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Inhibition of type I histone deacetylase increases resistance of checkpoint-deficient cells to genotoxic agents through mitotic delay

John P. Alao, Jeanette Olesch, and Per Sunnerhagen

Department of Cell and Molecular Biology, Lundberg Laboratory, University of Gothenburg, Göteborg, Sweden

Abstract

Histone deacetylase (HDAC) inhibitors potently inhibit tumor growth and are currently being evaluated for their efficacy as chemosensitizers and radiosensitizers. This efficacy is likely to be limited by the fact that HDAC inhibitors also induce cell cycle arrest. Deletion of the class I HDAC Rpd3 has been shown to specifically suppress the sensitivity of *Saccharomyces cerevisiae* DNA damage checkpoint mutants to UV and hydroxyurea. We show that in the fission yeast *Schizosaccharomyces pombe*, inhibition of the homologous class I HDAC specifically suppresses the DNA damage sensitivity of checkpoint mutants. Importantly, the prototype HDAC inhibitor Trichostatin A also suppressed the sensitivity of DNA damage checkpoint but not of DNA repair mutants to UV and HU. TSA suppressed DNA damage activity independently of the mitogen-activated protein kinase–dependent and spindle checkpoint pathways. We show that TSA delays progression into mitosis and propose that this is the main mechanism for suppression of the DNA damage sensitivity of *S. pombe* checkpoint mutants, partially compensating for the loss of the G_2 checkpoint pathway. Our studies also show that the ability of HDAC inhibitors to suppress DNA damage sensitivity is not species specific. Class I HDACs are the major target of HDAC inhibitors and cancer cells are often defective in checkpoint activation. Effective use of these agents as chemosensitizers and radiosensitizers may require specific treatment schedules that circumvent their inhibition of cell cycle progression. [Mol Cancer Ther 2009;8(9):2606–15]

Introduction

A functional DNA damage checkpoint pathway is essential for maintaining genomic integrity by facilitating the repair of damaged DNA. In *S. pombe*, the DNA damage checkpoint is regulated in a *rad3*$^+$-dependent (ATM/ATR homologue) manner by a signaling cascade that involves Rad26, Rad17, Rad9, Rad1, Hus1, Crb2, Mrc1, Chk1, and Cds1 (1). Activation of this pathway in response to DNA damage ultimately leads to inactivation of Cdc2 activity and cell cycle arrest (the DNA damage checkpoint). At the same time, the repair of DNA lesions is also activated and cell cycle arrest allows time for DNA damage repair to take place. The importance of this pathway is underscored by the extreme sensitivity of DNA damage checkpoint and repair mutants to DNA-damaging agents. It has also become clear that the regulation of chromatin architecture is important for the detection/recognition and repair of DNA damage and the recovery/resetting of higher order chromatin structure following repair (2).

Histone acetyl transferases and histone deacetylases (HDAC) regulate gene expression and chromatin structure by modulating the acetylation status of lysine residues in core histones within chromatin (3). Early studies reported that UV-induced DNA damage resulted in an increase in global histone acetylation levels (4). Genetic studies have also shown that both histone acetyl transferase and HDAC mutants are sensitive to DNA-damaging agents and impaired in their ability to repair damaged DNA (5, 6). It is likely that the global changes in acetylation following DNA damage modulate gene expression and allow DNA repair proteins to access the damaged DNA within chromatin. HDAC activity is commonly deregulated in cancer cells. Small molecule inhibitors of HDAC activity have proven effective at inhibiting cancer cell proliferation *in vitro* and *in vivo*. As a consequence, several HDAC inhibitors (HDACi) have entered clinical trials with encouraging results (7, 8). Of particular interest has been the observation that HDACis can sensitize cancer cells to the effects of conventional DNA-damaging agents used in the treatment of cancer (9–11). Similarly, *S. pombe* mutants that have mutations within the *clr6*$^+$ HDAC gene, or fail to express its associated cofactors, are sensitive to DNA-damaging agents (6). Interestingly, inhibition of HDAC activity has been shown to suppress the sensitivity of *Saccharomyces cerevisiae* checkpoint mutants to DNA damage and replication stress. Scott and Plon (12) observed that deletion of the *RPD3* HDAC

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Requests for reprints: Per Sunnerhagen, Department of Cell and Molecular Biology, Lundberg Laboratory, University of Gothenburg, P.O. Box 462, S-405 30 Göteborg, Sweden. Phone: 46-31-786 3830; Fax: 46-31-786 3801. E-mail: Per.Sunnerhagen@cmb.gu.se

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gene or its cofactor *SIN3* enhanced the viability of the *mec1*Δ and *rad9*Δ checkpoint mutants following exposure to hydroxyurea (HU) or UV radiation. The suppression of sensitivity was shown to involve a *RAD53*-independent checkpoint and require a functional spindle checkpoint pathway. In many cancer cells, the ability to effectively detect and repair DNA damage is impaired. Indeed, mutations within genes that regulate DNA damage repair have been causally liked to the development and progression of cancer and the response to therapy (13, 14). The use of HDACis as hemosensitizers and radiosensitizers may thus be contraindicated in cancer cells in which the DNA damage response pathway is impaired.

In the current study, we set out to (*a*) determine if inhibition of HDAC activity suppresses the sensitivity of *S. pombe* DNA damage checkpoint and repair mutants to DNA damage, (*b*) to identify the specific HDAC proteins involved in the suppression of sensitivity, and (*c*) to characterize the molecular mechanisms that underlie the suppression sensitivity to DNA damage. We present evidence that the HDACi trichostatin A (TSA) suppresses the sensitivity of *S. pombe* checkpoint mutants to UV-induced DNA damage. A *clr6-1 rad1*Δ double mutant displayed significantly reduced sensitivity to UV radiation compared with *rad1*Δ single mutants. In contrast, deletion of the $clr3^+$ and $hos2^+/hda1^+$ genes did not suppress sensitivity to DNA damage. In *rad1*Δ mutants, TSA attenuated UV-induced mitotic stimulation (15) but did not restore a normal DNA damage checkpoint. Furthermore, TSA did not induce Chk1 or Cdc2 phosphorylation in UV-exposed *rad3*Δ mutants and suppressed the UV sensitivity of *cds1*Δ *chk1*Δ double mutants. Similarly, *clr6-1 rad1*Δ double mutants underwent a reduced rate of cell division compared with *rad1*Δ mutants following UV exposure. HDAC inhibition did not, however, suppress the UV sensitivity of *rad13*Δ and *rhp51*Δ mutants that are defective in nucleotide excision repair (NER) and recombination, respectively. Our studies indicate that suppression of Clr6 activity modulates global histone acetylation and delays cell cycle progression. Together, these effects significantly suppress the UV sensitivity of *S. pombe* DNA damage checkpoint mutants.

Materials and Methods

All strains (Table 1) were cultured on YES agar or in YES broth (0.5% yeast extract, 3% glucose, and 225 mg/liter each of adenine, histidine, leucine, lysine, and uracil) at 30°C except where indicated. DNA constructs and chromosomal disruptions were done as previously described (16, 17). Stock solutions of TSA (10 mg/mL; Errant Gene Therapeutics) in ethanol were stored at −20°C. Bleomycin stock solution (Sigma Aldrich) was made up according to the manufacturer's instructions and stored at −20°C. Stock solutions of HU (1 mol/L; Sigma-Aldrich) were stored at −20°C.

Measurement of Survival after UV Irradiation

Cells were grown overnight in liquid medium until early or mid-log phase was reached. Cells were pelleted by brief centrifugation and resuspended in 25 mL of sterile water. A UVGL-58 short wave UV lamp with an emission peak of 254 nm was used as a UV source under conditions of continuous agitation. Following irradiation, cells were collected by centrifugation and resuspended in liquid medium with or without 20 μg/mL of TSA for 2 or 4 h. The cells were then equilibrated to an $OD_{600\ nm}$ of 0.2, serially diluted, spotted on plates, and incubated for 2 to 3 d at the indicated temperature. Alternatively, cells were grown to stationary phase and then diluted into fresh medium at an $OD_{600\ nm}$ of 0.2. The cells were then cultured at 30°C for 4 h to allow reentry into the cell cycle, serially diluted, and spotted on YES agar plates supplemented with the indicated compounds. The spotted cultures were allowed to dry in air and were then exposed to the appropriate dose of UV.

Measurement of Survival after Exposure to HU or Bleomycin

Early- to mid-log phase cultures were treated with 20 mmol/L HU alone or with 20 μg/mL TSA and incubated for 4 h with shaking at 30°C. Alternatively, cells were incubated with 1.5 mU/mL bleomycin alone or with 20 μg/mL TSA for 2 h. Following incubation, the cultures were equilibrated to an $OD_{600\ nm}$ of 0.2 to 0.3, serially diluted, spotted, and incubated for 2 to 3 d at the indicated temperature.

Immunoblot Analyses

Cells were harvested by centrifugation and processed immediately or snap frozen in an ethanol bath and stored at −80°C. The cells were lysed in buffer A [50 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl, 0.2% Triton X-100, and 1% NP40] supplemented with Complete protease inhibitor and phosphatase inhibitor cocktails (Roche), using a FastPrep SP120 apparatus (Savant, Inc.) with a speed setting of 5.0 for 20 s. Lysates were resolved on 7%, 10%, or 15% SDS-polyacrylamide gels and transferred to nitrocellulose membranes using a semidry blotting apparatus. Proteins were detected using an anti-HA or anti-Myc probe (Santa Cruz Biotechnology), anti–phospho-Cdc2, anti–α tubulin (Sigma), anti-Cdc2 (Abcam), anti-acetyl H3 (K9, K14), and anti-acetyl H4 (K5, K8, K12; Upstate Biotechnology, Millipore AB). Horseradish peroxidase–conjugated secondary antibodies and the enhanced chemiluminescence Western blotting system (General Electric Bio-Sciences) were used for detection.

4′, 6′-Diamidino-2-phenylindole and Calcofluor Staining for Fluorescence Microscopy

Cells were washed, fixed in 70% ethanol, and mounted in Vectashield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories Ltd.) to counterstain DNA. Calcofluor staining of septa was carried out as described (18). Briefly, washed and fixed cells were mounted in 50 μg/mL calcofluor (Sigma-Aldrich). Stained cells were examined by fluorescence microscopy and at least 300 cells were scored for septation assays.

Fluorescence-Activated Cell Sorting

Approximately 10^7 cells were harvested at the desired time points, resuspended in 70% ethanol, and stored at 4°C until use. Fluorescence-activated cell sorting (FACS)

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Table 1. *S. pombe* strain list

Strain	Genotype	Source
L972	*h⁻*	Paul Nurse
rad3-136	*h? rad3-136 ura4 leu1*	Anwar Nasim
rad3Δ	*h⁻ rad3::KanMX6*	This study
rad1Δ	*h⁻ his3 leu1-32 rad1::ura4*	(48)
hus1Δ	*hus1::LEU2*	Anthony Carr
cds1Δ	*cds1::ura4⁺*	Hiroto Okayama
chk1Δ	*h⁻ chk1::kanMX6*	This study
clr6-1	*clr6-1*	(6)
clr6-1 rad1Δ	*clr6-1 rad1::kanMX6*	This study
clr3Δ	*h⁺ clr3::kanMX6*	(49)
clr3Δ rad1Δ	*h⁺ clr3::kanMX6 rad1::hphMX6*	This study
hda1 (hos2) Δ	*h⁺ hda1::LEU2 leu1-32 ade6-M210*	(50)
hda1Δ rad1Δ	*h⁺ hda1::LEU2 leu1-32 ade6-M210 rad1::kanMX6*	This study
sty1Δ	*h⁺ sty1::ura4 ura4D-18 leu1-32*	Jonathan Millar
sty1Δ rad1Δ	*h⁺ sty1::ura4 ura4D-18 leu1-32 rad1::kanMX6*	This study
mkp1Δ	*h⁻ leu1 ura4 his3 mkp1::ura4⁺*	(16)
mkp1Δ rad1Δ	*h⁻ leu1 ura4 his3 mkp1::ura4⁺ rad1::hphMX6*	This study
mad2Δ	*h⁻ leu1 ura4 mad2::ura4⁺*	YGRC
mad2Δ rad1Δ	*h⁻ leu1 ura4 mad2::ura4⁺ rad1::kanMX6*	This study
rad13Δ	*h⁻ leu1 ura4 ade6 rad13::ura4⁺*	YGRC
rhp51Δ cnd2-1	*h? leu1 ade6 ura4 cnd2-1 Δrhp51::ura4⁺*	YGRC
chk1-HA (NW222)	*h⁻ chk1:HA(3) ade6-216 leu1-32*	(41)
chk1-HA rad3 Δ	*h⁻ chk1:HA(3) ade6-216 leu1-32 rad3::kanMX6*	This study
cdc10-M17	*h⁺ cdc10-M17 mcm6-GFP:KanR ade6-M210 ura4-D18*	Erik Boye

Abbreviation: YGRC, Yeast Genetic Resource Centre, Osaka, Japan.

analyses were done according to the protocol of Sazer and Sherwood (19), using propidium iodide (32 μg mL^{-1}) as outlined on the Forsburg lab page.[1] Flow cytometry was done with a BD FACSAria cell sorting system (Becton Dickinson AB).

Results

TSA Suppresses UV and HU Sensitivity in *S. pombe* Checkpoint Mutants

Deletion of the class I HDAC gene, *RPD3*, has been shown to suppress DNA damage sensitivity in *S. cerevisiae* checkpoint mutants (12). To determine if the inhibition of HDAC activity in *S. pombe* checkpoint mutants would also suppress UV sensitivity, early-log phase cultures of wild-type (wt) and checkpoint mutant strains were UV irradiated and cultured with or without TSA (Fig. 1A). Wt cells exhibited little sensitivity to UV and this was not affected by TSA. As expected, the *rad3-136*, *rad3Δ*, *rad1Δ*, *chk1Δ*, and *wee1Δ* mutants were extremely sensitive to UV. When these mutants were cultured in YES medium containing TSA however, a significant increase in the survival was observed following UV exposure. The survival of *rad3-136*, *hus1Δ*, and *rad1Δ* mutants was also significantly enhanced when plated on YES agar containing TSA followed by exposure to UV (Supplementary Fig. S1A). Clr6, Hos2, and Clr3 are *S. pombe* class I/II HDACs, and of these, Clr6 has been shown to be sensitive to TSA (6). We generated *clr6-1 rad1Δ*, *hos2Δ rad1Δ*, and *clr3Δ rad1Δ* double mutants to compare their relative sensitivity to UV. Culture of the *clr6-1 rad1Δ* mutant at the semipermissive temperature (30°C) significantly suppressed UV sensitivity, an effect not observed at the permissive temperature, 25°C (Fig. 1B). In contrast, the *hos2Δ rad1Δ* and *clr3Δ rad1Δ* double mutants failed to grow following exposure to UV (Fig. 1B). Inhibition of HDAC activity by TSA also suppressed the sensitivity of the *rad3Δ*, *rad1Δ*, *hus1Δ*, and *cdc2-3w* mutants to HU (Fig. 1C). Similarly, *clr6-1 rad1Δ* double mutants grown at the semirestrictive temperature (30°C) were considerably less sensitive to HU than *rad1Δ* single mutants (Fig. 1D). FACS analyses further showed that progression through mitosis is delayed in *clr6-1* and *clr6-1 rad1Δ* mutants at 30°C (Fig. 1D). However, TSA did not suppress the sensitivity of the *rad3-136* mutant to bleomycin (Supplementary Fig. S1B). *cds1* mutants undergo a Chk1-dependent cell cycle arrest when exposed to HU, but nevertheless lose viability due to their inability to resume DNA replication (20). In contrast, *cdc2-3w* mutants lose viability because they undergo mitosis in the absence of DNA replication when exposed to HU (21, 22). TSA suppressed the HU sensitivity of *cdc2-3w* but not *cds1Δ* mutants suggesting that the HDACi delays cell cycle progression (Fig. 1C). Accordingly, TSA also suppresses the UV sensitivity of *chk1Δ* and *wee1Δ* mutants (Fig. 1A), which are specifically defective in enforcing the G_2 DNA damage checkpoint (1).

[1] http://www-rcf.usc.edu/~forsburg/yeast-flow-protocol.html

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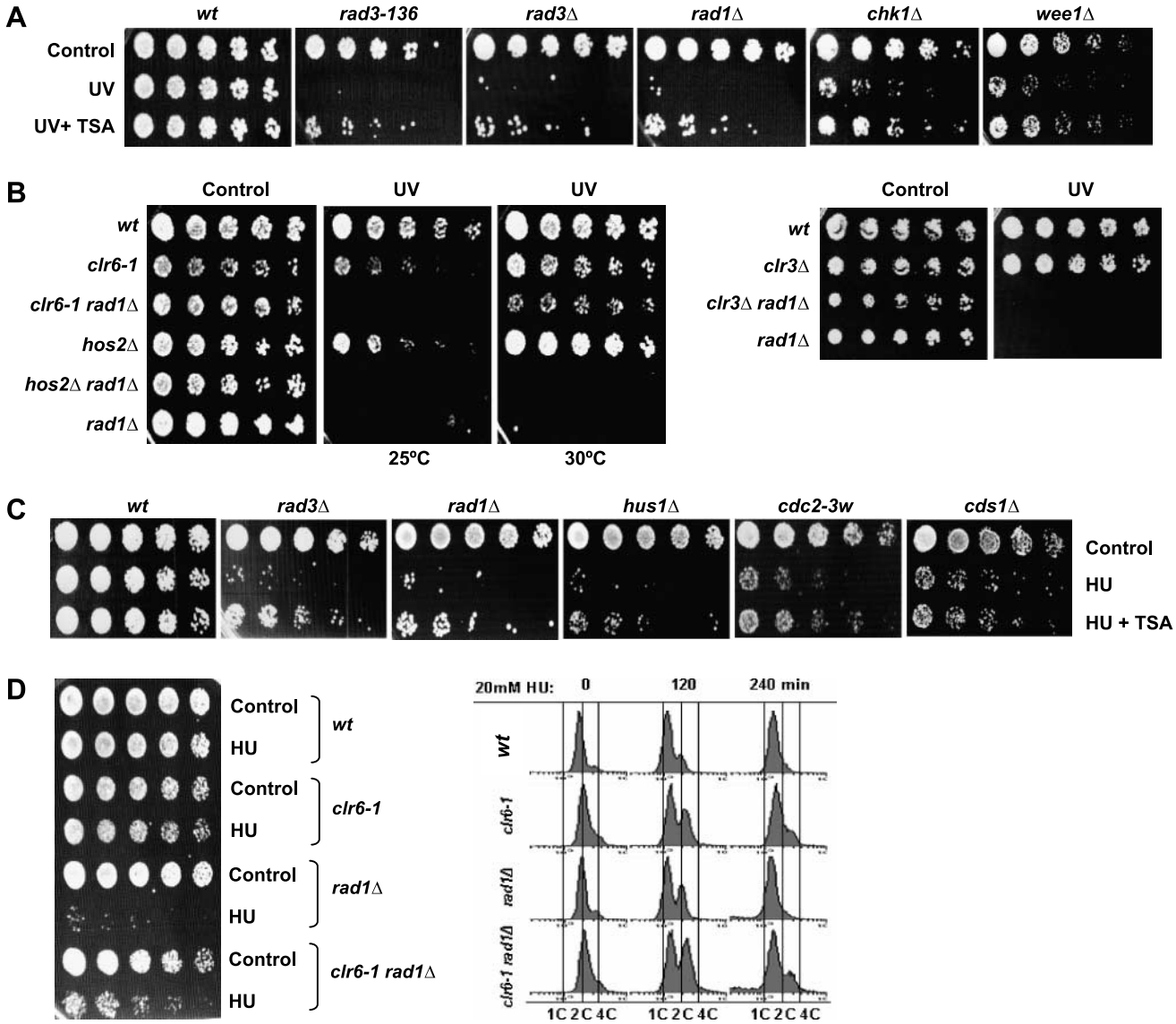

Figure 1. Inhibition of HDAC activity suppresses the UV sensitivity of *S. pombe* checkpoint mutants. **A,** the indicated strains were grown to mid-log phase, resuspended in sterile water, and exposed to 120 or 240 Jm^{-2} (*wee1*Δ) UV. Cells were then resuspended in YES medium ± 20 μg/mL TSA and incubated for 4 h at 30°C with shaking. Cells were then serially diluted, spotted onto YES plates, and incubated for 2 to 3 d at 30°C. **B,** cells were treated as in **A** and incubated at either 30°C (semipermissive temperature for *clr6-1*) or 25°C (permissive temperature for *clr6-1*). *clr3* and *clr3 rad1* mutants were treated as in **A. C,** the effect of TSA on HU sensitivity of *rad3*Δ, *rad1*Δ, *hus1*Δ, *cdc2-3w*, and *cds1*Δ cells was investigated. Strains were incubated with 20 mmol/L HU ± 20 μg/mL TSA for 4 h, serially diluted, and plated on normal media. Plates were incubated at 30°C for 2 to 3 d. **D,** the indicated strains were incubated with 20 mmol/L HU for 4 h at 30°C and treated as in C. Samples were also harvested at the indicated time points and analyzed by FACS.

TSA Delays Cell Cycle Progression of *S. pombe* Checkpoint Mutants

S. pombe DNA damage checkpoint mutants fail to undergo cell cycle arrest when exposed to DNA-damaging agents and accumulate as septated cells (23, 24). Septation indices were monitored to determine cell cycle progression following UV exposure (120 Jm^{-2}) with or without 20 μg/mL TSA (Fig. 2A). Mitotic division decreased rapidly in wt cells as monitored by the decrease in the number of septating cells and was not affected by TSA (Fig. 2A, *I*). In contrast, exposure of the *rad3-136, rad1*Δ, and *chk1*Δ mutants to UV did not result in cell cycle arrest (Fig. 2A, *II–IV*). As previously reported, exposure of *rad3-136, rad1*Δ, and *chk1*Δ mutants to UV resulted in an increase in the number of septated cells (Fig. 2A, *II–IV*). Addition of TSA to the culture medium prevented the accumulation of septated cells but did not restore a wt checkpoint in *rad3-136, rad1*Δ, and *chk1*Δ mutants; instead, the septation profiles were suggestive of a mitotic delay as the peak of septated cells was delayed or absent (Fig. 2A, *II–IV*). Similarly, attenuation of Clr6 activity also

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prevented the accumulation of septated cells when *clr6-1 rad1*Δ double mutants were exposed to UV (Fig. 2B). TSA did not restore Chk1 phosphorylation in a *rad3*Δ mutant or influence Cdc2 phosphorylation in *rad1*Δ mutants following UV exposure (Fig. 2C and D). Rather, the Chk1 level was lowered in *rad3*Δ mutants exposed to TSA. Together, these observations suggest that the suppression of Clr6 HDAC activity delays cell cycle progression independently of Cdc2 phosphorylation.

To determine where in the cell cycle the TSA-induced delay takes place, we next undertook analyses of cell cycle progression by microscopy, septation assays, and flow sorting. To determine if HDAC inhibition delays progression through G_2, we released *cdc25-22* mutants, synchronized in early G_2 by incubating for 4 h at 36°C, into the cell cycle in the presence or absence of 20 μg/mL TSA. In the absence of TSA, *cdc25-22* cells rapidly proceeded through mitosis into the subsequent G_1-S phase as indicated by the large number of binuclear and/or septated cells detectable between 80 and 140 minutes after release. In the presence of TSA, progression into mitosis was clearly delayed and the mitotic index did not peak until 140 to 160 minutes after release (Fig. 3A and B). FACS analyses further showed a marked difference in cell cycle kinetics between TSA-treated and untreated cells from Fig. 3A and B (Fig. 3C). In contrast, TSA did not affect progression into S phase when *cdc10-M17* mutants, synchronized in G_1 by incubation at the restrictive temperature, were downshifted to 25°C (Fig. 3D).

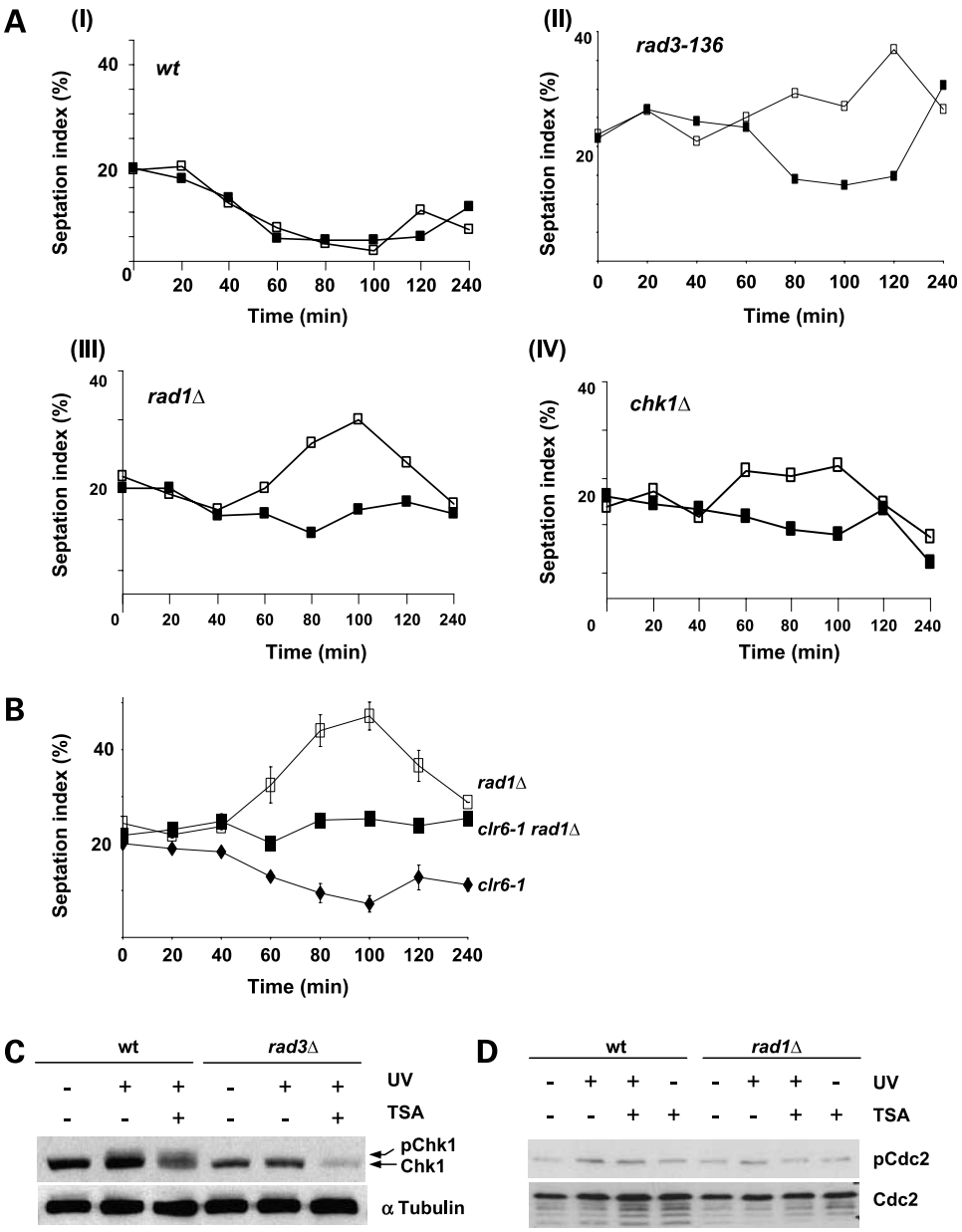

Figure 2. HDAC inhibition delays cell cycle progression in checkpoint mutants exposed to UV. **A,** the indicated strains were irradiated in sterile water and then incubated in YES medium ± 20 μg/mL TSA and incubated for 4 h at 30°C with shaking. Samples were harvested at the indicated time points, stained with calcofluor, and the septation index determined by microscopy. **B,** *clr6-1, clr6-1 rad1*Δ, and *rad1*Δ strains were irradiated in sterile water and then incubated in YES medium and incubated for 4 h at 30°C with shaking. Samples harvested at the indicated time points were treated as in **A. C,** Wt and *rad3*Δ mutant strains expressing HA epitope-tagged Chk1 were irradiated as in **A** and incubated for 2 h at 30°C with shaking. Total protein lysates were resolved by SDS-PAGE and probed with antibodies directed against HA and α-tubulin. **D,** Wt and *rad1*Δ mutants were treated as in **C** and probed with antibodies directed against phospho- and total Cdc2.

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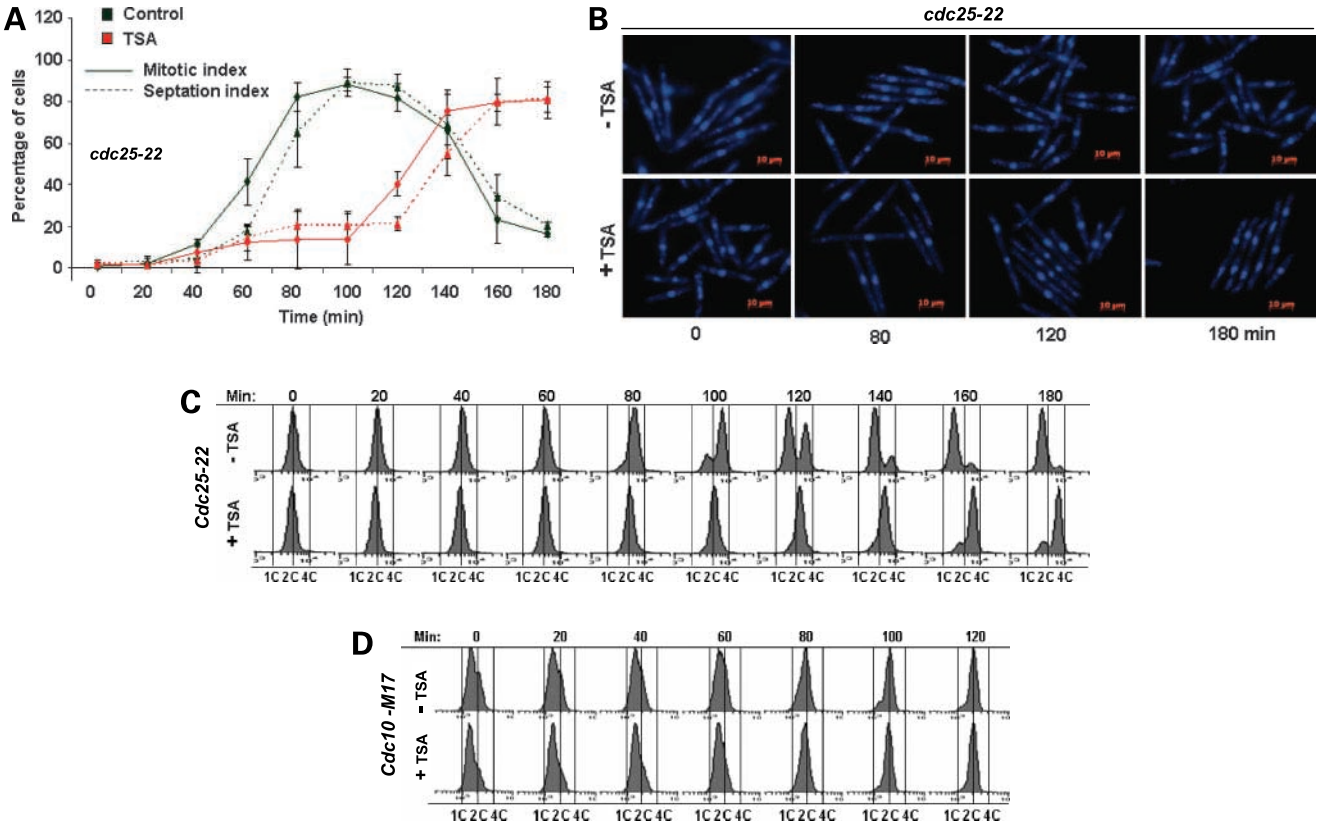

Figure 3. TSA delays entry into mitosis. **A,** *cdc25-22* cells were synchronized in early G_2 by incubation at 36°C for 4 h and released into the cell cycle by incubating at 25°C ± 20 μg/mL TSA, and samples were harvested at the indicated time points. Cells stained with calcofluor or DAPI and the mitotic and septation indices determined by fluorescence microscopy. *Points,* mean of three experiments; *bars,* SD. **B,** *cdc25-22* cells were treated as in **A**, stained with DAPI, and examined by fluorescence microscopy. **C,** *cdc25-22* cells from A were harvested at the indicated time points after release and analyzed by FACS. **D,** *cdc10-M17* cells were synchronized in G_1-S by incubation at 36°C for 4 h and released into the cell cycle by incubating at 25°C ± 20 μg/mL TSA. Samples were harvested at the indicated time points and analyzed by FACS.

In *S. pombe*, S phase, septum formation, and cytokinesis are dependent on the completion of the preceding mitosis (25, 26). Our results thus strongly suggest that TSA delays entry into and/or progression through mitosis. Because cytokinesis occurs after the completion of S phase in *S. pombe* however, we could not rule out the possibility that TSA also delays cell cycle progression between the completion of mitosis, the subsequent G_1-S phase and/or cytokinesis. When asynchronous populations of *rad3*Δ mutants were exposed to HU and monitored by FACS (Fig. 4A), TSA delayed progression into S phase. In the presence of 20 mmol/L HU alone, within 2 hours, a majority of the cell population had proceeded through one round of mitosis and daughter cells accumulated with a predominantly <2C DNA content. In the presence of TSA however, a significant proportion of the cell population remained in G_2 at 2 hours after drug exposure, indicating that these cells had not yet progressed through mitosis and/or cytokinesis. Coculture with TSA also reduced the number of cells with chromosome segregation defects (cut phenotype) when *rad3-136* or *cdc2-3w* mutants were exposed to HU (Fig. 3B and D). We noted that *rad3-136* mutants cocultured with HU and TSA were longer at cell division when compared with cells cultured in the presence of HU alone (Fig. 3C). In cells with a defective G_2 checkpoint attempting mitosis in the presence of DNA damage, chromosomal missegregation will occur. Taken together, our findings suggest that a mitotic delay is the main mechanism by which HDAC inhibition suppresses the DNA damage sensitivity of checkpoint mutants. This delay provides additional time for DNA repair, thus reducing the number of cells with chromosomal segregation defects.

TSA-Mediated Suppression of DNA Damage Sensitivity Occurs Independently of the Mitogen-Activated Protein Kinase or Spindle Checkpoint Pathways

HDAC inhibition has been shown to delay mitosis in a manner dependent on the p38 mitogen-activated protein kinase (27). Deletion of *sty1*$^+$ (the p38 homologue in *S. pombe*) in a *rad1*Δ background did not prevent the suppressive effect of TSA following exposure to UV (Fig. 4A). Interestingly, TSA also suppressed the UV sensitivity of *sty1*Δ mutants (Fig. 5A). Srk1, a downstream target of Sty1, has also been shown to delay G_2 progression in *S. pombe* (28). Deletion of *srk1*$^+$ in a *rad1*Δ background did not suppress the protective effect of TSA, however (Fig. 5A).

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In *S. cerevisiae*, HDAC inhibition induces an alternative Mad1-dependent cell cycle delay when checkpoint mutants are exposed to agents that damage DNA (12). Codeletion of the *S. pombe* spindle checkpoint regulator gene *mad2*$^+$ did not affect the ability of TSA to suppress UV sensitivity in *rad1*Δ mutants (Fig. 5A). Our findings indicate that HDAC inhibition delays cell cycle progression in *S. pombe* independently of Sty1, Srk1, and the spindle checkpoint pathway.

TSA-Mediated Suppression of UV Sensitivity Occurs Independently of the NER and Recombination Repair Pathways

We investigated the ability of TSA to suppress the UV sensitivity of *S. pombe* mutants that were checkpoint competent but deficient in nucleotide excision or recombination repair. For these experiments, we used the *rad13* mutant that is defective in NER and *rhp51* mutants defective in recombination repair. Exposure of both mutants to 120 Jm^{-2} UV resulted in a significant loss of viability. Culture of these mutants with TSA following UV exposure failed to suppress UV sensitivity (Fig. 5A). TSA nevertheless did suppress the DNA damage sensitivity of a *rad13*Δ *rad1*Δ double mutant indicating that the NER pathway is not required for the protective effects observed with HDAC inhibition. These observations suggest that TSA suppresses the UV sensitivity checkpoint mutants but not that of mutants defective in the NER and recombination repair pathways.

Role of Acetylation in the TSA-Mediated Suppression of UV Sensitivity

In *S. cerevisiae*, exposure to UV results in the rapid increase of global histone H3 and H4 acetylation levels (29, 30). In contrast to *S. cerevisiae*, exposure of wt *S. pombe* cells to 120 Jm^{-2} UV resulted in a rapid decline of global histone H3 and H4 acetylation levels and persisted for up to 2 hours (Fig. 5B and C). The global levels of histone H3 acetylation declined similarly in *rad3*Δ mutants following exposure to UV (Fig. 5D). TSA induced acetylation of H3 in both wt and *rad3*Δ mutants following exposure to UV (Fig. 5D). Our observations suggest that global increases in histone acetylation (at least of histones H3 and H4) are not part of the normal response to UV in *S. pombe*.

Discussion

In the current study, we have extended previous findings from *S. cerevisiae* (12), demonstrating that inhibition of

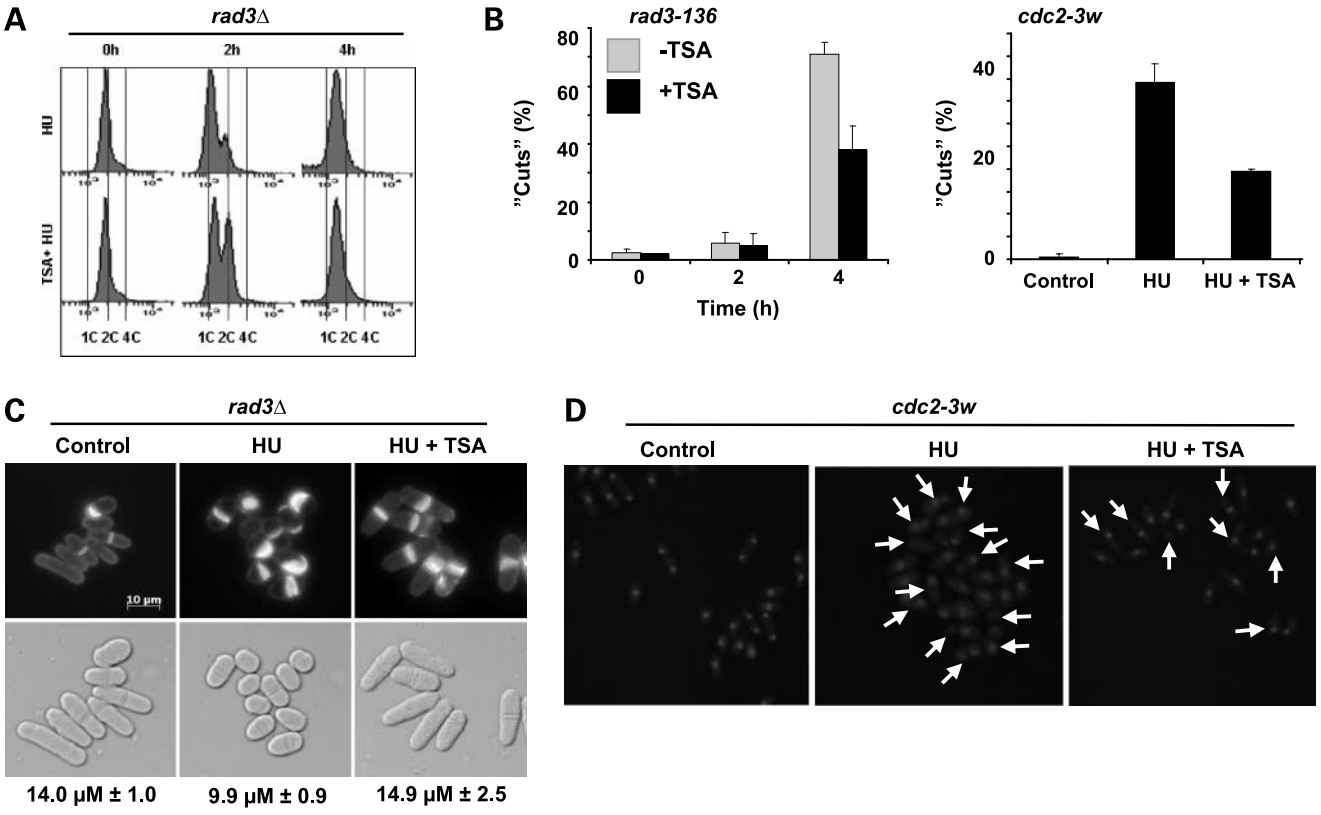

Figure 4. TSA delays entry into mitosis. **A,** *rad3*Δ cells were incubated with 20 mmol/L HU ± 20 μg/mL TSA. Samples were harvested at the indicated time points and analyzed by FACS. **B,** *rad3-136* cells were incubated with 20 mmol/L HU ± 20 μg/mL TSA and harvested at the indicated times. Cells were fixed, stained with DAPI, and the percentage of cells with chromosome missegregation (*cuts*) was determined by fluorescence microscopy. *cdc2-3w* cells were incubated for 4 h at 30°C as indicated, fixed, and analyzed in the same manner as the *rad3-136* cells. *Columns,* mean of three experiments; *bars,* SD. **C,** *rad3*Δ cells were treated as in **B** for 4 h, fixed, stained with calcofluor, and examined by fluorescence and DIG microscopy. Values indicate average cell length at division ± 1 SD. **D,** *cdc2-3w* cells were treated as in **B**. *White arrows,* cut cells.

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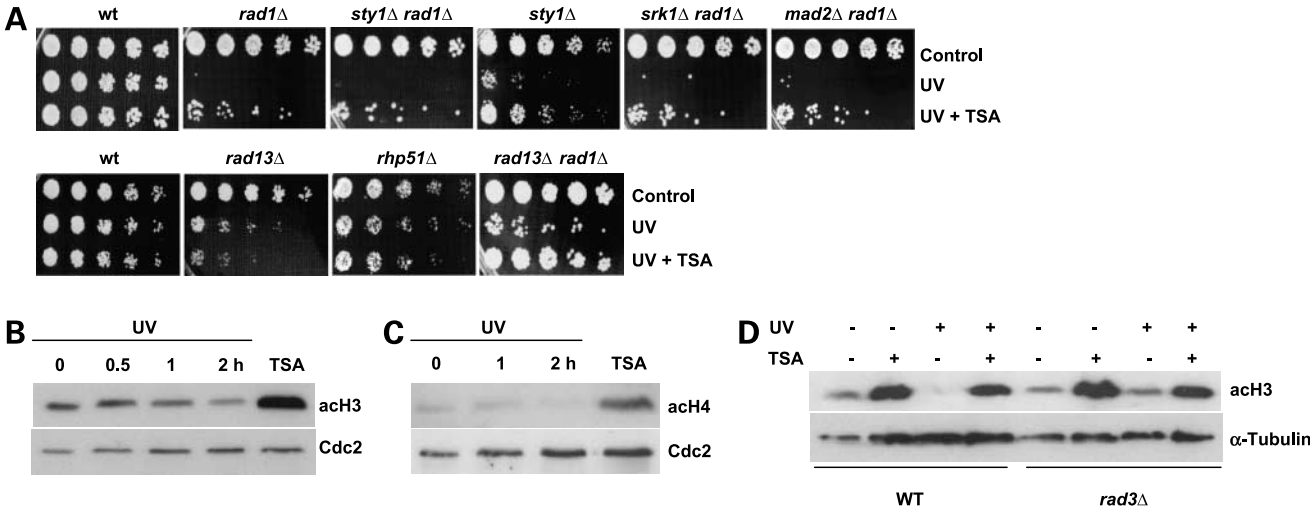

Figure 5. Role of checkpoints and DNA repair in TSA-mediated suppression of UV sensitivity. **A,** indicated strains were treated as in Fig. 1 except that the *sty1*Δ *rad1*Δ double mutant was exposed to 60 Jm^2 UV. **B** to **C,** Wt cells were exposed to 120 Jm^{-2} UV and incubated in YES medium for the indicated times at 30°C. Untreated cells are indicated by the 0 h time point and cells treated with 20 μg/mL TSA for 1 h served as a positive control. Global histone H3 and H4 acetylation was monitored using a polyclonal antibody against acetylated H3 Lys9, 14 or H4 Lys5, 8, and 12. Total Cdc2 levels were used to confirm equal gel loading. **D,** *chk1-HA* and *chk1-HA rad3*Δ mutant cells were treated as indicated and cultured at 30°C for 1 h. Histone H3 acetylation was monitored as in **B**. Cells were exposed to 120 Jm^{-2} UV and incubated ± 20 μg/mL TSA.

HDAC activity suppresses the sensitivity of checkpoint deficient strains to DNA damage. Our findings suggest that a major mechanism underlying this is that HDAC inhibition delays progression into mitosis in *S. pombe* cells, which in a cycling population are largely in G_2, and can partially suppress the need for a functional DNA damage checkpoint pathway. In contrast to *S. cerevisiae*, however, this mitotic delay occurs independently of a functional spindle checkpoint pathway. Furthermore, we have shown that chemical inhibition of HDACs by TSA similarly suppresses the sensitivity of checkpoint mutants to DNA damage, and that the HDAC Clr6 is the TSA target responsible for this effect. HDACis are currently in clinical trials to determine their efficacy as chemosensitizers and radiosensitizers (31). A proper understanding of how HDACis influence the response of cancer cells to DNA damage is required to facilitate effective use of these agents as modulators of drug sensitivity.

Numerous studies have shown that HDACis sensitize cancer cells to the cytotoxic and genotoxic effects of conventional cancer agents (31, 32). The precise mechanisms whereby HDACis sensitize cancer cells to these treatments remain unclear but may involve prevention of effective DNA repair (31). Cancer cells often harbor defects in DNA damage checkpoint pathways and thus have a limited repertoire of responses to deal with genotoxic insults (14). Conceptually, chemosensitization and radiosensitization stratagems seek to attenuate or inhibit the activity of the remaining checkpoints in cancer cells, thus enhancing their sensitivity to genotoxins. Accordingly, agents that abolish DNA damage checkpoints sensitize cancer cells to genotoxic agents (33). HDACis induce cell cycle arrest in both normal and cancer cells (34). This fact raises the possibility that the cell cycle effects of HDACis counteract their chemosensitizing activity. Disruption of an HDAC gene in *S. cerevisiae* has previously been shown to suppress the sensitivity of checkpoint mutants to UV and HU (12). It is currently unclear if HDAC inhibition will similarly compensate for the loss of checkpoint function in cancer cells. Given that cancer cell progression often selects for the loss of checkpoint pathways (35, 36), a better understanding of the interactions between HDACs and DNA damage response pathways is desirable.

The effects of HDAC inhibition on *S. pombe* DNA damage checkpoint and repair mutants have not been reported earlier. In the current study, we have characterized the effect of HDAC inhibition with TSA on the sensitivity of *S. pombe* checkpoint mutants to UV. TSA effectively suppressed the UV sensitivity of *rad3, rad1, hus1, chk1*, and *cds1 chk1* mutants to UV and HU. Deletion of the $clr6^+$ HDAC gene, but not of other HDAC genes, similarly suppressed the HU and UV sensitivity of *rad1*Δ mutants.

Inhibition of HDAC activity in *S. pombe* mutants may suppress UV sensitivity through the following mechanisms: (*a*) activation of an alternative checkpoint, (*b*) expression of DNA repair proteins not expressed in mutants exposed to UV alone, or (*c*) enhanced repair resulting from changes in chromatin structure due to increased histone acetylation. Our observations indicate that the suppression of UV sensitivity by TSA neither requires the damage recognition nor the cell cycle checkpoint activities of checkpoint proteins, because the sensitivity of *rad3* mutants is also suppressed (Fig. 1). We noted that TSA also suppressed the characteristic increase in septating cells following UV exposure that occurs in *rad3, rad1*Δ, and *chk1*Δ mutants (Fig. 2A; refs. 23, 24). The mitogen-activated protein kinase–activated protein

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kinase Srk1 has been shown to be capable of inducing cell cycle arrest in G_2 (37). Srk1 is activated by the Sty1 stress-activated protein kinase, a homologue of mammalian $p38^{SAPK2}$, following cellular exposure to stress (37, 38). Sty1 has itself been shown to be involved in the response of *S. pombe* cells to UV exposure and *sty1* mutants are moderately sensitive to this agent (39). Deletion of $sty1^+$ in a *rad1*Δ mutant to UV did not prevent the suppressive effect of TSA following exposure. Similarly, deletion of $srk1^+$ in a *rad1*Δ background did not suppress the protective effect of TSA or result in increased sensitivity to UV (Fig. 5). In *S. cerevisiae*, the suppression of UV sensitivity induced by the inhibition of HDAC activity requires an intact spindle checkpoint (12). Codeletion of the spindle checkpoint regulator gene $mad2^+$ did not affect the ability of TSA to suppress UV sensitivity in *S. pombe rad1*Δ mutants (Fig. 5). We conclude therefore that in fission yeast, the suppression of UV sensitivity by TSA does not involve activation of either of these alternative cell cycle checkpoints. TSA failed to suppress the sensitivity of *rad13*Δ mutants, defective in NER, and of an *rhp51*Δ mutant, defective in recombination repair (Fig. 5). These mutants arrest normally following exposure to UV but fail to repair UV-induced DNA damage (40). TSA, thus specifically suppresses the DNA damage sensitivity of checkpoint mutants, but not of DNA repair mutants.

TSA suppressed the UV sensitivity of *chk1*Δ mutants (Fig. 1). According to current models, the UV sensitivity of *chk1* mutants results from their inability to undergo G_2 arrest following DNA damage. These mutants thus undergo mitosis in the presence of damaged DNA (41). We postulate that TSA suppresses UV sensitivity by delaying progression into mitosis, thus abrogating the need for checkpoint induction. The precise mechanisms by which TSA delays mitotic progression remain unclear but are clearly linked to the increased global acetylation induced by this agent. Studies in mammalian cells suggest, however, that the prevention of entry into mitosis as well as its deregulation is indeed a common consequence of HDAC inhibition (42). TSA has been shown to suppress the expression of mitotic regulators such as cyclin B1 and Plk1 in human cancer cells (43). In *S. pombe*, osmotic stress has been shown to delay mitosis independently of $mad2^+$ and $rad3^+$ by enhancing the affinity of Cut2 (securin) for Cut1 (separase; ref. 44). We are currently investigating if HDACis prevent the initiation of mitosis by similar mechanisms in *S. pombe*.

It is increasingly evident that the modulation of histone acetylation by histone acetyl transferases and HDACs is important for the repair of DNA damage (45). Histone acetylation enhances DNA repair, and *S. cerevisiae gcn5*Δ mutants are mildly sensitive to UV (46). In *S. pombe*, loss of Clr6 HDAC activity or deletion of its associated cofactors results in increased sensitivity to higher doses of UV (6). Histone acetylation is believed to allow efficient access of repair proteins via its effects on transcription and chromatin architecture (45). Interestingly, we observed that exposure to UV does not induce increased, but rather a moderately decreased global histone acetylation in *S. pombe*. Indeed, global deacetylation seems to accompany cell cycle arrest in *S. pombe* irrespective of cell cycle phase (Supplementary Fig. S1C; ref. 47). Our studies show that HDAC inhibition does not suppress the sensitivity of mutants with DNA repair defects. They do not, however, exclude the possibility that TSA enhances the rate of DNA repair as a consequence of global histone acetylation. We are currently investigating this possibility.

In mammals, a defect within the ATM-regulated checkpoint pathway sensitizes cells to DNA damage but also predisposes toward the development of cancer. It is possible that the modulation of HDAC activity in cells with defective checkpoint pathways may contribute toward genetic instability by facilitating their survival. Our findings also provide further support for the notion that the use of HDACis as chemosensitizers or radiosensitizers may be contraindicated in tumors with defective DNA damage checkpoints (12).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Therapeutics

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John P. Alao, Jeanette Olesch and Per Sunnerhagen

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