Stress Activated Protein Kinase Pathway Modulates Homologous Recombination in Fission Yeast

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Abstract

Rad52 is a key player in homologous recombination (HR), a DNA repair pathway that is dedicated to double strand breaks repair and recovery of perturbed replication forks. Here we show that fission yeast Rad52 homologue is phosphorylated at specific amino acids in response to oxidative stress. This phosphorylation is dependent on a stress activated protein kinase (SAPK) pathway and can be partially prevented by anti-oxidant treatment. Importantly, impairing HR by deletion of the gene encoding the recombinase Rhp51 leads to Sty1 dependent Rad52 phosphorylation. Thus, SAPK pathway impinges on early step of HR through phosphorylation of Rad52 in cells challenged by oxidative stress or lacking Rhp51 and is required to promote spontaneous gene conversion and recovery from blocked replication forks.

Introduction

UVA radiation is the most abundant solar UV radiation that reaches earth's surface. UVA is able to penetrate human skin deeper than UVB and reaches the basal layer of skin where actively replicating keratinocytes are present. Different from UVB, UVA is weakly absorbed by DNA and mainly acts through interaction with endogenous photosensitizers resulting in generation of reactive oxygen species (ROS), predominantly singlet oxygen, which can damage all cellular components [1,2,3]. UVA induced ROS have been linked to skin photoaging [4] and increasing evidence suggest contribution to skin carcinogenesis [5].

More generally, ROS-induced cellular damage is linked to pathological conditions such as cancer, diabetes, atherosclerosis, neurodegenerative diseases and premature aging [6,7,8,9,10]. Because UVA induced biological effects are oxygen dependent, UVA is an inducer of oxidative stress and thus, cellular response to this radiation is complex. As many stresses, UVA-induced oxidative stress activates the SAPK pathways that are characterized by a cascade of kinases highly conserved: MAPK (mitogen activated protein kinase) kinase kinases (MAPKK), MAPK kinases (MAPKKK) and MAPKs. Eventually, activation of SAPK pathways results in protection of cells from injuries through appropriate regulation of gene expression and protein translation [11,12]. In human keratinocytes the p38 MAP kinase and JNK (c-Jun N-terminal kinase) pathways are activated by UVA radiation [13].

In Schizosaccharomyces pombe (S. pombe), Sty1/Spc1 is the principal MAPK, mainly related to human p38, which is activated by phosphorylation following a variety of stresses including high osmolarity, oxidative stress, UVC exposure [14,15] and metabolic inputs such as caloric restriction and histidine starvation, two situations generating endogenous oxidative stress [16,17]. Sty1 is also required for sexual differentiation by regulating Ste11 expression, the major transcription factor required to induce meiotic genes [18]. Activation of Sty1 results in its nuclear localization and induction of gene expression mainly through the Aft1 transcription factor. Such modulation of the gene expression program will provide cells with the necessary to face stress [19]. Furthermore, Sty1, but not its major target Aft1, controls mitotic entry upon nutritional stress [20,21]. However, different degrees of Sty1 activation impact on mitotic commitment in opposite ways: basal level of Sty1 activity promotes mitotic onset while high level of Sty1 activity delays mitotic entry [22]. This function is also conserved in mammals where the extracellular signal-related kinase (ERK) is required for mitotic entry and p38 activation by stress is required for mitotic delay [23,24,25]. In addition, different ways of Sty1 activation according to the stimulus applied have been reported, implying that Sty1, similarly to mammalian SAPKs, is finely tuned in the cell [26,27,28].

Nowadays, it is well established that UVA solar radiation is a biological relevant genotoxic agent [29]. At the DNA level UVA induces formation of oxidized bases, mainly 8-oxoguanine (8-
oxoG), cyclobutane pyrimidine dimers (CPDs), pyrimidine (6-4) pyrimidine photoproducts (6-4PPs), single strand breaks and DNA protein cross-linking [2]. 8-oxoguanine DNA glycosylase OGG1, which removes 8-oxoG, efficiently prevents UVA-induced mutagenesis in yeast *Saccharomyces cerevisiae* [30].

HR is a DNA repair pathway dedicated to DSBs repair and recovery of blocked replication forks. Failure to repair DSBs results in cell death while inaccurate repair results in genome instability. HR must be tightly regulated to avoid dangerous outcomes that challenge genome stability and to prevent accumulation of DNA structures that are toxic for the cell [31]. At stalled replication forks HR allows to resume replication following fork collapse, but this mechanism can potentially induce gross chromosomal rearrangements and thus genomic instability [32].

*S. pombe* encodes a Rad52 homologue called spRad22. Here we will refer to the fission yeast protein as Rad52. This protein, a so-called mediator protein central to HR, is essential for both Rad51 (spRhp51) dependent and independent DSB repair pathways [33,34]. Rad52 is required to replace Replication protein A (RPA) bound to single stranded DNA (ssDNA) by Rad51 (called Rhp51 in fission yeast), which will in turn promote homology search and D-loop formation [35]. However, Rad52 can promote HR in a Rad51 independent way because of its ability to anneal complementary single stranded DNA [36].

Recombination proteins, including Rad52, localize at double strand breaks to form discrete foci that occurs either spontaneously in S phase cells or after induction of DNA damage. In budding and fission yeast, Rad52 foci formation is a marker of ongoing HR [37,38].

We previously reported that, in both mammals and fission yeast, DNA replication is perturbed by UVA exposure and that, despite activation of checkpoint pathways, UVA induced delay in DNA synthesis is largely checkpoint and Sty1 independent [39,40]. In addition, we have shown that cells lacking Sty1 are UVA sensitive, indicating that the SAPK pathway plays an important role in response to this radiation also in fission yeast. Furthermore, cells exposed to UVA during S phase accumulate HR foci and this DNA repair pathway is required for survival to UVA [39].

We sought to further investigate on the interplay between HR and SAPK pathways in response to oxidative stress using UVA and hydrogen peroxide (H$_2$O$_2$). Here we report that Rad52 is phosphorylated in cells exposed to exogenous oxidative stress or in cells lacking Rhp51 and that phosphorylation is dependent on efficient SAPK pathway. Disabling the SAPK pathway delays resolution of UVA-induced Rad52 foci, affects spontaneous HR and HR occurring at blocked replication forks.

### Materials and Methods

#### Yeast strains, media, growth conditions

The strains used in this study are listed in Table 1. Standard techniques were used for yeast growth and strains construction. Strains were grown in YE-rich medium (DIFCO) containing 2% glucose and supplemented with adenine, leucine, uracile, arginine and histidine [41]. Strains containing the RuraR substrate were grown in EMM glutamate (MP Biomedical) medium with or without thiamine as described in [32].

#### Genotoxic, oxidative and anti-oxidant treatments

Synchronization in early S phase was achieved by 4 hours treatment with 12 mM hydroxyurea (HU) (Sigma). Cells were then collected, resuspended in H$_2$O and UVA irradiated as described in [39]. UVC irradiation was achieved using Strata-linkerTM (Stratagene) as described in [42]. Camptothecin (CPT) (Sigma) and H$_2$O$_2$ treatment (Sigma) was performed by releasing HU synchronized cells into fresh medium containing the chemical at the indicated concentrations. Anti-oxidant treatment was achieved adding 30 mM of N-acetylcysteine (NAC) (Sigma).

#### SDS-PAGE, immunoblot and phosphatase treatment

Protein extracts were done according to [42]. To analyze Rad52, 80 mg (unless differently stated) of each protein extract was separated by electrophoresis at 40 Volts over-night on 7.5% acrylamide SDS-PAGE (acrylamide : bis-acrylamide 37.5: 1) using the STURDIER vertical SE 400 gel unit (Hoefer Scientific Instruments). Proteins were transferred for 2 hours at 120 Volts on nitrocellulose membrane (PROTRAN Whatman) using a Biorad Trans-Blot® Cell system. Membranes were probed with mouse anti-GFP antibody (Roche).

Equal amount of protein extracts were incubated for 1 hour at 30°C with or without I phosphatase (New England Biolabs) according to manufactury instructions.

#### Microscopy and flow cytometry

Percentage of cells with Rad52 foci was scored on microphotographs as described in [37]. DNA content was analyzed by staining fixed cells with sytox green (Invitrogen) followed by flow cytometry analysis with FACSCalibur flow cytometer (Becton Dickinson). Data were plotted using CellQuest software. Percentage of cells with 2 nuclei was scored by staining fixed cells with DAPI (4',6-diamidino-2-phenylindole, Sigma).

Intracellular peroxide levels were measured as described in [16]. Briefly, 1 ml of exponentially growing cells was incubated with 30 mM DHR123 (Dihydrorhodamine 123, Invitrogen) and 4.4 mM PI (Propidium Iodide, Sigma) for 30 minutes in the dark at 30°C. ROS production (DHR123) and dead cells (PI) were simultaneously analyzed using a FACSCalibur flow cytometer. ROS levels of unstained PI cells (living cells) were normalized to cell size.

#### Fluctuation test and HR rate estimation

Fluctuation test was done as follow: 9 Ade- His+ colonies were independently inoculated in 10 ml of YE-rich medium and incubated at 30°C with agitation till cultures were around 5×10$^7$ cells/ml for reasons explained in the text. Cells were plated on YE-rich medium to estimate viability. About 3×10$^5$ cells were plated on EMM medium lacking adenine to estimate the frequency of Ade+ recombinants. Plates were replicated on EMM lacking adenine and histidine to estimate frequency of Ade+ His+ recombinants. Frequencies were analyzed by MSS-ML extensional maximum likelihood estimator method with the program FALCOR (Fluctuation AnaLysis CalculatOR) to estimate the rate of recombination [43]. For each strain at least three independent experiments of nine cultures each were performed.

#### RuraR assay, RFLA and PFGE

Procedure for RFLA (Restriction Fragment Length Analysis) to determine % of recombination at blocked replication forks and PFGE (Pulse Field Gel Electrophoresis) are extensively described in [44,45]. Briefly, one single colony of each strain was grown in 10 ml of EMM + thiamine (OFF) for 24 hours at 30°C. Cells are then washed twice and inoculate in EMM + thiamine (OFF) and EMM - thiamine (ON) for 24 hours. Recombination between the RTS1 inverted repeats is detected by Southern blot on genomic DNA digested either with Ase I or with EcoV restriction enzyme using the *wad* probe. % of recombination was estimated on the
basis of three independent experiments. Analysis of acentric chromosome III by PFGE was done using rng3 probe.

### Results

Rad52 is phosphorylated in cells undergoing S phase upon UVA exposure

We have previously shown that cells irradiated with UVA in early S phase delay DNA replication and accumulate Rad52 foci, a marker of HR, in a dose dependent manner. We also showed that Rad52 is required for survival of cells irradiated in S phase, indicating that HR is necessary to repair some of the DNA lesions induced by UVA radiations [39].

We took advantage of the strain rad52YFP, which expresses the Rad52 protein tagged in C-terminal with YFP (Yellow Fluorescent Protein) [37], to analyze potential post-translational modifications of Rad52 protein by immunoblot with anti-GFP antibodies in cells exposed to UVA in early S phase. This strain was previously shown to delay DNA replication upon UVA exposure in early S phase similarly to act cells [39].

Cells were synchronized in early S phase by HU treatment and released into cell cycle after UVA irradiation at 0 or 400 kJ/m2. Cell cycle progression was followed by FACS analysis. Time 0 corresponds to the moment where cells were released into cell cycle immediately after irradiation. As expected, DNA replication was delayed in UVA treated cells if compared to untreated ones (Figure 2A). However, despite delaying S phase, DNA replication in UVA irradiated cells did not display the slower migrating form of Rad52 if compared to untreated ones (Figure 2A). However, despite delaying S phase, DNA replication was delayed in UVA treated cells if compared to untreated ones (Figure 2A). Thus, this experiment shows that Rad52 is phosphorylated in response to UVA radiation.

Rad52 phosphorylation is observed upon oxidative stress, but not after UVC or CPT treatment

In order to establish if Rad52 phosphorylation is specific to UVA treatment, we examined it in S phase cells exposed to different DNA damaging agents such as UVC radiation and CPT.

UVC radiation directly damages DNA producing mainly CPDs that block replication fork progression [46]. Thus, we analyzed the Rad52 protein in cells synchronized in early S phase and exposed to 100 J/m2 UVC radiation prior release into cell cycle. DNA replication progression was followed every 30 minutes by FACS analysis. Time 0 corresponds to the moment where cells were released into cell cycle immediately after irradiation. As expected, DNA replication was delayed in UVC treated cells if compared to untreated ones (Figure 2A). However, despite delaying S phase, UVC irradiated cells did not display the slower migrating form of Rad52 indicating that UVC radiation does not induce detectable Rad52 phosphorylation (Figure 2A).

CPT is a topoisomerase I inhibitor that, at the concentration of 20 mM, generates DSBs in S phase cells and formation of Rad52 foci. Indeed, similarly to UVA [39], HR is required for cell survival to CPT treatment and cells activate Chk1 kinase to delay G2/M transition [47,48,49]. Thus, we analyzed S phase progression and Rad52 phosphorylation in HU synchronized cells released into cell cycle in the presence of 20 mM CPT. Under these conditions and in contrast to UVA and UVC, S phase progression was not delayed as judged by FACS analysis (Figure 2A) but, as expected, entry into mitosis was delayed by the treatment as judged by the persistence of cells with one nucleus in CPT treated culture (Figure 2A). However, similarly to UVC exposure, Rad52 phosphorylation was not detected (Figure 2A).

Then, we asked if an oxidative agent different from UVA would induce replication delay and Rad52 phosphorylation, and if both would depend on the presence of ROS. Thus, cells synchronized in early S phase were released into cell cycle in the presence of 250 mM of H2O2 or in the presence of H2O2 and 30 mM of NAC, an anti-oxidant able to counterbalance H2O2 effects. Cell cycle progression and Rad52 phosphorylation were assessed at different time points (Figure 2A). Thus, this experiment shows that Rad52 is phosphorylated in response to UVA radiation.
different time points by FACS analysis and western blot, respectively. In cells exposed to H$_2$O$_2$ DNA replication was delayed (compare FACS panel in figure 2B to FACS panel "untreated" in figure 2A) and Rad52 protein was phosphorylated starting at 30 minutes from release (Figure 2B). When cells were released into cell cycle in presence of both oxidant and NAC, cell cycle delay was diminished if compared to cell exposed only to H$_2$O$_2$, although it was not completely abolished. Indeed, in cells treated with H$_2$O$_2$ the first round of replication ended around 150 minutes post release (Figure 2B), in the presence of H$_2$O$_2$ and NAC it was completed at 90 minutes (Figure 2C), while in untreated cells DNA replication ended around 60 minutes (Figure 2A “untreated”). Furthermore, Rad52 phosphorylation was barely visible in cells exposed to H$_2$O$_2$ along with NAC (Figure 2C).

In conclusion, UVC and CPT did not induce Rad52 phosphorylation, while treatment with 250 mM H$_2$O$_2$ resulted in DNA replication slow down and Rad52 modification, as for UVA radiation. Furthermore, NAC prevented in part both cell

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Figure 1. Rad52 is phosphorylated in S phase cells exposed to UVA radiations. (A) Protein extracts analyzed by immunoblot with anti-GFP antibodies (upper panel) to detect Rad52 protein from rad52YFP cells synchronized in early S phase by HU (block), collected in water and released in fresh medium after 0 or 400 kJ/m$^2$ UVA for different time points. “asyn” indicates cells in log phase. Star refers to the same membrane colored with Rouge Ponceau to serve as loading control. An aliquot of cells at different time points was used to estimate cell cycle progression by FACS analysis and by scoring the percentage of cells that passed mitosis (cells with 2 nuclei). 1C and 2C indicate DNA content. (B) The change in mobility shift of Rad52 protein upon UVA is abolished by treatment with 1 phosphatase (PP).

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cycle slow down and Rad52 phosphorylation, pointing to the possibility that both events rely on ROS.

Rad52 phosphorylation is Sty1 dependent

We previously showed that checkpoint Chk1 kinase is phosphorylated in S phase by HU (block), collected in water and released into fresh medium either without treatment (untreated), or after exposure to 100 J/m² of UVC radiation, or in the presence of 20 mM of CPT. 1C and 2C indicate DNA content. Percentage of cells with 2 nuclei scored in untreated and CPT treated cells shows the delay in mitotic entry imposed by the treatment (right panel). Time 0 is immediately after release into cell cycle in presence or not of CPT. Aliquots of cells at different time points were processed for protein extracts that were analyzed by immunoblot with anti-GFP antibodies to detect Rad52 protein. (B) Cell cycle progression and Rad52 phosphorylation at different time points of rad52YFP cells synchronized in early S phase (block) and released into cell cycle in the presence of 250 mM of H₂O₂. (C) Cell cycle progression and Rad52 detection at different time points of rad52YFP cells synchronized in early S phase (block) and released into cell cycle in the presence of 250 mM of H₂O₂ and 30 mM of anti-oxidant (+ NAC).

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Figure 2. H₂O₂, but not UVC radiation or CPT, induces Rad52 phosphorylation. (A) FACS analysis (left panel) of rad52YFP cells synchronized in early S phase by HU (block), collected in water and released into fresh medium either without treatment (untreated), or after exposure to 100 J/m² of UVC radiation, or in the presence of 20 mM of CPT. 1C and 2C indicate DNA content. Percentage of cells with 2 nuclei scored in untreated and CPT treated cells shows the delay in mitotic entry imposed by the treatment (right panel). Time 0 is immediately after release into cell cycle in presence or not of CPT. Aliquots of cells at different time points were processed for protein extracts that were analyzed by immunoblot with anti-GFP antibodies to detect Rad52 protein. (B) Cell cycle progression and Rad52 phosphorylation at different time points of rad52YFP cells synchronized in early S phase (block) and released into cell cycle in the presence of 250 mM of H₂O₂. (C) Cell cycle progression and Rad52 detection at different time points of rad52YFP cells synchronized in early S phase (block) and released into cell cycle in the presence of 250 mM of H₂O₂ and 30 mM of anti-oxidant (+ NAC).
either 0 or 400 kJ/m² UVA. and released into cell cycle for 0 and 90 minutes after treatment with protein from rad52YFP strain was analyzed at 0 and 90 minutes after release. (Figure 3C). At 180 minutes, about 85% of cells lacking Sty1 had Rad52 foci, in contrast to 55% in control strain (Figure 4C). It is to note that the % of cells with Rad52 foci in sty1-d cells released from the HU block without exposure to UVA radiation is greater than in rad52YFP cells up to 60 minutes, but then reaches set levels suggesting that the first round of replication after HU is perturbed in cyld lacking Sty1.

In addition, as shown in figure 4D, deletion of Sty1 also abolished Rad52 phosphorylation in asynchronous cells exposed to UVA and lacking Rad3 kinase (rad3-d sty1-d rad52YFP).

It has been shown in fission yeast that the stress responsive protein Srr2 is phosphorylated in a Sty1 dependent manner upon stress, resulting in nuclear translocation and binding to Rd4 (TopBP1) [51], a scaffold protein that plays a role in both DNA replication and checkpoint response [52]. Thus, we asked if Srr2 might be required for the Sty1 dependent phosphorylation of Rad52 upon UVA. To answer this question we constructed a strain deleted for srr2 gene and expressing Rad52YFP protein (srr2-d rad52YFP). This strain was synchronized in early S phase, irradiated or not with UVA and released into cell cycle. Immediately (0) and at 90 minutes after release, the Rad52 phosphorylation was examined. As shown in Figure 4E, absence of Srr2 protein did not prevent Rad52 phosphorylation. Then we asked if phosphorylation of Rad52 is Sty1 dependent in cells exposed to H2O2. Treating sty1-d cells with 250 mM of oxidant repetitively resulted in almost undetectable Rad52 protein (data not shown), likely due to the Sty1 requirement to support protein synthesis upon exposure to H2O2 [53]. Thus, we assessed Rad52 phosphorylation upon exposure to lower concentration of oxidant (100 mM) in both Sty1 proficient and deficient cells. 80 and 120 mg of protein extracts were analyzed for rad52YFP and sty1-d rad52YFP cells, respectively. As shown in Figure 5, Rad52 phosphorylation was detected in rad52YFP but not in sty1-d cells although the treatment with 100 mM H2O2 delayed cell cycle progression in both strains as judged by FACS analysis and by scoring the percentage of cells that passed mitosis (cells with 2 nuclei).

In conclusion, Rad52 phosphorylation in cells exposed to either UVA or H2O2 is Sty1 dependent.

Lack of Sty1 affects spontaneous rate of gene conversion

One possibility explaining the persistence of Rad52 foci in sty1-d cells is that HR is less performing in cells lacking the Sty1 kinase. Thus, we assessed the rate of spontaneous HR by fluctuation test in set and sty1-d cells using the system described in Osman et al. [54]. This system allows measuring the frequency of recombination between non-tandem hetero-allelic duplications of the ade6 gene separated by a region of DNA carrying the his3" gene. With this substrate two classes of Ade+ recombinants can be assessed immediately after release (time 0) and after 90 minutes (Figure 3B). At both temperatures Rad52 phosphorylation was detected indicating that Cdc2 is unlikely the kinase required for such modification.

We then asked if Rad3 (ATR), the main upstream kinase in DNA damage checkpoint, or Tel1 (ATM) kinases were involved in Rad52 phosphorylation. Because cells lacking Rad3 (rad3-d) cannot be synchronized by HU in early S phase, we used asynchronous cultures to assess the phosphorylation state of Rad52 in this genetic background. Differently, cells lacking Tel1 (tel1-d) were synchronized by HU and released into cell cycle after exposure to either 0 or 400 kJ/m² UVA. We found that upon UVA exposure Rad52 phosphorylation is still detected in both rad3-d and tel1-d cells (Figure 3C).

Because UVA treated cells experience oxidative stress that elicits activation of the SAPK pathway, we asked if Sty1 kinase would be involved in Rad52 phosphorylation upon UVA. At first, we compare Rad52 expression from exponentially growing cells expressing or not Sty1 (sty1+ rad52YFP and sty1-d rad52YFP strains) (Figure 4A) and found no differences between the two strains. Then we analyzed Rad52 in sty1-d rad52YFP cells exposed to UVA radiation. Rad52 protein was detected prior HU addition (asyn), after 4 hours of HU treatment (block), and every 30 minutes from time 0 till 120 minutes after irradiation. As a control Rad52 protein from rad52YFP strain was analyzed at 0 and 90 minutes from release after UVA exposure. As shown in Figure 4B, Rad52 phosphorylation was not detected in UVA-treated cells lacking Sty1, although cell cycle progression was clearly delayed by radiations as judged by the delay in the appearance of cells that have passed mitosis.

Because S phase cells exposed to UVA radiation accumulate Rad52 foci in a dose dependent manner [39] and we showed that Rad52 phosphorylation is Sty1 dependent, we asked if the kinetics of HR foci formation would be affected in sty1-d cells. To determine the % of cells having Rad52 foci, rad52YFP and sty1-d rad52YFP cells were synchronized in early S phase, exposed or not to 200 kJ/m² of UVA and then released into cell cycle. Percentage of cells with Rad52 foci was scored at different time points. Rad52 foci were induced by UVA in both strains, however they persisted longer in sty1-d rad52YFP cells if compared to rad52YFP strain (Figure 4C). At 180 minutes, about 85% of cells lacking Sty1 had Rad52 foci, in contrast to 55% in control strain (Figure 4C). It is to note that the % of cells with Rad52 foci in sty1-d cells released from the HU block without exposure to UVA radiation is greater than in rad52YFP cells up to 60 minutes, but then reaches set levels suggesting that the first round of replication after HU is perturbed in cells lacking Sty1.

We then asked if Rad3 phosphorylation upon UVA is independent of Chk1, Cdc2, Rad3 and Tel1 kinases. (A) Cells expressing or not the Chk1 kinase were irradiated with either 0 or 400 kJ/m² UVA after synchronization in early S phase and Rad52 protein was detected by immunoblot with anti-GFP antibodies at 0 and 90 minutes after release. (B) Rad52 protein was detected at the indicated time points in the cdc2-33ts strain synchronized by HU treatment at permissive temperature (25°C) and released into cell cycle upon UVA radiation either at permissive or non permissive (37°C) temperature. (C) Rad52 protein detected in rad3-d asynchronous cells after exposition (+) or not (–) to UVA radiation and in tel1-d cells synchronized by HU treatment (block) and released into cell cycle for 0 and 90 minutes after treatment with either 0 or 400 kJ/m² UVA.

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distinguished: Ade+ His+ resulting from gene conversion and Ade+ His− resulting from deletion of the his3+ gene located between the two ade6 alleles (Figure 6A). Because sty1-1 cells loose viability when reaching saturation in medium containing standard glucose concentrations, HR frequencies were estimated in cultures not yet in stationary phase and used to calculate the rate of spontaneous HR using the MSS-MLE method [43]. As shown in Figure 6B, a significant two fold decrease in the rate of spontaneous HR was unveiled in sty1-1 cells. While the rate of deletion types (Ade+ His−) was comparable to that of sty1+ cells, 

Figure 4. Rad52 phosphorylation upon UVA is Sty1 dependent, but Srr2 independent. (A) Rad52 expression detected by anti-GFP antibodies in cells proficient or not for Sty1. Stars indicate aspecific bands serving as loading control. (B) Protein extracts at the indicated time points were prepared from cells lacking or not Sty1 and probed with anti-GFP antibodies to detect Rad52 protein (upper panel). Cells were synchronized in early S phase by HU (block) and released into cell cycle after irradiation with either 0 or 400 kJ/m². “asyn” indicates cells in log phase. Delay in cell cycle progression upon UVA was monitored by scoring the percentage of cells with 2 nuclei (lower panel). Time 0 is immediately after irradiation. (C) % of cells with Rad52 foci monitored in the indicated strains released from the HU block and exposed or not to UVA radiation. Time 0 is immediately after irradiation. (D) Immunoblot with anti-GFP antibodies of protein extracts from asynchronous cells of the indicated strains exposed (+) or not (−) to UVA. (E) Immunoblot with anti-GFP antibodies of protein extracts from cells depleted of Srr2 responsive element upon UVA irradiation in early S phase. doi:10.1371/journal.pone.0047987.g004
the rate of conversion types (Ade+ His+) in sty1-d cells was significantly diminished.

This experiment indicates that Sty1 modulates spontaneous intrachromosomal recombination by promoting one of the mechanisms leading to gene conversion. It also suggests that Sty1 acts on HR also in the absence of exogenous oxidative stress.

HR at blocked replication forks is diminished in sty1-d cells

The results obtained using UVA and H2O2 suggest that the Sty1 pathway impinges on HR at least in cells undergoing S phase. We took advantage of the RuraR inducible system (Figure 7A) described in Lambert et al. [32] to assess recombination induced by replication fork arrest. This system allows replication fork to be blocked at the RTS1 fork barrier near the ura4+ gene (RuraR substrate) on chromosome 3. Induction of replication fork arrest is obtained by controlled expression of Rtf1 protein that binds RTS1 sequence creating a barrier to fork progression: expression is shut down in medium containing thiamine (OFF) and is induced in medium depleted of thiamine (ON) leading to fork arrest. Restart of arrested replication forks requires HR and, occasionally, fork arrest induced recombination will result in genome rearrangements (Figure 7A) [32]. A strain deleted for sty1 and containing the RuraR substrate and the inducible sty1+ gene was constructed (RuraR sty1-d). Growth rate of this strain was comparable to the one of RuraR sty1+ (not shown). Survival of RuraR sty1-d strain was compared to the RuraR sty1+ and to RuraR rad52-d by growing cells over night in the absence of thiamine (ON) and spotting serial dilutions on plate containing (OFF) or not (ON) thiamine. The RuraR sty1-d strain behaved as RuraR sty1+ (Figure 7B).

Restriction fragment length analysis (RFLA) showed that rearrangements were induced by replication forks arrest (ON) in both sty1+ and sty1-d strains. However, RuraR sty1-d cells had decreased levels of ura4+ marker switch and of acentric chromosome formation if compared to strain of reference. Quantification from three independent experiments indicated a significant two fold decrease in % of HR occurring at blocked replication forks in RuraR sty1-d cells (Figure 7C). The reduced formation of acentric chromosome in RuraR sty1-d cells was confirmed by PFGE where chromosome 3 containing the substrate was identified by hybridizing with rng3 probe (Figure 7D).

Thus, in line with the results of the experiment measuring spontaneous recombination rates, HR occurring at blocked replication forks is less efficient in cells with disabled SAPK pathway suggesting that it impinges on HR even in the absence of external oxidative stress. Both the lower spontaneous HR rate and the lower percentage of HR at blocked replication forks are consistent with the suggestion that HR is less performing in sty1-d cells exposed to UVA, where Rad52 foci persist longer.

Rad52 is constitutively phosphorylated in a Sty1 dependent manner in cells lacking Rhp51

The results presented above indicate that Sty1 modulates HR even in the absence of exogenous oxidative stress and that Rad52 phosphorylation might be implicated in recombination processes. However, Rad52 phosphorylation was not observed in cells not
exposed to oxidative stress. As we suggest in the discussion, a Sty1 dependent phosphorylation of Rad52 might be difficult to detect in untreated cells because of low basal levels of HR. Thus, we looked at Rad52 phosphorylation in cells lacking the Rhp51 recombinase \( \text{rhp51-d rad52YFP} \) where early steps of HR are impaired \[35\]. We found that a slower migrating band of Rad52, which can be reversed by IPP treatment, was detected in cells lacking Rhp51 in the absence of any external insult (Figure 8A, first and third lanes), indicating that Rad52 is constitutively phosphorylated when early step of HR is prevented. More importantly, deletion of \text{sty1} gene abolished Rad52 phosphorylation in \text{rhp51-d} cells (Figure 8A, second lane). In addition, the intracellular concentration of ROS in \text{rhp51-d rad52YFP} cells, measured using the redox sensitive fluorescent probe DHR123,
levels using the fluorescent probe DHR1,2,3 in shift of Rad52 protein in rhp51-d rad52YFP cells. Bar represents the SEM of two independent experiments.

Figure 8. Sty1 dependent Rad52 phosphorylation is constitutively detected in cells lacking Rhp51. (A) Immunoblot with anti-GFP antibodies of protein extracts from strains rhp51-d rad52YFP (left lane) and rhp51-d sty1-d rad52YFP (middle lane). The change in mobility shift of Rad52 protein in rhp51-d rad52YFP cells is abolished by treatment with I PP (right lane). (B) Measurement of intracellular ROS levels using the fluorescent probe DHR1,2,3 in rad52YFP and rhp51-d rad52YFP cells. Bar represents the SEM of two independent experiments.

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was similar to the control strain (Figure 8B), indicating that intrinsic oxidative stress is unlikely what is triggering the Sty1 dependent Rad52 phosphorylation in rhp51-d cells.

Thus, these results support the notion that Sty1 pathway impinges on HR through phosphorylation of Rad52 even in the absence of treatments inducing oxidative stress.

Discussion

Here we present evidence that the SAPK pathway phosphorylates Rad52, a protein central to HR repair, when cells are subject to oxidative stress either by exposure to UVA or to H$_2$O$_2$. We also show that in the absence of exogenous treatment the Sty1 kinase promote spontaneous mitotic gene conversion and recombination induced by replication fork arrest. Importantly, we show that cells lacking the Rhp51 recombinase display constitutive Rad52 phosphorylation that is also dependent on Sty1 kinase.

A role for fission yeast Sty1 in HR has been shown for meiotic hotspot recombination where Sty1 regulates in a phosphorylation-independent manner the positioning of transcription factor Atf1 at the M26 hotspot. The Sty1 role in meiotic hot spot recombination together with the observation that it is dispensable for basal meiotic recombination, suggest that Sty1 does not regulate general key elements of recombination during meiosis [55]. Rather, chromatin remodeling in meiosis triggered by Sty1 activation in response to nitrogen starvation allows large protein complexes, such as the HR machinery, to be recruited at the hotspot recombination sites [56].

It has also been proposed that all three major MAPK pathways affect mitotic HR in human malignant glioma, but the molecular basis of the MAPK impact on HR remain unknown, and it was concluded, based on the use of SAPKs inhibitors, that ERK and JNK positively regulate HR while p38 has an opposite impact on it [57].

In the first part of our work we studied Rad52 modification in replicating cells exposed to different exogenous treatments. Sty1 dependent Rad52 phosphorylation was observed upon UVA and H$_2$O$_2$ treatments in replicating cells. Both agents induce a delay in DNA replication progression, but this is not likely the reason leading to Rad52 phosphorylation, since we show that UVC radiation, which also delays replication, did not induce Rad52 phosphorylation. In addition, induction of replication stress in S phase by CPT that leads to requirement for HR also did not result in Rad52 phosphorylation, suggesting that collapse of replication forks because of replisome loss is not a requirement for Rad52 modification. Rather, Rad52 phosphorylation correlated with ROS production since concomitant treatment of cells with oxidant and antioxidant reduced the replication delay and Rad52 phosphorylation. However, Rad52 phosphorylation is not required for replication delay upon oxidative stress, since cells lacking Sty1, and thus Rad52 phosphorylation, still delay replication (this work and [39]).

These observations suggest that the Sty1 dependent phosphorylation of Rad52 is induced by oxidative stress rather than by DNA lesions. Though, we didn’t observed Rad52 phosphorylation after UVC although it has been shown that UVC radiation induces the Sty1 oxidative stress response explaining the hypersensitivity to UVC of sty1-d cells [58]. This discrepancy might outline the greater requirement for HR repair after UVA and H$_2$O$_2$ than after UVC radiation or might reflect different responses to different levels of oxidative stress.

According to our results, the response to oxidative stress is required to promote HR since Rad52 foci persist longer in cells lacking Sty1 where Rad52 is not phosphorylated. We do not know if Rad52 is a direct substrate of Sty1 and we cannot exclude that another kinase acting downstream of Sty1 phosphorylates Rad52 in the nucleus. Although we didn’t succeed in identifying by 2-dimensional electrophoresis followed by mass spectrometry the sites of phosphorylation because the Rad52YFP protein failed to separate by isoelectric focusing under different experimental conditions, we ruled out the contribution of kinases Chk1, Cdc2, Rad3 and Tel1 in Rad52 phosphorylation.

We have shown that the rate of spontaneous HR is reduced in sty1-d cells that are not exposed to exogenous oxidative stress, and that loss of recombinants is at charge of gene conversion types. Differently from meiotic recombination, mitotic HR is rarely associated with crossovers and different published data suggest that SDSA (Synthesis-Dependent Strand Annealing) is the major non-crossovers mechanism in DSBRs repair during mitosis. In SDSA as well as in DSBR (DSB Repair) model, after resection at DSB, a 3’ single strand DNA tail is generated that will be coated by Rad51 through a process requiring Rad53. This permits strand invasion and D loop formation allowing DNA synthesis using the homologous unbroken DNA sequence as a template. In SDSA, differently from DSBR, the D loop is displaced and newly synthesized DNA pairs to the other end of DSB. If the homologous sequences are heteroallelic, then the paired DNA will contain a mismatch. Alternatively, a mismatch between the invading strand and the unbroken molecule is formed during D loop extension. Mismatches are detected by the mismatch repair system (MMR) that will either reject the invading strand or correct the mismatch possibly leading to gene conversion.

Similarly, the recombination dependent genome rearrangements upon replication fork arrest in the RuraR system result from a mechanism of template exchange where the newly synthesized strand at blocked replication fork will engage in formation of D...
loop by pairing on the homologous noncontiguous sequence \cite{44}. Since we observed in cells experiencing exogenous oxidative stress a correlation between absence of Sty1 dependent Rad52 phosphorylation and persistence of Rad52 foci, it might be that lower levels of rearrangements at blocked replication forks as well as reduced spontaneous gene conversion in sty1Δ cells result from incapacity to form a stable D loop. According to this hypothesis, a Sty1 dependent phosphorylation of Rad52 should occur also in cells not exposed to oxidants, however it might be difficult to detect it because of low basal levels of HR. Consistently, we didn’t observed Rad52 phosphorylation using the \textit{RupaR} system where one replication fork per cell is blocked (not shown). Alternatively, Rad52 phosphorylation by Sty1 occurs only upon Sty1 activation by oxidative stress while basal levels of Sty1 affect spontaneous HR and HR at blocked replication forks without Rad52 modification. However, we have shown that blocking D loop formation by inactivation of Rhp51 recombinase results in Sty1 dependent Rad52 phosphorylation. Because \textit{rhp51Δ} cells don’t seem to have an altered redox state as judged by measurement of intracellular ROS, it is unlikely that Rad52 phosphorylation in this genetic background results from activation of Sty1 by oxidative stress. Nevertheless, our results point to a possible role of phosphorylated Rad52 in early step of HR. The question rises about the signal bringing cytosolic Sty1 to promote HR in the nucleus of cells lacking Rhp51 or bearing either the intrachromosomosomal recombination substrate or the \textit{RupaR} system.

In conclusion, we bring evidence that SAPK pathway influences HR, a DNA repair pathway with implications in both protecting and promoting genome rearrangements that can drive oncogenesis.

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Author Contributions

Conceived and designed the experiments: PMG SF. Performed the experiments: AB SL LT SF. Analyzed the data: PMG ES SF. Wrote the paper: SF.

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