[Cell Cycle 7:3, 1-8; 1 February 2008]; ©2008 Landes Bioscience

Report

MAPK mediated cell cycle regulation is associated with Cdc25 turnover in *S. pombe* after exposure to genotoxic stress

Geetanjali Sundaram, Santanu Palchaudhuri,† Swati Dixit‡ and Dhrubajyoti Chattopadhyay*

B.C. Guha Centre for Genetic Engineering and Biotechnology; Department of Biochemistry; University of Calcutta; Kolkata, India

†Present affiliation: Northwestern University; Feinberg School of Medicine; Chicago, Illinois USA

‡Present affiliation: Heslop School of Biotechnology; Nagpur, India

Abbreviations: CSE, cigarette smoke extract; HU, hydroxyurea; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Key words: *S. pombe*, cigarette smoke, Sty1, Cdc25, cell cycle, MAPK, genotoxic stress

Genotoxic stress caused by carcinogens like Cigarette smoke activate both the MAPK pathway and the S phase checkpoint in *Schizosaccharomyces pombe*. But the cross talk between these two pathways has not been investigated in great detail in fission yeast. This study deals with the molecular mechanism of co-ordination between the two regulatory pathways. We show that both the pathways have a common effector molecule, namely Cdc25, the cell cycle regulatory phosphatase. We demonstrate that the MAPK Sty1 interacts with Cdc25 and prevents mitotic entry in *S. pombe* cells exposed to CSE. To our knowledge, this is the first demonstration of interaction between Sty1 and Cdc25 in *S. pombe*. The functional significance of this interaction lies in effecting Cdc25 turnover after CSE exposure in *S. pombe*. We show that Cdc25 turnover after CSE treatment is dependent on the presence of Rad3 activity and Sty1-Cdc25 interaction. Our study suggests that the Cigarette Smoke Extract (CSE) induced stress is counteracted by the simultaneous activation of a mitotic checkpoint in addition to the previously described S phase checkpoint. We also show that Sty1 activity is not essential for activation of the S phase checkpoint.

Introduction

The DNA damage detection, repair and checkpoint activation pathways are evolutionarily conserved from yeast to human to an appreciable extent, as are the stress responsive MAPK pathways. Specifically the major players in these pathways in mammals have their well described homologues in *S. pombe*. This makes *S. pombe* a very good model system for studying the cellular responses to various stress conditions including DNA damage, as the extrapolation of the results to mammalian systems is sufficiently relevant. We have already established *S. pombe* as a model system for studying the cellular responses to Cigarette Smoke Extract (CSE) (Chaudhuri et al.[1]).

Cigarette Smoke (CS) has been classified as a group A carcinogen and smoking is known to be associated with a variety of diseases like emphysema, chronic obstructive pulmonary disease, atherosclerosis, cardiovascular diseases, neurodegenerative diseases and different types of cancer, most importantly lung cancer (Sherman CB[2]). Thus elucidation of the intricate molecular interactions that help the cell to survive after being exposed to CS are necessary to understand the mechanism of CS induced disease progression. Cigarette smoke contains 4,700 chemical compounds including sixty known carcinogens and many oxidants and free radicals that are capable of initiating or promoting oxidative damage. Apart from the oxidants present in cigarette smoke, the reactive oxygen species generated by the activated phagocytes following smoking, may also be responsible for the observed oxidative damage (Chow CK[3]). The high level of oxidative potency of cigarette smoke accounts for its capacity to oxidize proteins (reviewed in refs. 4–6;), lipids (reviewed in refs. 5 and 7), and DNA (reviewed in refs. 8–10). It has been demonstrated to induce chromosomal aberrations (Hirao et al.[11]), sister chromatid exchange (Lambert et al.[12]) and DNA damage including single strand break (reviewed in refs. 13 and 14). Cumulatively, these effects can cause extensive damage to tissues and organs (Panda et al.[15]) and potentially contribute to different diseases associated with smoking.

Our previous studies have shown that treatment with sub-lethal doses of CSE leads to S phase checkpoint activation as well as the MAPK Sty1 activation in *S. pombe*. The S phase checkpoint activation is dependent on Rad3, which is homologous to the mammalian ATM/ATR family of protein kinases. The activation of Rad3 initiates the activation/inhibition of a series of downstream effector molecules including Cds1, which ultimately leads to cell cycle arrest and damage repair. Cell cycle progression in *S. pombe* requires the activity of the cyclin dependent kinase Cdc2, which is very tightly regulated. The tyrosine kinases Wee1 and Mik1 can phosphorylate Cdc2 on Tyr15 thereby inhibiting its activity (reviewed in refs. 16–18). Cdc25 phosphatase can remove this inhibitory phosphorylation and activate Cdc2 (reviewed in refs. 19–21). In turn Cdc25 activity is regulated by

*Correspondence to: Dhrubajyoti Chattopadhyay; Dr. B. C. Guha Centre for Genetic Engineering and Biotechnology; Department of Biochemistry; University of Calcutta; 35 B. C. Road; Kolkata 700019; West Bengal INDIA; Tel.: 091.033.2461.4983; Fax: 091.033.2476.4419; Email: d_jc@sify.com

Submitted: 08/21/07; Revised: 11/02/07; Accepted: 11/02/07

Previously published online as a *Cell Cycle* E-publication:
http://www.landesbioscience.com/journals/cc/article/5266

a negative regulatory phosphorylation by the kinases Chk1 and Cds1 under the conditions of incomplete replication or DNA damage (Furnari et al.[22]). The MAPK Sty1 on the other hand, responds to a wide spectrum of stress stimuli. It is the homolog of mammalian p38 and *S. cerevisiae* Hog1. Interestingly many extracellular stimuli like UV or gamma-radiation, DNA damaging compounds etc. have been shown to effect cell cycle checkpoint activation in a p38 dependent manner in mammalian cells (reviewed in refs. 23–29). Sty1 also has been shown to link cell cycle progression with environmental stress in *S. pombe* (reviewed in refs. 30–32), but the exact molecular mechanism is not known, except when the inhibition of Cdc25 occurs through the activity of the Srk1 kinase which is dependent on Sty1 for its activation (Lopez-Aviles et al.[33]). Srk1 however cannot regulate Cdc25 when cells are exposed to genotoxic stress.

In this report we provide evidences, which suggest that both the Rad3-Cds1 dependent S phase checkpoint pathway and the Sty1 MAPK pathway independently regulate the activity of Cdc25 to synergize the cellular responses towards CSE treatment, thus ensuring full protection. We show that Sty1 can physically associate with Cdc25 in vivo in CSE treated *S. pombe* cells and inhibit mitotic entry of CSE exposed fission yeast cells, and that this association is required for degradation of Cdc25 after CSE treatment. Importantly, association of Cdc25 and Sty1 was also found in fission yeast cells treated with HydroxyUrea also indicating a more general role for this association. The investigation of possible cross talks between MAPK activity and cell cycle regulation also shows that neither Sty1 nor its downstream effector Atf1 can inhibit Cds1 activation in CSE treated cells.

Results

Δ*rad3*Δ*sty1* cells are highly sensitive to CSE. The first indications for a role of Sty1 in cell cycle modulation after CSE treatment came from the observations on the sensitivities of the respective mutants to CSE. Wild type, Δ*sty1,* Δ*rad3* and Δ*rad3*Δsty1 cells were exposed to 40 ul CSE and cell death was determined by the extent of PI uptake (Fig. 1A). The Δ*rad3*Δ*sty1* cells exhibited extreme sensitivity to CSE, much higher than the additive sensitivity of the Δ*rad3* and Δ*sty1* cells. Chronic exposure to CSE, rendered the Δrad3Δ*sty1* mutant completely non—viable (Fig. 1B). ROS generation profiles of the CSE treated mutants showed similar trends (Fig. 1C).

S phase checkpoint is intact in Δ*sty1* cells. The cell sensitivity data strongly indicated the existence of a synergistic interaction between the Rad3 and Sty1 mediated pathways. So we next investigated whether Sty1 could affect the Rad3 dependent S phase arrest in *S. pombe* cells exposed to CSE. Wild type and Δ*sty1* cells treated with 40 ul CSE were fixed at definite time intervals, processed (as described in Materials and Methods) and the cell cycle progression was monitored. Intriguingly we observed that both the wild type and Δ*sty1* mutants showed activation of the S phase checkpoint, wherein CSE treatment causes a fraction of the cells to accumulate with a DNA content intermediate between 1N and 2N (Fig. 2A). The biochemical hallmark of S phase checkpoint activation in CSE treated *S. pombe* cells is the activation of the checkpoint kinase Cds1 (downstream of Rad3) (Chaudhuri et al.[1]). We found that increase in Cds1 activity after CSE treatment was not affected by the deletion of either Sty1 or its downstream effector Atf1 (data not shown). Hence loss of sty1 activity does not compromise the CSE induced S phase delay in *S. pombe*. So any possible synergistic interaction between the two pathways is not affecting the S phase checkpoint activation after CSE treatment.

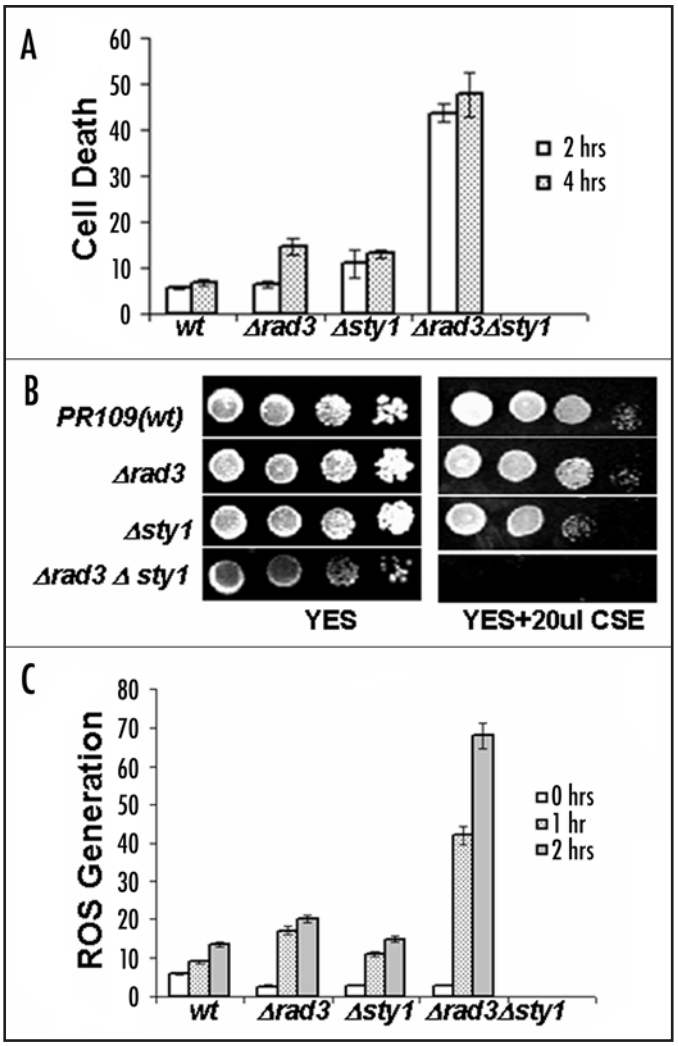

Figure 1. Potential synergism between the Rad3 and Sty1 mediated signaling. (A) Wild type, Δ*rad3*,Δ*sty1*,Δ*rad3*Δ*sty1 S. pombe* cells were treated with CSE and the sensitivity of these different cell populations to CSE was Flow Cytometrically determined by Propidium Iodide (PI) uptake at 2 and 4 hrs after CSE treatment. The data was normalized to the PI uptake values before treatment in each of the cell populations. (B) Viability of *S. pombe* wild type, Δ*rad3,* Δ*sty1* and Δ*rad3*Δ*sty1* cells in the presence or absence of a chronic exposure to CSE was determined by spotting serial 10-fold dilutions of the different cells types in YES-Agar plates containing CSE and compared with the viability of cells spotted similarly on YES-Agar plates not containing CSE. The plates were incubated at 30°C for three days before being photographed. (C) *S. pombe* cells were treated with CSE for the indicated time intervals, washed in YES and then incubated for 30 mins in the dark with the dye H_2DCF-DA. Intracellular ROS levels in *S. pombe* wild type, Δ*rad3,* Δ*sty1,* Δ*rad3*Δ*sty1* cells before and after CSE treatment was then measured flow cytometrically through H2DCF fluorescence.

Sty1 regulates mitotic progression in CSE treated cells. Now that we knew that Sty1 was not affecting the S phase delay, we went on to investigate what else could be the overlapping role of Sty1 with the cell cycle regulatory pathway in *S. pombe*. To this end we searched for any enhanced cell division defects in CSE treated Δ*sty1* cells. It was observed that the CSE treated Δ*sty1* cells exhibit multiple and abnormal septation (Fig. 3A, part iii), a phenotype that is highly

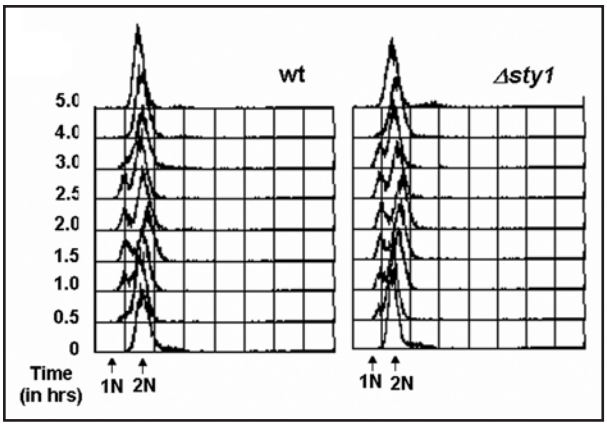

Figure 2. Activation of S phase checkpoint in Δsty1 cells. Wild type and Δsty1 *S. pombe* cells were treated with CSE, cells were collected at definite intervals after CSE treatment, fixed, and then the cell cycle progression in these cells was flow cytometrically monitored by staining with PI and determining the DNA content of these cells.

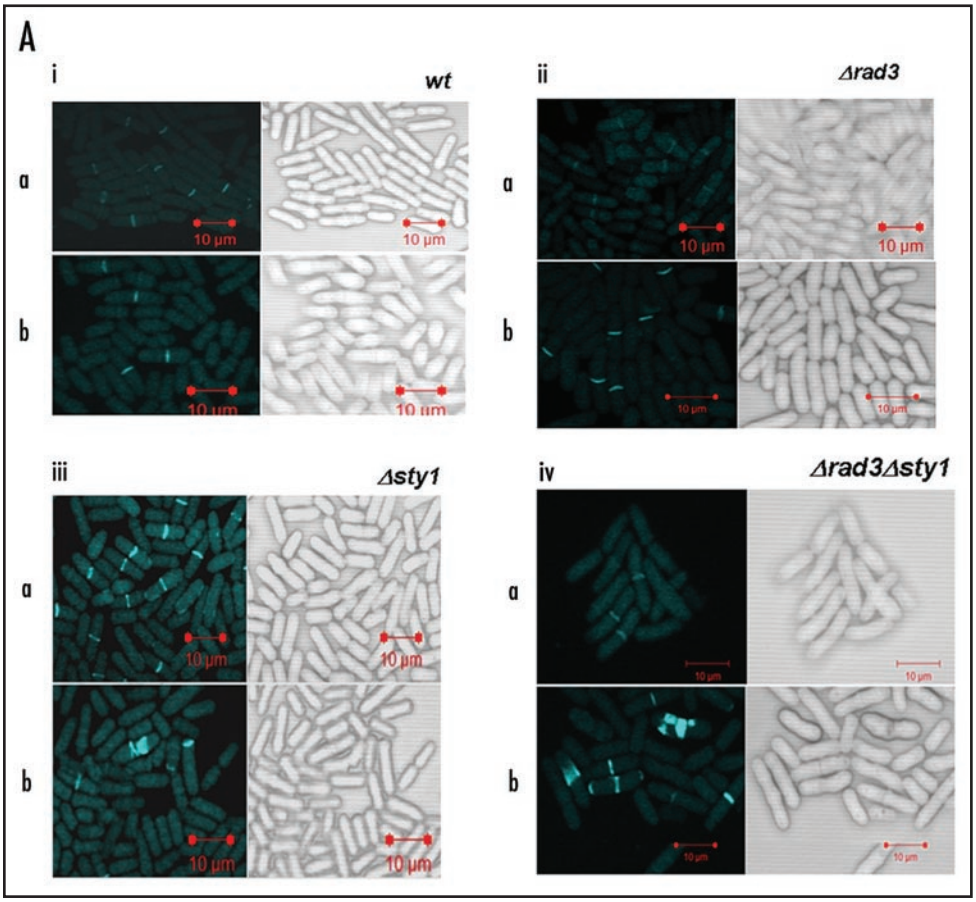

Figure 3. For figure legend, see page 4.

aggravated in the Δrad3Δ*sty1* mutants (Fig. 3A, part iv). Such abnormalities are not observed in either wild type (Fig. 3A, part i) or the Δ*rad3* (Fig. 3A, part ii) cells. Absence of abnormal septation indicated that CSE treated wild type cells have a mechanism to ensure that the cells do not accumulate with mitotic abnormalities. CSE induced S phase checkpoint is absent in Δ*rad3* cells (Chaudhuri et al.[1]). However CSE treated Δ*rad3* cells also did not undergo abnormal mitosis. Hence the mechanism for repair or prevention of aberrant mitosis does not appear to be dependent on the activation of the S phase checkpoint following CSE treatment. However this repair appeared to be severely impaired in both Δ*sty1* and Δ*sty1*Δ*rad3* cells. Hence Sty1 must be having a role in ensuring that mitotic aberrations did not accumulate in CSE treated *S. pombe* cells. To investigate what this role was, asynchronously growing wild type and Δ*sty1* cells were treated with CSE and the growth and mitotic progression of both cell populations was monitored. We observed that there is a significant growth arrest in both wild type and Δ*sty1* cells after CSE treatment (Fig. 3B). Mitotic progression was determined and expressed as the Mitotic index, i.e., the percentage of single, non-septate, binucleate cells in the population. We found that CSE treatment causes an appreciable decrease in mitotic index in the wild type population within 30 mins of exposure (Fig. 3C, part i), whereas a comparable decrease in the mitotic index of CSE treated Δ*sty1* wasn't seen before at least 1.5 hrs post CSE exposure (Fig. 3C, part ii), although in case of both the cell types the cells number ceases to increase after CSE treatment (Fig. 3B). These results clearly show that Sty1 is required for slowing down mitotic progression in CSE treated cells and in absence of this Sty1 mediated delay, the cells undergo aberrant mitosis, thus leading to mitotic catastrophe. So *S. pombe* cells respond to CSE treatment by activating two defense strategies, namely the slowed mitotic progression and the slowing of S phase. Absence of both these pathways in the Δrad3Δsty1 renders them extremely sensitive to CSE.

Sty1 associates with Cdc25 in vivo. The next logical step was obviously to probe into the molecular mechanism by which Sty1 activates a mitotic checkpoint. Now, if Sty1 has to bring about a cell cycle delay, it has to directly or indirectly affect the regulation of any of the important cell cycle regulatory molecules. One prime suspect was the cell cycle regulatory phosphatase Cdc25. The reasons behind implicating Cdc25 was that Sty1 being a Ser/Thr Kinase had the potential to inhibit Cdc25, as the latter is known to be inactivated after Ser phosphorylation. Chances were that if Sty1 could function in Cdc25 inhibition then we might find Cdc25 associated with Sty1 in CSE treated *S. pombe* cells. So, total Sty1 was immunoprecipitated from CSE treated wild type KS1376 *S. pombe* cells (which have a HA-tagged genomic copy of Sty1) at different times after treatment and transferred onto PVDF membrane. Imunoblotting with anti Cdc25 antibodies revealed the presence of associated Cdc25 with the immunoprecipitated Sty1 (Fig. 4A), thus confirming our suspicions. The extent of this association peaked at 10 mins after treatment. Sty1 activation after CSE treatment has been shown to be maximal around this time also (Chaudhuri et al.[1]).

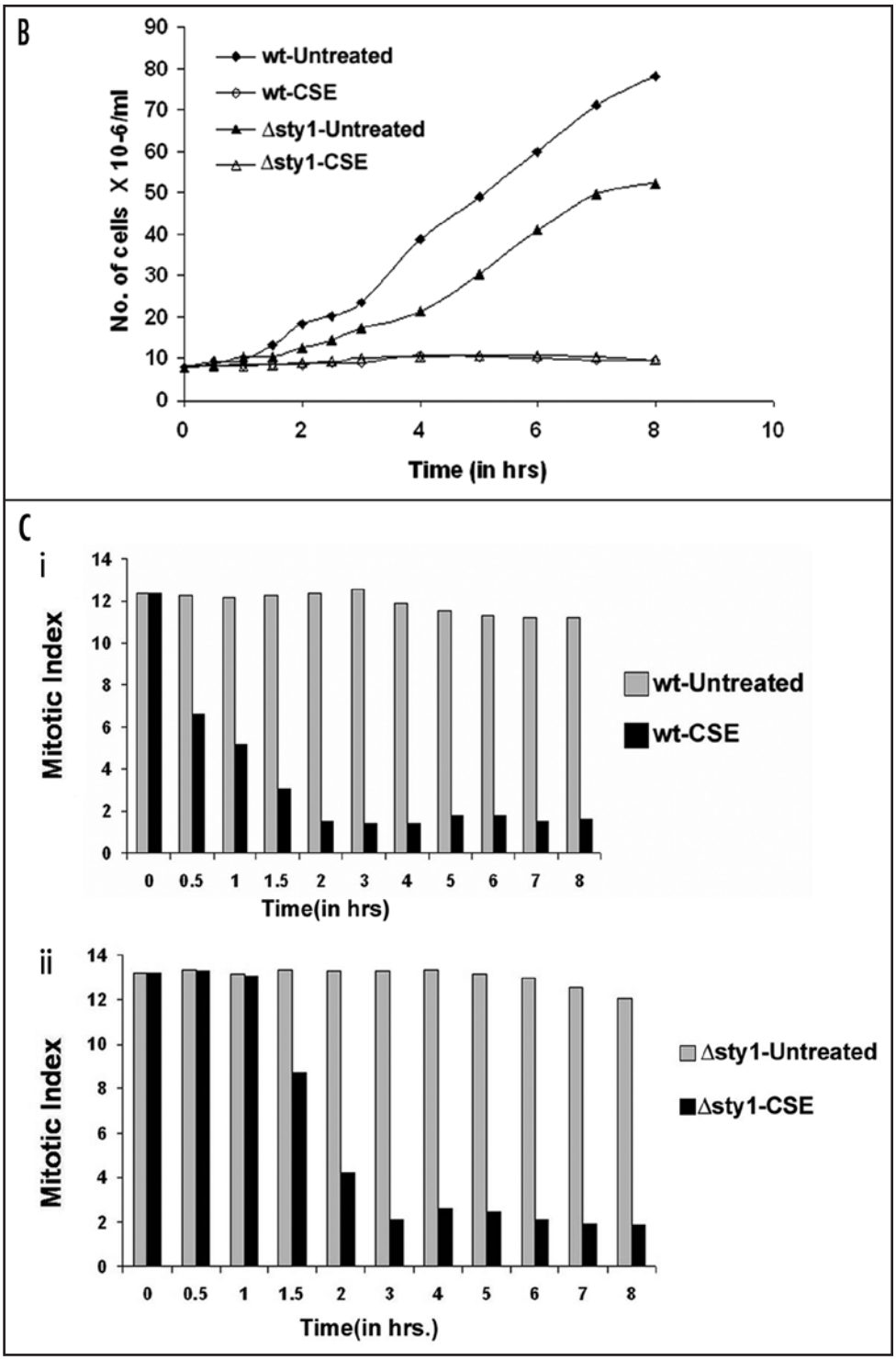

Figure 3. Sty1 dependent mitotic delay in *S. pombe*. (A) Wild type and Δsty1, Δ*rad3 and* Δ*rad3*Δsty1 cells were treated with CSE and then stained with calcoflour and the observed through a fluorescence microscope at a magnification of 63X. Abnormal septation in Δsty1(iii) and Δrad3 Δsty1(iv) cells was seen after CSE treatment for 2 hrs. No such defects are seen in wt(i) and Δrad3(ii) cells. (B) Wild type and Δsty1cells were grown in the presence and absence of CSE and the cell number in these populations was determined at regular time intervals to monitor the growth of these cells. (C) Wild type and Δsty1cells were grown in the presence and absence of CSE and the Mitotic index of these cells was determined by calculating the % of single binucleate non-septate cells. In all the figures (a) represents the untreated cells and the rest represent cells treated with CSE for 2 hrs.

Thus CSE treatment activates Sty1, which can then associate with Cdc25 and inhibit its activity, thus blocking the cells' entry into mitosis. Genetic studies in *S. pombe* have earlier pointed to a link between Sty1 and Cdc25 activity but this study, to our knowledge, is the first report of direct interaction between Sty1 and Cdc25 in *S. pombe* in vivo. Further investigation is however needed to confirm whether this interaction leads to any direct or indirect modification of Cdc25. Reverse immunoprecipitation experiments where Cdc25 was immunoprecipitated from CSE treated wild type cells and then immunoblotting was done using anti p38 antibodies confirmed this interaction (data not shown). So we knew now how Sty1 was bringing about the delayed mitotic progression. It was necessary to find out whether this function of Sty1 was in any ways modulated by Rad3. While investigating such a possibility we actually found that Cdc25 immunoprecipitated from Δ*rad3* cells treated with CSE was still found to be associated with Sty1 but unlike the case for the wild type cells, there was a significant continuation of this association upto 30 mins after treatment (Fig. 4B). Hence although Rad3 activity is not essential for initiating the interaction between Sty1 and Cdc25, but temporal regulation of this interaction definitely depends on Rad3 activity. Wild type *S. pombe* cells were then subjected to stress condition other than CSE and it was found that the association of Sty1 with Cdc25 is not CSE specific. The Sty1 -Cdc25 interaction was present in HU treated cells and to a much lesser extent in H_2O_2 treated cells (Fig. 4C).

CSE induced Cdc25 degradation depends on Sty1-Cdc25 interaction. The demonstration of this novel interaction between Sty1 and Cdc25 made it important to look for its functional significance. Wild type, Δ*sty1 and* Δ*rad3* cells were separately treated with CSE and cells were harvested at 0, 10, 20, 30 and 60 minutes after treatment. Denatured extracts were prepared and equal amounts of total protein from these extracts were resolved on 10% SDS-PAGE. Subsequently Cdc25 levels were monitored by immunoblotting. It was observed that when wild type *S. pombe* cells are treated with CSE, there is a rapid decrease in the total Cdc25 levels (Fig. 5A). Hence treatment with CSE leads to degradation of Cdc25 resulting in the observed cell cycle arrest. Interestingly under similar conditions there is no Cdc25 degradation at all in Δ*sty1* cells (Fig. 5B). Even in Δ*rad3* cells there is only a slight decrease in total Cdc25 levels, at 60 mins after

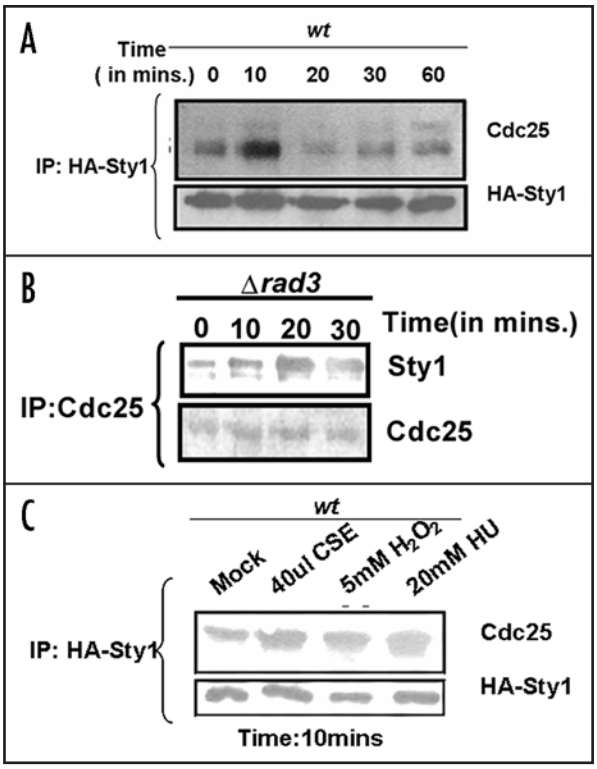

Figure 4. Sty1 inhibits Cdc25 in CSE treated *S. pombe* cells. (A) Sty1 was immunoprecipitated from wild type cells treated with CSE at 0, 10, 20, 30 and 60 mins after treatement, resolved on 10% SDS-PAGE and then immunoblotted with Anti Cdc25 antibodies. Physical Association of Sty1 with Cdc25 in vivo was observed in wild type *S. pombe* cells treated with CSE. (B) Δrad3 cells were also treated with CSE and Cdc25 was immunoprecipitated at similar time intervals after treatment. The immunoprecipitated complexes were resolved on 10% SDS-PAGE and then immunoblotted with anti p38 antibodies to reveal associated Sty1 (C) Wild type *S. pombe* cells were treated with CSE, 20 mM Hydroxyurea(HU), or 5 mM Hydrogen Peroxide(H_2O_2) and the extent of association between Cdc25 and Sty1 was determined similarly as in (A).

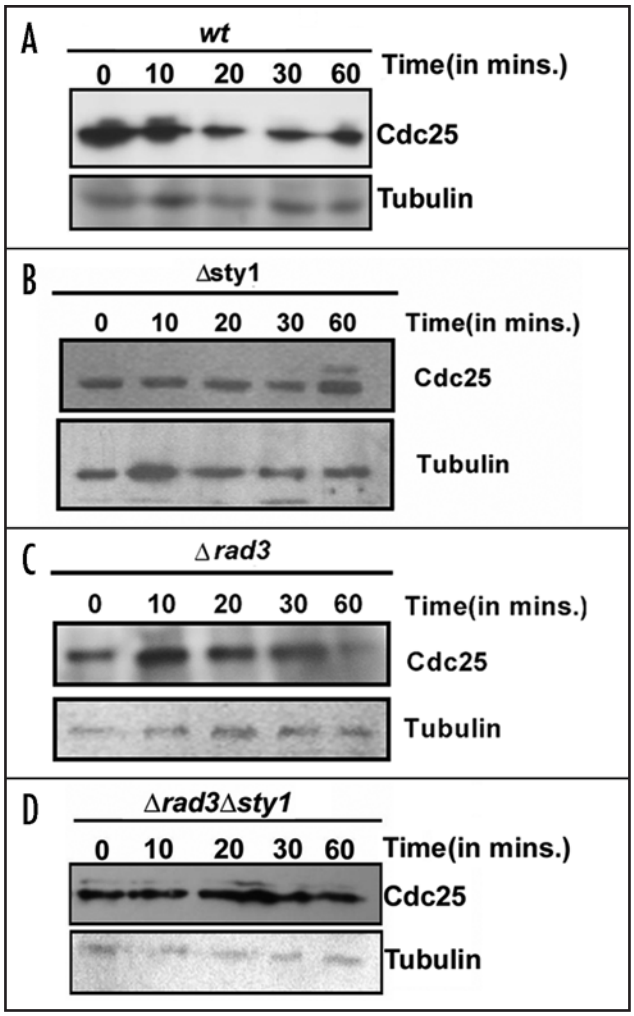

Figure 5. Sty1 activity regulates Cdc25 turnover after CSE treatment. (A) Wild type (B) Δsty1(C) Δrad3 and (D) Δrad3Δsty1 cells were treated with CSE. Cells were harvested at 0, 10, 20, 30 and 60 mins after treatment, denatured extracts were prepared and Cdc25 levels were determined by immunoblotting with anti Cdc25 antibodies.

CSE treatment (Fig. 5C). Expectedly, in Δ*rad3*Δ*sty1* cells the Cdc25 levels remain fairly constant (Fig. 5D).

Hence the CSE mediated degradation of Cdc25 was impaired in the absence of either Sty1 or Rad3 activity. Rad3 mediated Cdc25 degradation in *S. pombe* during S phase arrest is well documented. Here we show that both Rad3 dependent inhibition of Cdc25 and the interaction between Sty1 and Cdc25 are equally important for effecting the Cdc25 degradation and cell cycle arrest resulting from CSE treatment.

We investigated whether this Cdc25 degradation is reflected in Cdc2 activity. We found that the levels of inhibitory Tyr 15 phosphorylation on Cdc2 show an oscillating pattern in CSE treated wild type cells (Fig. 6A), while it remains constant in Δ*sty1* (Fig. 6B) cells under similar conditions. This shows that there might be a more complex role of Sty1 in the regulation of Cdc2 activity, probably mediated through Wee1 or Mik1 kinases. This possibility however needs further investigation.

Discussion

In this report we demonstrate a novel pathway of modulation of cell cycle regulatory molecules in *S. pombe*, namely, the activation of a mitotic checkpoint by the MAPK Sty1.

Interstingly many extracellular stimuli like UV or gamma-radiation, DNA damaging compounds etc have been shown to activate cell cycle checkpoints in a p38 dependent manner in mammalian cells. p38 has been demonstrated to bind and phosphorylate Cdc25B, Cdc25C and Cdc25A (Bulavin et al.,[24] Lemaire et al.,[25] Kittipatarin et al.[36]). ERK has also been shown to phosphorylate Cdc25C (Eymin et al.[37]). Our data shows that a similar mechanism is present in *S. pombe* also. We show that MAPK activity is related to Cdc25 turnover in *S. pombe*. Although a role for Sty1 has long been implicated in Cell cycle regulation in *S. pombe*, this is the first study illustrating the molecular mechanism behind this regulation.

This is the first report demonstrating the direct physical association of Sty1 and Cdc25. We show that CSE treated *S. pombe* cells face two different checkpoints in order to counteract the damage caused by CSE. We had previously shown that CSE treated *S. pombe* cells are arrested in S phase in a Rad3—Cds1 dependent manner (Chauduri et al.[1]). We now add that CSE treated *S. pombe* cells also exhibit a delay in mitotic progression. The activation of this mitotic checkpoint requires the activity of the MAPK Sty1. Sty1 activated

in response to CSE slows down mitotic progression in CSE treated *S. pombe* cells and thus prevents a mitotic catastrophe. Based on the above findings we propose a modified model for cellular response to CSE in *S. pombe* (Fig. 7). We show that Sty1 is necessary for the CSE induced delay in mitotic progression in *S. pombe* cells. This is achieved through the in vivo association of Sty1 with the cell cycle regulatory phosphatase Cdc25 and the resulting degradation of Cdc25. This interaction between Sty1 and Cdc25 is not specific to CSE but is also present in *S. pombe* cells treated with Hydroxyurea. Thus this mechanism represents a general mode of cell cycle regulation in *S. pombe*. Both CSE and Hydroxyurea are potent genotoxins. They cause extensive DNA damage which also leads to generation of free radicals inside the cells. Either the DNA damage or the resulting generation of free radicals or both might be responsible for the observed Sty1-Cdc25 interaction. We show that Rad3 activity is important for temporal regulation of the interaction between Sty1 and Cdc25. Our observations suggest that the cell cycle regulatory and the MAPK signaling pathways converge at the common effector molecule, namely the Cdc25 phosphatase. Absence of either of these two pathways increases the cells' sensitivity to CSE and the absence of both is extremely deleterious to cell survival. We also show that there is huge ROS accumulation in the Δsty1Δrad3 double mutant after CSE treatment. It is possible that failure to activate cell cycle checkpoints leads to accumulation of DNA damage in these cells after CSE treatment. This in turn would lead to generation of free radicals as manifested by an increase in intracellular ROS levels. This may also lead to the observed high sensitivity of the Δsty1Δrad3 cells to CSE.

It needs to be mentioned here that Sty1 mediated Cdc25 degradation does not co-relate directly with the levels of inhibitory Tyr15 phosphorylation on Cdc2 (Fig. 6). This indicates the probable presence of a more complex regulation of Cdc2 activity downstream of or parallel to the Sty1—Cdc25 interaction. The differential levels of phospho Cdc2(Tyr15) in CSE treated Δsty1 and wild type cells suggests that apart from regulating Cdc25 degradation, Sty1 may influence the activity of Cdc2 also. However this possibility needs further investigation.

This study also investigates whether the CSE induced S phase delay is in anyway dependent on MAPK activity. We clearly show that Sty1 does not affect the S phase checkpoint activation. ATF2 the mammalian homologue of *S. pombe* Atf1, a Sty1 dependent transcription factor has been earlier reported to have a role in regulating the activity of Chk2 (mammalian homologue of *S. pombe* Cds1) during DNA damage response (Bhoumik et al.[38]), but we observed that neither Sty1 nor Atf1 activity is essential for Cds1 activation during cellular response to CSE treatment (data not shown).

The association of Sty1 with Cdc25 in CSE treated *S. pombe* cells implicates a novel role of Sty1 in Cell cycle regulation upon genotoxic stress. The functional significance of this association is seen at the level of Cdc25 degradation in response to CSE. Our studies have clearly demonstrated that both Rad3 dependent inhibition of Cdc25 and the interaction between Sty1 and Cdc25 are significantly important for Cdc25 degradation and cell cycle arrest in response to CSE treatment. This interdependence of Sty1 and Rad3 in regulating Cdc25 turnover is the hallmark of the synergism between the two signaling cascades regulated by the individual proteins. These findings also give an extended insight into how human cells may potentially react to Cigarette smoke exposure. Interaction between their respective homologs in humans, p38 and Cdc25A/B/C respectively in cells treated with CSE has not been reported earlier, but our findings strongly suggest that the activation of p38 might lead to the activation of a similar mitotic checkpoint in human cells exposed to Cigarette smoke. Together these findings elucidate the importance of Sty1/p38 in counteracting genotoxic stress and strengthen the ideas behind the emerging role of p38 as a tumor suppressor (Timofeev et al.[39]).

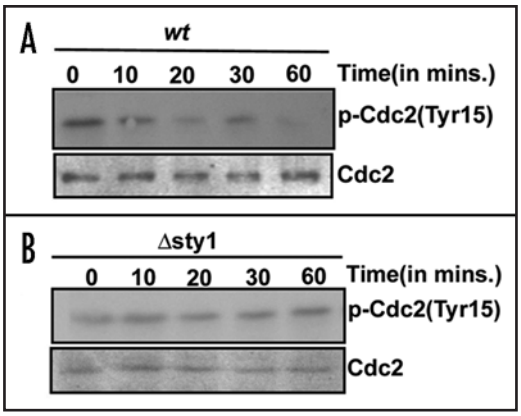

Figure 6. (A) wild type and (B) Δsty1cells were treated with CSE. Cells were harvested at 0, 10, 20, 30 and 60 mins after treatment, denatured extracts were prepared and the levels of inhibitory phosphorylation on Cdc2 was determined by immunoblotting with anti phosphoCdc2 (Tyr15) antibodies.

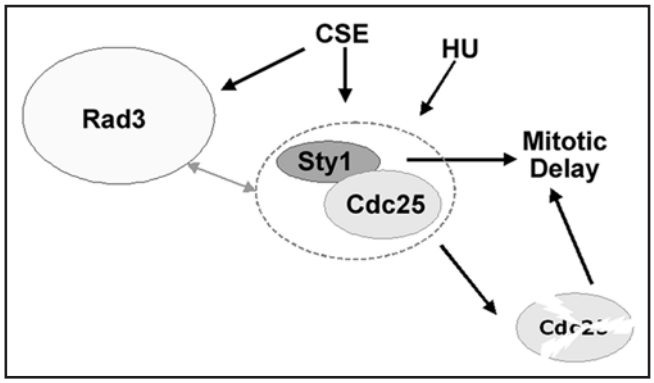

Figure 7. Proposed model for Cellular responses to CSE. CSE treatment leads to activation of both Rad3 and Sty1.The activated Sty1 then associates with the cell cycle regulatory phosphatase Cdc25. This interaction is not CSE specific and is present in *S. pombe* cells treated with HU as well. Temporal regulation of Sty1-Cdc25 interaction is Rad3 dependent. The Sty1-Cdc25 interaction is responsible (along with Rad3 activity) for Cdc25 turnover required for cell cycle arrest in *S. pombe* cells exposed to CSE.

Materials and Methods

Fission yeast strains, media and growth conditions. *S. pombe* strains used in this study are listed in Table 1. All cells were grown at 30°C in the rich medium (YES), as described previously (Moreno et al.[34]).

Preparation of cigarette smoke extract (CSE) and CSE treatment. Preparation of CSE and treatment was done as described before (Chaudhuri et al.[1]). Unless otherwise mentioned all treatments with CSE were done at a dose of 40 ul CSE/8 x 10^6 cells.

Table 1 ***S. pombe* strains used**

Strain	Genotype	Source
PR109	*h⁻ leu1-32 ura4-D18*	Paul Russell
KS1366	*h⁻ sty1:: ura4⁺ leu1-32 ura4-D18*	K. Shiozaki
KS1376	*h⁻ sty1::HA6H (ura4⁺) leu1-32 ura4-D18*	Paul Russell
TE570	*h⁻ rad3::ura4⁺ ade6-704 leu1-32 ura4-D18*	Tamar Enoch
Δrad3Δsty1	*h⁻ rad3::ura4⁺ sty1::ura4⁺ leu1-32 ura4-D18*	Tim Humphrey

Fluorescence microscopy. Fission yeast nuclei and septum were stained with 4', 6'-diamidino-2- phenylindole (DAPI) and Calcofluor, respectively, as described previously (Moreno et al.[34]). After staining, cells were examined using Zeiss LSM 510 Meta Laser Scanning Confocal Microscope at 63X magnification. The mitotic index was determined by counting the percentage of single (non septate) binucleate cells. All images were taken and processed with the use of identical parameters. For all quantitative experiments at least 400 cells were examined in each sample.

Flow cytometry. For cell cycle monitoring, asynchronously growing fission yeast cells were treated with CSE as described above and were fixed in 70% ethanol at indicated time points and stored at 4°C. For measuring DNA content, cells were washed once with 50 mM sodium citrate, then resuspended in the same buffer containing 0.1 mg/ml RNase A (Bangalore Genei) and incubated at 37°C for at least 2 hrs. Cells were then stained with the same buffer supplemented with 4 μg/ml Propidium Iodide and analyzed by a Becton Dickinson FACS Calibur. Data were analyzed by CellQuest software for Macintosh.

For cell death measurement cells treated with CSE were collected at the indicated time points and stained with Propidiun Iodide (without fixing). Only dead cells take up PI. So PI uptake can be used to asses cell death. % of cells having higher PI fluorescence than unstained cells in each case was determined. The data so obtained was normalized with respect to the cell death in untreated cell populations in each strain. The data represent mean of three independent experiments. For ROS level determination cells treated with CSE were collected at the indicated time points, washed in YES and incubated for further 30 mins with 10 uM H_2DCF-DA. H_2DCF-DA is a cell permeable substance. Cellular esterases can cleave the ester linkage and convert it into a flourophore trapped inside the cell. Intracellular ROS can then oxidize H_2DCF to a fluorescent molecule and its fluorescence is thus comparable to intracellular ROS levels. % of cells having higher fluorescence than the unstained cells was determined for each sample. The data represent mean of three independent experiments.

Immunoprecipitations and immunoblotting. Cells were collected by rapid filtration following CSE exposure at indicated time points and cell extracts were made under native conditions. Sty1 was immunoprecipitated from wild type cells using the strain KS1376, which carries a chromosomal copy of Spc1 having a C-terminal tag with two copies of the influenza virus hemagglutinin (HA) epitope followed by six consecutive histidine residues (reviewed in refs. 31 and 35), using anti-HA(12CA5) antibodies (1:100, Santa Cruz). Cdc25 was immunoprecipitated using polyclonal Cdc25A antibodies (1:100, Santa Cruz). Immunocomplexes were resolved by SDS-PAGE and electroblotted to PVDF membrane. Immunoblotting was done with anti Cdc25A antibodies (1:500; Santa Cruz), or anti p38 (1:500, Cell Signalling) as the case may be and then with HRP conjugated Secondary antibody (1:5000; Bangalore Genei) and detected by Chemiluminiscence using 20X LumiGLO® Reagent and 20X Peroxide (Cell Signalling Technology).

Acknowledgements

We gratefully acknowledge Paul Russell (Scripps Research Institute), Kazuhiro Shiozaki (University of California, Davis), Tamar Enoch (Harvard Medical School, Boston), Tim Humphrey for generously providing the strains. We are also thankful to Keith Gull for providing the TAT-1 antibodies. G.S. and S.P.C. thank Council for Scientific and Industrial Research, India for providing fellowship during the course of the work. We thank DSA, University Grants Commission India, NCMP grant and DST FIST programme for infrastructural support.

References

1. Chaudhuri SP, Sundaram G, Bhattacharya A, Ray P, Ray A, Chatterjee IB, Chattopadhyay D. Activation of S phase checkpoint by cigarette smoke extract in Schizosaccharomyces pombe. Yeast 2005; 22:1223-38.
2. Sherman CB. The health consequences of cigarette smoking. Pulmonary diseases. Med Clin North Am 1992; 76:355-75.
3. Chow, C. K. Cigarette smoking and oxidative damage in the lung. Ann NY Acad Sci 1993; 686:289-98.
4. Reznick, A. Z., Cross, C. E., Hu, M. L., Suzuki, Y. J., Khwaja, S., Safadi, A., Motchnik, P. A., Packer, L. and Halliwell, B. Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. Biochem J 1992; 286:607-11.
5. Santanam, N., Sanchez, R., Hendler, S. and Parthasarathy, S. Aqueous extracts of cigarette smoke promote the oxidation of low density lipoprotein by peroxidases. FEBS Lett 1997; 414:549-51.
6. Panda, K., Chattopadhyay, R., Ghosh, M. K., Chattopadhyay, D. J. and Chatterjee, I. B. Vitamin C prevents cigarette smoke induced oxidative damage of proteins and increased proteolysis. Free Radic Biol Med 1999; 27:1064-79.
7. Frei, B., Forte, T. M., Ames, B. N. and Cross, C. E. Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma. Protective effects of ascorbic acid. Biochem J 1991; 277:133-8.
8. Kiyosawa, H., Suko, M., Okudaira, H., Murata, K., Miyamoto, T., Chung, M. H., Kasai, H. and Nishimura, S. Cigarette smoking induces formation of 8-hydroxydeoxyguanosine, one of the oxidative DNA damages in human peripheral leukocytes. Free Radic Res Commun 1990; 11:23-7.
9. Shah, P. K. and Helfant, R. H. Smoking and coronary artery disease. Chest 1998; 94:449-52.
10. Muller T, Gebel S. The cellular stress response induced by aqueous extracts of cigarette smoke is critically dependent on the intracellular glutathione concentration. Carcinogenesis 1998; 19:797-801.
11. Hirao, T., Nelson, H. H., Ashok, T. D., Wain, J. C., Mark, E. J., Christiani, D. C., Wiencke, J. K. and Kelsey, K. T. Tobacco smoke-induced DNA damage and an early age of smoking initiation induce chromosome loss at 3p21 in lung cancer. Cancer Res 2001; 61:612-5.
12. Lambert, B., Berndtsson, I., Lindsten, J., Nordenskjold, M., Soderhall, S., Holmstedt, B., Palmer, L., Jernstrom, B. and Marsk, L. Smoking and sister chromatid exchange. Prog Clin Biol Res 1982; 109:401-14.
13. Bernardini, S., Adessi, G. L., Chezy, E., Billerey, C., Carbillet, J. P. and Bittard, H. Influence of cigarette smoking on P53 gene mutations in bladder carcinomas. Anticancer Res 2001; 21:3001-4.
14. Leanderson, P. and Tagesson, C. Cigarette smoke-induced DNA damage in cultured human lung cells: role of hydroxyl radicals and endonuclease activation. Chem Biol Interact 1992; 81:197-208.
15. Panda, K., Chattopadhyay, R., Chattopadhyay, D. J. and Chatterjee, I. B. Vitamin C prevents cigarette smoke-induced oxidative damage in vivo. Free Radic Biol Med 2000; 29:115-24.
16. Russell, P. and Nurse, P. Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell 1987; 49:559-67.
17. Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. Mik1 and Wee1 cooperate in the inhibitory tyrosine phosphorylation of Cdc2. Cell 1991; 64:1111-22.
18. Aligue, R., Wu, L. and Russell, P.Regulation of Schizosaccharomyces pombe Wee1 tyrosine kinase. J Biol Chem 1997; 272:13320-5.
19. Russell, P. and Nurse, P. cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell 1986; 45:145-53.
20. Gautier, J., Solomon, M.J., Booher, R.N., Bazan, J.F. and Kirschner, M.W. Cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell 1991; 67:197-211.
21. Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R. and Russell, P.p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. EMBO J 1991; 10:4301-9.

22. Furnari B, Blasina A, Boddy MN, McGowan CH, Russell P. Cdc25 inhibited in vivo and in vitro by checkpoint kinases Cds1 and Chk1. Mol Biol Cell 1999; 10:833-45.
23. Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., Appella, E. and Fornace, A. J., Jr. Initiation of a G_2/M checkpoint after ultraviolet radiation requires p38 kinase. Nature 2001; 411:102-7.
24. Bulavin DV, Amundson SA, Fornace AJ.p38 and Chk1 kinases: different conductors for the G(2)/M checkpoint symphonyCurr Opin Genet Dev 2002; 12:92-7.
25. Mathieu Lemaire, Carine Froment, Rose Boutros, Odile Mondesert, Angel R. Nebreda, Bernard Monsarrat and Bernard Ducommun. CDC25B Phosphorylation by p38 and MK-2. Cell Cycle 2006; 5:1649-53.
26. Goldstone S, Pavey S, Forrest A, Sinnamon J, Gabrielli B. Cdc25-dependent activation of cyclin A/cdk2 is blocked in G_2 phase arrested cells independently of ATM/ATR. Oncogene 2001; 20:921-32.
27. Wang X, McGowan CH, Zhao M, He L, Downey JS, Fearns C, Wang Y, Huang S, Han J. Involvement of the MKK6-p38gamma cascade in gamma-radiation-induced cell cycle arrest. Mol Cell Biol 2000; 20:4543-52.
28. Mikhailov A, Shinohara M, Rieder CL.Topoisomerase II and histone deacetylase inhibitors delay the G_2/M transition by triggering the p38 MAPK checkpoint pathway. J Cell Biol 2004; 166:517-26.
29. Matsusaka T, Pines J. Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. J Cell Biol 2004; 166:507-16.
30. Millar JB, Buck V, Wilkinson MG. Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. Genes Dev 1995; 9:2117-30.
31. Shiozaki K and Russell P. Cell cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. Nature 1995; 378:739-43.
32. Gachet Y, Tournier S, Millar JB, Hyams JS.A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. Nature 2001; 412:352-5.
33. Lopez-Aviles S, Grande M, Gonzalez M, Helgesen AL, Alemany V, Sanchez-Piris M, Bachs O, Millar JB, Aligue R. Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast. Mol Cell 2005; 17:49-59.
34. Moreno S., Klar A. and Nurse P. Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol 1991; 194:795-823.
35. Degols G., Shiozaki K. and Russell P. Activation and regulation of the Spc1 stress-activated protein kinase in Schizosaccharomyces pombe. Mol Cell Biol 1996; 16:2870-7.
36. C. Kittipatarin, WQ. Li, D. Bulavin, S.K. Durum and A.R. Khaled. Cell Cycling through Cdc25A: Transducer of Cytokine Proliferative Signals. Cell Cycle 2006; 5:907-12.
37. Beatrice Eymin, Paule Claverie, Caroline Salon, Christian Brambilla, Elisabeth Brambilla and Sylvie Gazzeri.p14ARF Triggers G_2 Arrest Through ERK-Mediated Cdc25C Phosphorylation, Ubiquitination and Proteasomal Degradation. Cell Cycle 2006; 5:759-65.
38. Bhoumik A, Takahashi S, Breitweiser W, Shiloh Y, Jones N, Ronai Z. ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. Mol Cell 2005; 18:577-87.
39. Timofeev O, Lee TY, Bulavin DV. A subtle change in p38 MAPK activity is sufficient to suppress in vivo tumorigenesis Cell Cycle 2005; 4:118-20.