dNTP pools, little is known about the consequences of reduced Rad53 levels on the various cellular endpoints or about its roles in dealing with chronic vs. acute genotoxic challenges. Using a tetraploid gene dosage model in which only one copy of the yeast *RAD53* is functional (simplex), we found that the simplex strain was not sensitive to acute UV radiation or chronic MMS exposure. However, the simplex strain was sensitized to chronic exposure of the ribonucleotide reductase inhibitor hydroxyurea (HU). Surprisingly, reduced *RAD53* gene dosage did not affect sensitivity to HU acute exposure, indicating that immediate checkpoint responses and recovery from HU-induced stress were not compromised. Interestingly, cells of most of the colonies that arise after chronic HU exposure acquired heritable resistance to HU. We also found that short HU exposure before and after treatment of G₂ cells with ionizing radiation (IR) reduced the capability of *RAD53* simplex cells to repair DSBs, in agreement with sensitivity of *RAD53* simplex strain to high doses of IR. We propose that a modest reduction in Rad53 activity can impact the activation of the ribonucleotide reductase catalytic subunit Rnr1 following stress, reducing the ability to generate nucleotide pools sufficient for DNA repair and replication. At the same time, reduced Rad53 activity may lead to genome instability and to the acquisition of drug resistance before and/or during the chronic exposure to HU. These results have implications for developing drug enhancers as well as for understanding mechanisms of drug resistance in cells compromised for DNA damage checkpoint.

Keywords

RAD53; Hydroxyurea; Rnr1; Double strand breaks

1. Introduction

Persistent DNA damage often leads to blockage of DNA replication which can be lethal to dividing cells. Therefore, elaborate mechanisms have evolved that detect and repair DNA

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Conflict of interest
None declared.

Appendix A. Supplementary data

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damage. The damage response is coordinated by protein kinases that can transduce a genotoxic stress signal to various effector molecules, eventually leading to repair of the lesion or allowing DNA replication to proceed even in the presence of stress [1,2]. The central DNA damage transducer in yeast is Rad53, a homolog to mammalian Chk2 and Chk1 [3]. It controls several pathways including cell cycle arrest, prevention of replication fork collapse and resolution of stalled replication forks as well as activation of repair proteins [1,2,4].

Among the important roles of Rad53 is an increase of dNTP pools in response to genotoxic stress by elevating activity of the ribonucleotide reductase (RNR) catalytic subunit Rnr1 [5–7]. Rnr1 catalyzes the reduction of ribonucleotides to deoxyribonucleotides, the rate limiting step in dNTP biogenesis, thereby controlling the amount and balance of dNTPs. The activity of RNR is tightly regulated. It is kept low before S phase [8] but increases during S or in response to DNA damage; failure to induce RNR is lethal [9]. Rad53 activates signal transduction cascades that elevate RNR activity by targeting the RNR inhibitor Sml1 for degradation, increasing components of RNR transcription and importing some of them into the nucleus [6,7,10,11].

Hydroxyurea (HU) is a cancer chemotherapeutic agent that acts by inhibiting Rnr1 and is also used in the treatment of several chronic diseases [12–14]. Reduction of dNTPs below the level sufficient for stable DNA replication leads to formation of single strand gaps [15] which can trigger a DNA damage response and activate Rad53, leading to cell cycle arrest and impeding firing of late replication origins [16–18]. Replication forks that have been initiated are stabilized and can resume after removal of the drug [16]. The Rad53 mediated DNA damage response leads to increases in RNR activity, as described above. However, the extent to which reduction in Rad53 activity can affect the various Rad53-dependent pathways is unknown. Furthermore, information about cellular responses to HU comes primarily from results with acute or short-term exposures. Little is known about toleration of chronic HU exposure especially under conditions of reduced Rad53 activity. This is relevant to addressing chronic administration of HU in patients. Given the many roles of Rad53 in the cell even partial inhibition of Rad53 might be expected to cause increased sensitivity to prolonged genotoxic stress.

Here, we employed our previous approach for addressing the genetic consequences of reduced levels of essential DNA metabolic genes in tetraploid yeast with only one (simplex) out of four copies of a gene [19]. We find that the *RAD53* simplex cells are sensitized to chronic HU exposure. This study provides the first direct evidence that reduction in Rad53 activity reduces double strand break repair (DSBR), especially when RNR is additionally inhibited. These findings have implications for drugs that might target Chk1/Chk2 in order to sensitize cancer cells during DNA damage-based chemotherapy [20,21].

2. Materials and methods

2.1. Strains

The tetraploid yeast cells presented in this study were constructed by crosses of opposite mating type diploids as shown in Fig. S1. Detailed description of how those diploid were constructed is found in [19]. The genotype of the diploid strains are as follows: CS2064: MAT α / α , ade5-1 his7-2 leu2-3,112, trp1-DEL, ura3-Del, met2-DEL, tyr1 300-1359. CS2065: MAT α / α , ade5-1 his7-2 leu2-3,112, trp1-DEL, ura3-Del, met6-DEL, tyr1 1-700.

2.2. RAD53 simplex strain construction

The following oligonucleotides were used to amplify G418^R and Hygromycin^R deletion/disruption cassettes from plasmids pFA6 and pAG32 by PCR [22], respectively for targeted

replacement of *RAD53* open reading frame: 5'
 GCATTCGATTTTCTTAAGCTTTAAAAGAGAGAATAGTGAGAAAAGATAGTGTTA
 CACAACATCAACTAAAA-CGTACGCTGCAGGTTCGAC and 5'
 TTCTGAGTATTGGTATCTACCATCTTCTCTCTTAAAAAGGGGCAGCATTCTAT
 GGGTATTTGTCCTTGG-ATCGATGAATTCGAGCTCG

The *RAD53* heterozygous diploid was constructed by transforming two opposite mating type diploid cells with the targeted G418^R and Hygromycin^R cassettes, respectively. Independent *RAD53* heterozygous isolates derived from those diploids were crossed to create *RAD53* duplexes (two WT alleles with one G418^R and one Hygromycin^R replacement alleles; see Fig. S1). Duplexes were transformed with a *URA3* cassette that was targeted to an internal portion of the *RAD53* ORF (23 aa in-frame) by amplifying *URA3* gene from pRS306 using primers: 5'

TGGAAAATATTACACAACCCACACAGCAATCCACGCAGGCTACTCAAAGGTTTT
 TGATTGAGAAGTTTTCT-CAGAGCAGATTGTACTGAGAGTGCACC and 5'
 ACGAAAATTGCAAATTCTCGGGGCCTTTTGAGGTTTGGTCCAATTTGCCCCTTT
 AACCTTCTTACTAGGA-CGCATCTGTGCGGTATTTACACCCG

Ura⁺ transformants were confirmed to be *RAD53* simplex if they maintained G418^R, Hygromycin^R, were non-mating and Tyr⁺ recombinants could be induced. In addition genomic DNA was purified from the putative simplex and the modifications to the *RAD53* locus were verified by PCR using the flanking primers 5' TGGTGTGGACGCGTTGATA and 5' GGTTACAGCCTCTCCATAGATTCA. Two transformants were isolated from each of 2 independent duplex strains, resulting in 4 independent simplex isolates.

2.3. Nocodazole arrest, gamma irradiation, and post irradiation incubation

The details of nocodazole arrest and gamma irradiation have been described [23,24]. Briefly, nocodazole (20 µg/ml, final concentration) was added to cells that were growing logarithmically at 30 °C in YPDA media (1% yeast extract, 2% Bacto-Peptone, 2% dextrose, 60 µg/ml adenine sulfate). G₂ arrest was monitored by cell morphology. Cells were collected by centrifugation, washed and resuspended in ice-cold sterile water. The cell suspensions were kept on ice while being irradiated in a ¹³⁷Cs irradiator (J. L. Shepherd Model 431) at a dose rate of 2.3 krad/min. Irradiated cells were harvested by centrifugation and resuspended in YPDA at 30 °C with nocodazole for post-irradiation incubation.

2.4. Pulsed field electrophoresis (PFGE) procedures

PFGE procedures were done as previously described [24]. Briefly, Contour-Clamped Homogeneous Electric Field (CHEF) systems were used for electrophoresis of yeast chromosomes using a CHEF Mapper XA system (Bio-Rad, Hercules, CA). Plugs were prepared in 0.5% LE agarose (Seakem, Rockland, ME) using 1–2 × 10⁷ G₂-arrested cells per 100 µl plug. They were cut to a thickness of ~2 mm and loaded in the bottom of a preparative well so that the entirety of the DNA migrated very close to the bottom surface of the CHEF gel. PFGE running conditions were according to the CHEF auto-algorithm to separate DNA's in the 250–1600 kb range.

3. Results

3.1. Rad53 activity is limiting for protection from some genotoxic stressors

Recently, we showed that tetraploid yeast with a reduced amount of Mcd1 cohesin subunit, i.e., *MCD1* simplex, are sensitive to ionizing radiation (IR) and exhibit increased IR-induced genome instability. Interestingly, a similar reduction in Rad50 or Rad51, which directly participate in DSB repair, did not affect responses to IR [19]. We suggested that the

sensitivity of the *MCD1* simplex cells was due to only a small amount of cohesin being available for the multiple tasks of DNA replication, chromosome segregation, transcription and repair, while Rad50 and Rad51, which are present at comparable amounts [25] function mainly in DSB repair.

Given that the Rad53 protein kinase is known to receive signals from a variety of cellular inputs and to transduce signals to several end points, we speculated, by way of analogy with the multifunctional cohesin, that Rad53 would also be limiting for genome maintenance and integrity. To test this hypothesis, we exposed a tetraploid yeast strain that carries only one functional copy of *RAD53* (simplex – for details see Fig. S1) to different genotoxic stressors. The *RAD53* simplex strain showed UV-survival similar to tetraploid cells carrying four copies of functional *RAD53* (wild type – WT) (Fig. 1A). However, the *RAD53* simplex strain was more sensitive to high doses of ionizing radiation (IR) than the WT (Fig. 1B). *RAD53* simplex strains were much more sensitive to chronic exposure to HU within a range of concentrations at which survival of WT was high (Fig. 1C). There was no difference in sensitivity between WT and simplex cells to chronic MMS exposure (Fig. 1D).

3.2. Rad53 activity is limiting in protecting genome stability and survival in response to ribonucleotide reductase inhibition

Upon exposure to HU, replication forks can stall due to an insufficient supply of dNTPs. Stalling can lead to fork collapse which can be rescued by homologous recombination. If homologous recombination involves sequences of homologous chromosomes or ectopic repeats, loss of heterozygosity (LOH, discussed in [19]) and chromosome rearrangements can result. Rad53 reduces the chance of replication fork collapse, thereby lowering levels of LOH and rearrangements via homologous recombination [1,26–30]. Therefore, we measured recombination (see schematic figure of HR assay in Fig. S2) between homologous chromosomes in WT and *RAD53* simplex cells exposed to non-lethal levels of HU (Fig. 2A; survival was more than 50%, Fig. 1). The recombination rate in the *RAD53* simplex, as compared to WT, was slightly higher after 50 mM HU exposure and ~6-fold higher after 100 mM HU exposure (Fig. 2A). This is in agreement with elevated recombination in mutants of *MEC1* that is functionally upstream of *RAD53* [31].

In light of the distinctive sensitivity of the *RAD53* simplex vs. WT to chronic HU exposure, we examined further the effects of HU. Increased HU sensitivity could arise from an inability to tolerate the inhibition of ribonucleotide reductase, resulting in insufficient dNTPs to support DNA replication. Consistent with this, a nonlethal dose of HU caused growth retardation (probably due to slowing cell division cycle) in the simplex as compared to the WT strains (Fig. S3). Unlike the simplex strain, colonies of WT cells were visible within one day of plating onto YPDA plates containing 50 mM HU. However, the *RAD53* simplex colonies became visible only on day 2 and by day 3 were hardly distinguishable from WT (Fig. S3). Moreover, ~70% of the *RAD53* simplex colonies arising on 200 mM HU plates were much smaller than the WT colonies, even after prolonged incubation (up to 7 days; Fig. S4). HU toxicity could also result from a failure of the DNA damage checkpoint to stop DNA replication, which could lead in turn, to improper replication of the centromeres [32]. Unlike *rad53* checkpoint mutants [32], recovery after 3–6 h exposure to 200 mM HU was at most slightly less for *RAD53* simplex as compared to WT, suggesting that both strains have robust checkpoint activity (Fig. 2B). However, when HU exposure was extended to 24 h, the recovery was less than 5% as compared to no loss in viability of the WT strain (Fig. 2B).

3.3. Rad53-mediated control of the level of ribonucleotide reductase activity is limiting for efficient double strand break repair

As shown in Fig. 1B, *RAD53* simplex cells are more sensitive to high doses of IR than WT tetraploid cells, suggesting a partial defect in double strand break repair (DSBR). DSBR of multiple lesions through homologous recombination requires a significant amount of DNA synthesis in order to restore resected DNA molecules. For example, there is approximately 2–4 kb resected DNA per radiation induced DSB in yeast [24]. Therefore, failure to repair multiple DSBs could stem from the same cause as the failure to survive high dose of HU exposure, i.e., inefficient RNR induction. We measured DSBR of broken yeast chromosomes using Pulsed Field Gel Electrophoresis (PFGE) analysis. Repair is calculated by the ability to restore chromosomes after exposure to ionizing radiation (the method was previously described in [23] and is briefly summarized in Section 2). As shown in Fig. 3A, a single copy of *RAD53* resulted in a slower rate of DSBR when G₂ nocodazole-arrested cells were irradiated with 80 krad and incubated further in nocodazole. Within 1 h, the number of DSB/Mb dropped from more than 5 (or ~125 DSBs per G₂ cell) to about 1 in WT cells. There was a modest decrease with further incubation to 0.6–0.7 breaks/Mb, in agreement with previously reports. In contrast, the number in *RAD53* simplex dropped to only 2 DSB/Mb (Fig. 3A and C). With increase in repair time *RAD53* simplex cells were able to repair most of the chromosomes as efficiently as WT. Nevertheless, restoration of a large chromosome (i.e., Chr 4, indicated by arrow in Fig. 3A) was inefficient in the simplex strain compared to WT, even after 4 h (Fig. 3A). The differences in repair kinetics may explain the differences in survival between WT and simplex due to the dominant lethal nature of DSBs (even 1 unrepaired break per cell can be lethal [33]). Since the cells were held in G₂, the reduced DSBR of the simplex cells cannot be related to any difference in capacity to arrest the cell cycle.

While Rad53 plays an important role in DNA damage-induced expression of RNR, it also phosphorylates several proteins involved in homologous recombination-associated DNA repair [34]. To address directly the effect that the level of RNR activity has on DSBR, G₂ nocodazole-arrested WT and *RAD53* simplex cells were exposed to HU beginning 1 h before irradiation and for an additional 4 h after exposure. For the WT, the HU-induced reduction in RNR activity clearly led to a decrease in rate of DSB repair (almost 2 DSB/Mb were left after 1 h of repair in the presence of HU instead of only 1 break/Mb without HU – Fig. 3B and C), although the repair reached the level of untreated cells by 4 h. Repair in the *RAD53* simplex strain was slowed even further by HU; at 2 h after irradiation, 2 breaks/Mb remained, while there were 0.7 breaks/Mb in the WT. Unlike global repair without HU (Fig. 3A), there was still a difference in repair even after 4 h when cells were exposed to HU (Fig. 3B). While HU-exposed WT cells had 0.5 breaks/Mb remaining after 4 h, there were 1.2 breaks/Mb in the simplex cells (Fig. 3C). Thus, not only does a reduction in RNR activity reduce DSBR in G₂ cells, the limited level of Rad53 in the simplex synergizes with HU to inhibit DSBR, suggesting that the repair defect in the simplex strain is likely due to a severe deficiency in dNTPs.

We also measured the effect of irradiation and HU exposure on survival. Cells that were first gamma-irradiated with 80 krad and held for 4 h in nocodazole and 200 mM HU were plated to YPDA. Interestingly, despite the effect on repair, the HU exposure did not have an effect on survival (Fig. S5A). This might be due to cells being able to complete double strand break repair after plated on to HU-free YPDA media. Subsequently, we transiently arrested cells with nocodazole, gamma-irradiated them, and then plated to YPDA containing 200 mM HU. Surprisingly even unirradiated cells exhibited a synergistic lethal effect of nocodazole and HU exposure (Fig. S5B). This may indicate a connection between the spindle assembly checkpoint and the DNA replication initiation checkpoint. We also irradiated logarithmically growing cells and plated them on to media containing 200 mM

HU. *RAD53* simplex, but not the WT cells, exhibited an approximate 10-fold reduction in viability below that expected for independent killing by radiation and HU (Fig. 3D). Thus, continuous RNR repression sensitizes Rad53-compromised cells to radiation. This finding is important for understanding how drug resistant variants of the *RAD53* simplex mutant arises (see below).

3.4. Drug resistance of survivors after HU treatment

While 97% of simplex cells failed to form colonies when plated on the medium with 200 mM HU, the survivors formed colonies of varying size, including very small colonies (Fig. S4). The HU-survivor colonies that grew for 7 days on 200 mM HU plates were streaked onto HU-free YPDA media. Descendants of the small HU-survivor colonies gave rise to normal size colonies on HU-free medium (Fig. S4 lower panel) indicating that the HU-survivors had not suffered from catastrophic chromosome instability and have functional mitochondria. Next, the ability of the descendants of HU-survivors to survive chronic HU exposure was determined. The *RAD53* simplex descendants from the small and big HU colonies showed much better growth than the parental simplex strain on HU (Fig. 4A) indicating that these colonies had inherited HU resistance (discussed below). Survival measurements for the 4 different cell types (*RAD53* simplex and WT parental strains as well as descendants of big and small *RAD53* simplex that survived 200 mM HU) revealed that descendants of big survivors had almost WT survival levels and that descendants of small colonies had slightly lower survival but still much higher than the *RAD53* simplex parental strain (Fig. 4B).

Next, we followed another set of descendants of *RAD53* simplex colonies that had survived 200 mM HU, for both HU and IR resistance. Interestingly, while two descendants showed both HU and IR survival, one exhibited high HU tolerance and low IR tolerance, while the other showed low HU, but high IR tolerance (Table in Fig. 4C). The higher survival of the HU-exposed descendent colonies is likely due to a pre-existing sub-population among the *RAD53* simplex cells that had acquired an extra copy (or copies) of *RAD53*, since chromosome increase is frequent in tetraploids [35,36]. If this were the case, then most of the *RAD53* simplex survivors of IR exposure should give rise to descendent colonies that tolerate IR like WT and not like *RAD53* simplex (Fig. 1B). However, unlike the results with HU, none of 6 IR survivors examined had acquired WT IR-survival (Fig. 4D). Surprisingly, at least one of the IR survivors showed enhanced HU resistance (Fig. 4D).

Taken together, our results suggest that inheritance of resistance to HU in *RAD53* simplex cells can occur in more than one way and it may be induced by the HU exposure itself. More experiments will be required to fully understand the nature of HU survivors in molecular terms. Importantly, the difference between HU-induced lethality of WT as compared to simplex cells is even greater than it appears from the survival curves, because nearly all survivors in the simplex are actually resistant variants.

4. Discussion

Of the multiple roles ascribed to Rad53 in cellular responses to chromosomal stress, protection against toxicity associated with HU inhibition of RNR activity turned out to be the most sensitive to reduction in levels of Rad53 (Fig. 1). This conclusion is in agreement with the hypomorphic mutant *rad53* HA which has low levels of Rad53 and is very sensitive to low doses of HU (10 mM; see [37]). Interestingly, *RAD53* simplex cells recover from short exposures to high doses of HU (Fig. 2B), indicating a robust capacity for checkpoint arrest.

Cellular levels of dNTPs are very limited; even during S phase, at any given instant, there are only enough dNTPs to replicate a tiny fraction of the genome [38]. Therefore, we propose that the hypersensitivity of *RAD53* simplex to HU is due to an inability to increase the activity of RNR, and consequently the dNTP pools, to levels sufficient for DNA replication. While *RAD53* simplex cells may be able to protect their genome from a temporary shortage in dNTP pools, prolonged exposure to HU may cause checkpoint adaptation, leading to cell division without complete DNA replication followed by cell death [4,39,40]. Surprisingly, we found that survivors of *RAD53* simplex strain exposed to 200 mM HU confer at least partial HU resistance to their descendants (Fig. 4 and Fig. S4). Interestingly, the *RAD53* simplex colonies that survived HU chronic exposure varied in size and their descendants varied in the extent of HU resistance. Transmission of the capacity to survive HU exposure to the daughter cells is likely due to a genetic change in the parental population, but this can only be established with the identification of such change(s). At this point we cannot determine if the genetic change pre-existed in the *RAD53* simplex population or was induced during the long exposure to the drug. The genomes of tetraploid yeast cells are unstable, exhibiting changes in chromosome number at frequencies that are 100–200 fold greater than in diploids [35,36]. Therefore, the HU-resistant variants of *RAD53* simplex are likely to have arisen through a change in gene dosage due to chromosome gain (or loss). Nevertheless, dominant mutations, or silencing of key genes in nucleotide metabolism such as *SML1* are possible. The *RAD53* simplex HU-resistant isolates also could have been due to increased copy number of the RNR1 wild type alleles or factors that assist RNR components import into the nucleus like Dif1 [11].

Since chronic administration of hydroxyurea is commonly used in the treatment of several diseases such as sickle cell anemia [13], it is important to understand sources of HU resistance. Moreover, HU is a model for DNA replication stress. Acquiring resistance to such stress is an important feature in a wide range of pathological conditions, including cancer cells and pathogenic microbial cells. Particularly relevant to this study is the acquisition of HU-resistance when cells are also exposed to inhibitors of the DNA damage response pathway, that may mimic the *RAD53* simplex phenotype [41–45].

We were able to show that Rad53 activity is limiting for DSB repair and an impediment to the induction of RNR activity is the most likely cause (Fig. 3). The tetraploid G₂ cells exhibited fast repair, fixing nearly 90% of DSBs within 4 h similar to the repair efficiency in diploid G₂ cells [23]. Pretreatment with HU slowed down repair, presumably because there is only a small supply of dNTPs. The completion of repair was likely due to the response to DNA damage which can lead to increased RNR. Wild type levels of Rad53 are important for RNR DNA damage activation since RNR inhibited by HU in *RAD53* simplex cells substantially slows down DSB repair (Fig. 3). These results are in agreement with the need for robust DNA synthesis during the gap filling step of homologous recombination. Previously, we had reported that up to 5% of the yeast genome would need to be re-synthesized to replace the nucleotides lost during DNA end resection after a dose of 80 krad to diploid G₂ cells [24]. Since the cellular nucleotide pool is sufficient for replication of only small percentage of the genome even at S phase, a significant increase in dNTP production after IR at G₂ is required. These results also suggest a strategy for radiation-based therapy that combines HU and DNA damage response inhibition [21] especially since the combined effect of HU and irradiation seems to reduce the total survivors and thus reduce the number of HU resistant cells (Fig. 3D).

The need for RNR activity in the repair of DSBs by homologous recombination is not confined to *Saccharomyces cerevisiae*. Recently, DNA damage induction of RNR activity via the ATR pathway was also shown to be important to recombinational repair of DSBs in *Schizosaccharomyces pombe* [46]. Also, mammalian cells deficient in non-homologous end

joining are extremely sensitive to HU, due to defects in HR [47]. The RNR subunit RRM2B is strongly induced in human cells by DNA damage in a p53-dependent manner. The p53 is stabilized through the DNA damage-dependent protein kinase ATM, suggesting a functional conservation of activation of RNR via DNA damage responses between yeast and humans. It will be interesting to determine if the DNA damage response in human cells is limiting for RNR activation as shown here for yeast.

Overall, this study provides a model based in tetraploid cells for addressing limiting components in DNA damage response especially if a gene is essential. Using this system, our results with HU have suggested how different therapeutic approaches might be effectively combined even if each of them has a partial inhibitory effect.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

RAD53 simplex is hypersensitive to IR and hydroxyurea. In all cases at least 6 cultures from each genotype were analyzed. (A) Liquid cultures were grown over night, diluted and pronged to YPDA plates (using a pronging that delivers 1 μ l per drop and 121 drops per plate as described on line at <http://m.pu.ru/images/stories/Perfect%20order%20plating.html>), then UV-irradiated; survival was determined after 3 days. (B) Overnight cultures were diluted and arrested with nocodazole for 3 h, then cells were resuspended in water, irradiated with the indicated doses using a Cesium source and plated on YPDA plates; survival was determined after 3 days. (C) Overnight cultures were diluted and pronged to YPDA plates containing the indicated amounts of HU, incubated for 4 days for lower doses and up to 7 days for the 200 mM dose. (D) As in (C), but cells were pronged to MMS YPDA plates and incubated for 4 days.

Fig. 2.

Rad53 is limiting in protection against toxicity associated with ribonucleotide reductase activity. (A) In order to determine the rate of HU-induced recombination between homologous chromosomes, overnight cultures were pronged on plates with the indicated HU concentrations, as described in Fig. 1C. After 3 days several independent colonies were collected re-suspended and spread to complete plates and plates lacking tyrosine. The rates of recombination were determined after 3 days (*TYR1* recombination assay is described in Fig. S2). In order to determine the rate of spontaneous recombination between homologous chromosomes, small cultures were grown on YPDA plates, resuspended and after appropriate dilutions spread to plates lacking tyrosine or complete plates. For each HU concentration at least 6 independent recombination measurements were made for each genotype; results are presented as mean $\pm$ SEM. B. WT and *RAD53* simplex strain cultures (5 cultures for each genotype) were grown overnight, diluted 100-fold and grown in YPDA for 30 min; then HU was added to a concentration of 200 mM. Samples were taken to assess colony forming units.

Fig. 3.

WT Rad53 amount are needed for Rnr1 activation to support efficient DSB repair. WT and *RAD53* simplex cultures were G₂-arrested with nocodazole; after 2 h the cultures were split and to half of each culture HU was added to a final concentration of 200 mM; cells were incubated further for another hour. The cultures were resuspended in water and irradiated with 80 krad (see conditions in Fig. 1). Finally, cells were resuspended in warm medium containing nocodazole with or without 200 mM HU and incubated for 4 h. Samples were taken from the cultures, before irradiation, just after radiation and following 1, 2 and 4 h of recovery. The samples were processed to agarose DNA plugs and the yeast chromosomes were separated using pulsed field gel electrophoresis (PFGE; see Section 2). Gels were stained with Cyber Gold dye. The amount of repair was calculated as described in [19,23,24]. (A) Representative gels demonstrating broken and repaired yeast chromosomes; cultures were not treated with HU. (B) As in (A) but cultures were treated with 200 mM HU. (C) Quantification of repair kinetics with or without HU exposure in WT and *RAD53* simplex strains (3 repeats; presented are the mean $\pm$ SEM). The determination of the initial DSB/Mb (time "0") was calculated from cultures irradiated in the same conditions but with 10 and 20 krad; a linear IR dose to DSB/Mb was assumed in agreement with previous results [23,24]. (D) Cultures grown to logarithmic phase were irradiated with 80 krad, then cells were spread on YPDA or YPDA + 200 mM HU plates and survival was determined after 4 days. The survival expected with independent killing by IR and HU treatment was calculated by multiplying survivals measured with each agent applied separately (0.2% for IR and 3% HU (Fig. 1 and Fig. S5)).

Fig. 4. Descendants of *RAD53* simplex HU survivor colonies are more resistant to the drug than the parental strain. (A) Colonies arising from cells that survived 200 mM (see examples in Fig. S4) were streaked or spread to YPDA plates. Next, independent descendant colonies (WT, *RAD53* simplex parental and descendants of small and big colony HU survivors) were collected, resuspended in 200 μ l water, diluted serially (10-fold dilutions) and pronged to YPDA containing 200 mM HU and YPDA only. Plates were examined after 4 days. (B) Descendants from small and big survivors of HU (*RAD53* simplex) were resuspended in 10 ml water and pronged on YPDA 200 mM HU and YPDA only plates; survival was calculated after 7 days, as described in Fig. 1. Presented are the survivals of cells from independent colonies. “P” is the *RAD53* simplex parental strain, “S” and “B” are descendants of small and big *RAD53* simplex HU survivors and “WT” is the wild type tetraploid (*note*: results for WT and *RAD53* simplex parental strains are the same as those presented in Fig. 1C, 200 mM HU). (C) Four *RAD53* simplex HU survivors were streaked on YPDA plates, single colonies were taken and resistance to 80 krad and 200 mM HU was determined in the same manner as in Fig. 1 (2 of the isolates were presented in panel B above and 2 were independently collected). The mean survival of 2 repeats is presented. (D) *RAD53* simplex single colonies that survived 80 krad IR were streaked on YPDA to obtain descendant colonies; the IR-resistance of cultures propagated from these colonies was then determined. Additional single colony isolates of the same IR survivors were resuspended in 200 μ l water, diluted in 10-fold serial dilutions and pronged to YPDA 200 mM HU plates. Colonies were examined after 4 days.