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Hyperosmosis enhances radiation and hydroxyurea resistance of *Schizosaccharomyces pombe* checkpoint mutants through the spindle checkpoint and delayed cytokinesis

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Summary

The DNA damage and stress response pathways interact to regulate cellular responses to genotoxins and environmental stresses. How these pathways interact in *Schizosaccharomyces pombe* is not well understood. We demonstrate that osmotic stress suppresses the DNA damage sensitivity of checkpoint mutants, and that this occurs through three distinct cell cycle delays. A delay in G2/M is dependent on Srk1. Progression through mitosis is halted by the Mad2-dependent spindle checkpoint. Finally, cytokinesis is impaired by modulating Cdc25 expression. These three delays, imposed by osmotic stress, together compensate for the loss of checkpoint signalling.

Introduction

The stress-activated MAP kinase Sty1 (Spc1) plays a central role in mediating the response of *Schizosaccharomyces pombe* cells to various environmental stresses (Millar *et al*., 1995; Shiozaki and Russell, 1995; Degols *et al*., 1996). Early studies suggested a role for Sty1 in regulating cell cycle progression. First, *sty1* mutants are elongated, indicating delayed progression from G2 into mitosis. Furthermore, the *spc1-1* mutation is synergistically lethal with the *cdc25-22* mutation at the semi-permissive temperature (Millar *et al*., 1995; Shiozaki and Russell, 1995). Following exposure to osmotic stress, *sty1* mutants undergo G2 arrest and lose viability. In contrast, *sty1*$^+$ cells undergo a transient cell cycle delay but then resume cell division resulting in the accumulation of small cells. Entry into mitosis following the resumption of cell division is dependent on Cdc25 as *cdc25-22* mutants fail to enter mitosis at the restrictive temperature under these conditions (Kishimoto and Yamashita, 2000). Together, these studies suggest that Sty1 and Cdc25 cooperate to facilitate cell cycle progression under osmotic stress conditions. In fact, *sty1* mutants, unlike wild-type (wt) cells, fail to accumulate Cdc25 following exposure to osmotic stress (Kishimoto and Yamashita, 2000).

In contrast to Sty1, the ATM homologue Rad3-regulated DNA damage checkpoint pathways are required to delay cell cycle progression following stalled replication or DNA damage. Checkpoint mutants attempt mitosis with incompletely replicated and/or damaged DNA, resulting in aberrant mitoses and a rapid loss of viability. Following its DNA damaged induced activation in G2, Rad3 activates Chk1 in a Crb2-dependent manner (Humphrey, 2000). Chk1 directly inhibits Cdc25 and also induces the activity of Wee1. This kinase in turn inhibits Cdc2 activity through inhibitory phosphorylation of tyrosine 15. In addition, Rad3 also functions via Cds1 to facilitate the resumption of stalled replication through a process generally referred to as 'recovery' (Murakami and Okayama, 1995; Lindsay *et al*., 1998). The effective enforcement of DNA damage checkpoints requires simultaneous inhibition of Cd25 and Cdc2 by a double lock mechanism (Raleigh and O'Connell, 2000).

Several studies have provided evidence that the Rad3- and Sty1-regulated pathways are co-activated in response to ultraviolet (UV) and ionizing radiation (reviewed in Alao and Sunnerhagen, 2008). Given their opposing roles on cell cycle progression, it is unclear how these pathways are integrated. Studies have shown that following exposure to hydroxyurea (HU), ionizing radiation or UV radiation, *rad1* and *rad3* mutants not only fail to delay cell cycle progression but appear to be advanced through the cell cycle (Jimenez *et al*., 1992; Kanter-Smoler *et al*., 1995; Alao *et al*., 2009). This cell cycle

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response is remarkably similar to that observed in *S. pombe* cells exposed to osmotic stress (Kishimoto and Yamashita, 2000). Indeed, the levels of reactive oxygen species are greatly elevated relative to wt cells, when these checkpoint mutants are exposed to HU (Marchetti *et al*., 2006). Furthermore, suppression of the stress cell cycle response increases the resistance of *cdc2-3w* mutants to HU (Humphrey and Enoch, 1998). It has thus been proposed that Sty1-mediated cell cycle acceleration contributes to the loss of viability observed in checkpoint mutants exposed to genotoxins (Humphrey and Enoch, 1998). Recent studies have demonstrated, however, that Sty1 activation inhibits entry into mitosis following exposure to osmotic stress. Sty1 induces the activation and accumulation of Srk1, which in turn phosphorylates Cdc25 and mediates its cytoplasmic sequestration (Lopez-Aviles *et al*., 2005; 2008). Osmotic stress also induces the hyperactivation of Cut2 (securin) and delays entry into mitosis for approximately 1 h (Kawasaki *et al*., 2006). Activation of the mammalian homologue of Sty1 (p38) has similarly been shown to inhibit entry into mitosis. The precise impact of Sty1 on cell cycle progression and DNA damage resistance of *S. pombe* checkpoint mutants exposed to genotoxins remains unclear (reviewed in Alao and Sunnerhagen, 2008).

To understand in more detail how the DNA damage and stress response pathways interact in *S. pombe*, we have investigated the effects of osmotic stress on the DNA damage sensitivity of checkpoint mutants. Osmotic stress suppressed the UV and HU sensitivity of mutants unable to fully activate and/or sustain the DNA damage and/or replication checkpoints. The suppression of DNA damage sensitivity was dependent on the osmotic stress-induced activation of alternative checkpoints within multiple cell cycle phases. This activity was only partially dependent on Srk1. We identified a novel role for the spindle checkpoint enforcer Mad2 in regulating cell cycle progression following exposure to osmotic stress. Finally, we demonstrate that osmotic stress delays cytokinesis and that this activity is sufficient to suppress DNA damage sensitivity in checkpoint mutants. Osmotic stress thus enforces multiple cell cycle delays that can compensate for the loss of checkpoint signalling.

Results

Osmotic stress suppresses DNA damage sensitivity

In order to determine the effect of osmotic stress on DNA damage sensitivity, cells were cultured in YES medium containing 0.6 M KCl prior to or immediately after exposure to UV. Osmotic stress did not affect the DNA damage sensitivity of wt cells. By contrast, exposure to osmotic stress following irradiation suppressed the UV sensitivity of *rad3*Δ, *rad3-136* and *rad1* checkpoint mutants (Fig. 1A). Similar results were obtained irrespective of whether exposure to osmotic stress was pre- or post-irradiation. Osmotic stress also suppressed the UV sensitivity of *chk1*Δ and *wee1*Δ mutants, which specifically lack a fully functional G2 checkpoint (reviewed in Humphrey, 2000) (Fig. 1B). Deletion of the spindle checkpoint enforcer *mad2*$^+$ (He *et al*., 1997) or *rad13*$^+$, required for nucleotide excision repair (NER) (McCready *et al*., 1989), in a *rad1*Δ background did not affect the ability of osmotic stress to suppress UV sensitivity (Fig. 1C and D). Osmotic stress can thus compensate specifically for the loss of the G2 checkpoint independently of the spindle checkpoint and the NER pathways.

Osmotic stress also suppressed the HU sensitivity of *rad3*Δ and *rad1*Δ mutants but did not affect the sensitivity of the *wt* strain to this agent (Fig. 1G). Osmotic stress likewise suppressed the HU sensitivity of the *cdc2-3w* and *cdc2-3w cdc25Δ* mutants (Fig. 1G) that specifically lack the replication checkpoint (Enoch and Nurse, 1990; Enoch *et al*., 1992). *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 (Murakami and Okayama, 1995). Osmotic stress did not, however, suppress the HU sensitivity of a *cds1*Δ mutant (Fig. 1I). Osmotic stress induced by 0.1 M $CaCl_2$ or 1.2 M sorbitol suppressed HU sensitivity similar to 0.6 M KCl (Fig. S1A and B). KCl did not influence the sensitivity of *sty1*Δ

Fig. 1. Osmotic stress suppresses the UV sensitivity of *S. pombe* DNA damage checkpoint mutants.
A. The indicated strains were plated on YES medium with or without 0.6 M KCl, exposed to increasing doses of UV as described in *Experimental procedures* and incubated at 30°C for 3 days.
B. *chk1*Δ and *wee1*Δ mutants were exposed to 120 Jm^{-2} and 240 Jm^{-2}, respectively, and cultured for 4 h in liquid YES medium with or without 0.6 M KCl. Equal cell numbers were spotted onto YES agar plates and incubated at 30°C for 3 days.
C, D. The indicated strains were exposed to 120 Jm^{-2} UV and treated as in (A).
E. The indicated strains were spotted onto YES agar plates, exposed to increasing doses of UV and incubated at 30°C for 3 days.
F. *sty1*Δ mutants were exposed to 150 Jm^{-2} UV and cultured for 4 h in liquid YES medium with or without 0.6 M KCl. Equal cell numbers were spotted unto YES agar plates and incubated at 30°C for 3 days.
G. The indicated strains were cultured in YES medium containing 20 mM HU with or without 0.6 M KCl and incubated at 30°C for 4 h. Equal numbers of cells were spotted on YES agar and incubated at 30°C for 3 days.
H. *sty1*Δ mutant cells were treated as in (G).
I. *cds1*Δ mutants were cultured in YES medium containing 20 mM HU with or without 0.6 M KCl, incubated at 30°C for 4 h and then treated as in G.

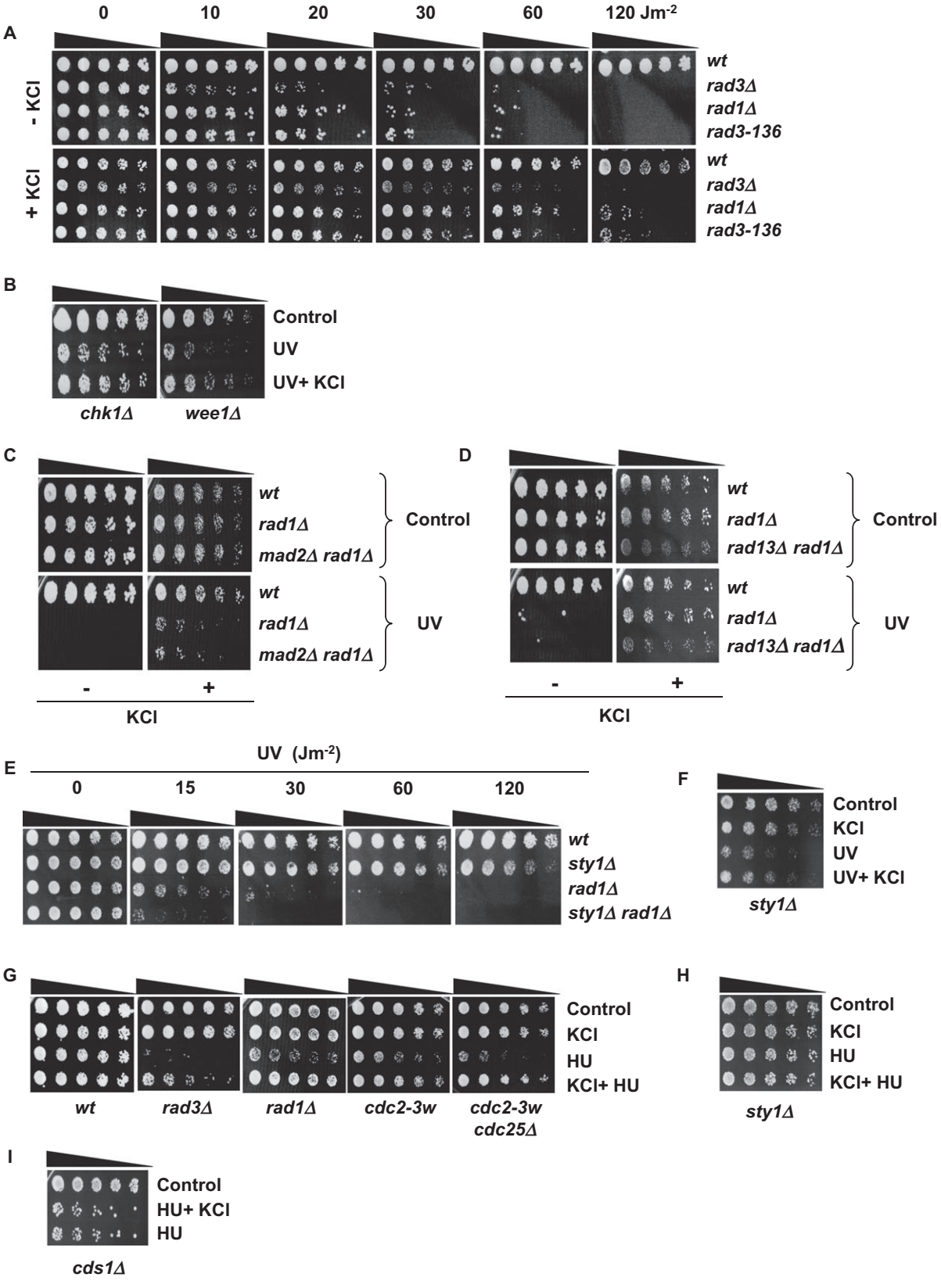

A
0
10
20
30
60
120 Jm-2
- KCl
+ KCl
wt
rad3Δ
rad1Δ
rad3-136
wt
rad3Δ
rad1Δ
rad3-136
B
Control
UV
UV+ KCl
chk1Δ
wee1Δ
C
wt
rad1Δ
mad2Δ rad1Δ
Control
wt
rad1Δ
mad2Δ rad1Δ
UV
-
+
KCl
D
wt
rad1Δ
rad13Δ rad1Δ
Control
wt
rad1Δ
rad13Δ rad1Δ
UV
-
+
KCl
E
UV (Jm-2)
0
15
30
60
120
wt
sty1Δ
rad1Δ
sty1Δ rad1Δ
F
Control
KCl
UV
UV+ KCl
sty1Δ
G
Control
KCl
HU
KCl+ HU
wt
rad3Δ
rad1Δ
cdc2-3w
cdc2-3w cdc25Δ
H
Control
KCl
HU
KCl+ HU
sty1Δ
I
Control
HU+ KCl
HU
cds1Δ

Fig. 2. Osmotic stress delays cell cycle progression in *S. pombe* cells exposed to UV.
A. wt (i), *rad3*Δ (ii) and *rad1*Δ (iii) cells were exposed to 120 Jm^{-2} UV as described in *Experimental procedures* and cultured in YES medium with or without 0.6 M KCl. Samples were harvested at the indicated time points, fixed in 70% ethanol, stained with aniline blue and examined by fluorescence microscopy. *rad1*Δ and *sty1*Δ *rad1*Δ (iv) mutants were exposed to 120 Jm^{-2} UV, cultured in YES medium and treated as in (A).
B. wt, *rad3*Δ and *rad1*Δ strains were cultured in YES medium containing 20 mM HU with or without 0.6 M KCl and samples harvested at the indicated time points and fixed in 70% ethanol prior to FACS analysis.
C. The *cdc2-3w* mutant strain was cultured as indicated for 4 h. Cells were stained with DAPI as described in *Experimental procedures* and examined by fluorescence microscopy to determine the number of cells with mis-segregated chromosomes. Results indicate the means from three independent experiments ± SE.

mutants to HU but partially suppressed sensitivity to UV (Fig. 1F and H). *sty1*Δ mutants did not, however, display sensitivity to 0.6 M KCl following a 4 h incubation (Fig. 1F and H) and were not advanced through mitosis under these conditions (Fig. S1I). Together, these data demonstrate that exposure to osmotic stress suppresses the DNA damage sensitivity of *S. pombe* checkpoint mutants independently of Sty1.

Osmotic stress delays cell cycle progression

Septation assays (Dunaway and Walworth, 2004) were used to monitor the effect of osmotic stress on cell cycle progression in wt and checkpoint mutants exposed to UV. Following exposure to UV, the septation index of wt cells rapidly declines due to activation of the G2 checkpoint (Fig. 2A, panel i). This effect was suppressed when UV irradiated cells were incubated in medium containing 0.6 M KCl, suggesting that osmotic stress delays cytokinesis because the septation index did not decline (Fig. 2A, panel i). By contrast, *rad3*Δ and *rad1*Δ mutants that lack a functional G2 checkpoint appear to be advanced into mitosis following exposure to DNA-damaging agents (Jimenez *et al*., 1992; Kanter-Smoler *et al*., 1995; Alao *et al*., 2009). Osmotic stress suppressed the accumulation of septated cells in these mutants suggesting that it delays cell cycle progression following exposure to UV (Fig. 2A, panels ii and iii).

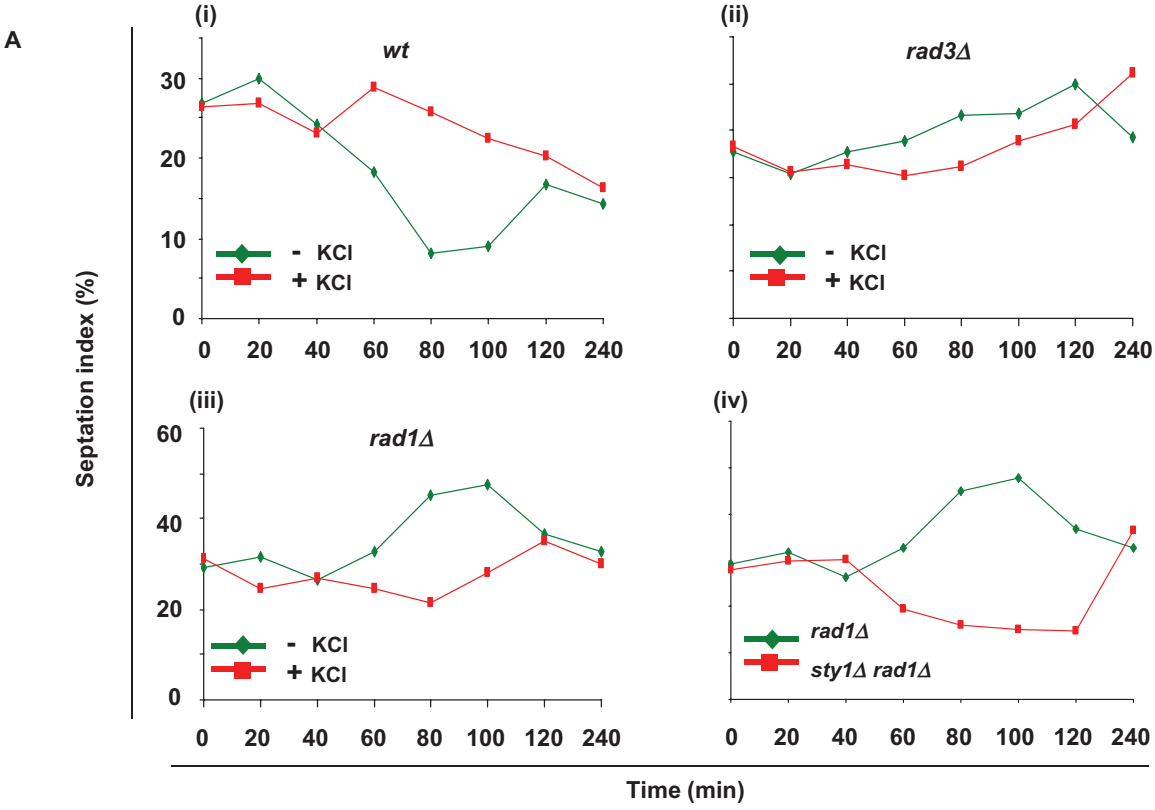
KCl-induced osmotic stress similarly delayed cell cycle progression in wt, *rad3*Δ and *rad1*Δ cells exposed to HU. Fluorescence-activated cell sorting (FACS) analyses showed that about 50% of asynchronous wt cells exposed to HU had progressed through mitosis, undergone cytokinesis and accumulated as single cells with a < 2C DNA content within 2 h (Fig. 2B). By contrast, the cell cycle progression of wt cells exposed to HU in the presence of 0.6 M KCl was delayed by approximately 3 h (Fig. 2B). Osmotic stress also delayed the cell cycle progression of *rad3*Δ and *rad1*Δ mutants under similar conditions (Fig. 2B). Further, osmotic stress suppressed the accumulation of cells with chromosome mis-segregation defects ('cuts') in *cdc2-3w* mutants exposed to HU (Fig. 2C). Osmotic stress thus suppresses the sensitivity of DNA damage checkpoint mutants, presumably by inducing delays in cell cycle progression.

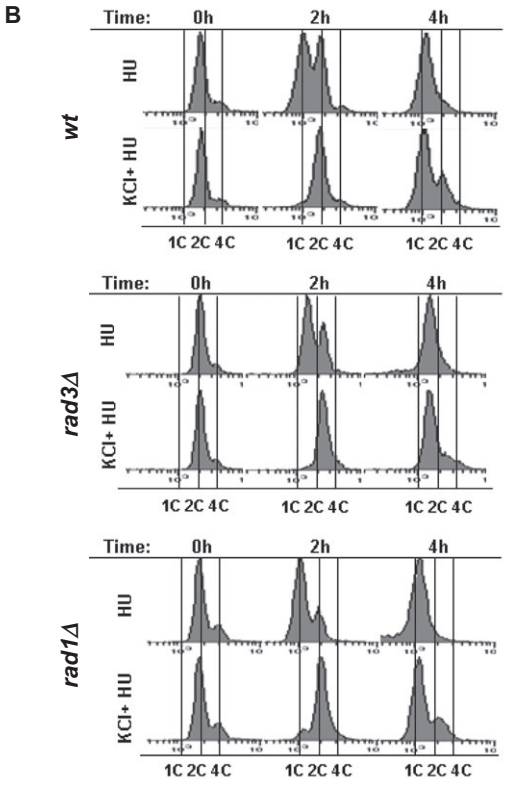
Paradoxical effect of Sty1 on DNA damage sensitivity

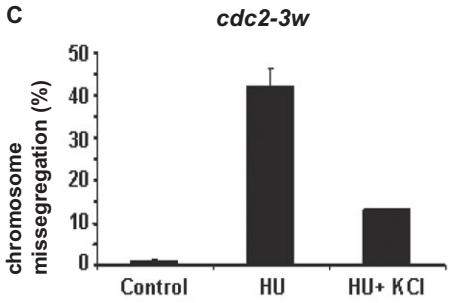
Sty1 activation has been proposed to increase the sensitivity of checkpoint mutants to DNA damage by advancing entry into mitosis (Humphrey and Enoch, 1998). Our observation that osmotic stress (which like UV activates Sty1; Fig. S1J) suppresses DNA damage sensitivity suggests that Sty1 activation is not lethal *per se* (Fig. 1F and H). Deletion of *sty1*$^+$ in a *rad1*Δ background partially restored checkpoint activation (Fig. 2A, panel iv), consistent with its role in regulating cell cycle re-entry (Degols and Russell, 1997). Co-deletion of *sty1*$^+$ and *rad1*$^+$ nonetheless synergistically enhanced UV sensitivity relative to *rad1*Δ single mutants (Fig. 1E). These findings suggest that Sty1 facilitates mitosis in *rad1*Δ mutants exposed to UV, but its transcriptional activity simultaneously enhances survival under these conditions (Degols and Russell, 1997).

Role of Srk1 in osmotic stress-induced cell cycle delays

Block and release experiments, using *cdc25-22* mutants synchronized in G2, demonstrated that KCl-induced osmotic stress suppresses progression through mitosis for at least 60 min (Fig. 3A and B). Srk1 has previously been proposed to activate a G2 checkpoint in *S. pombe* cells exposed to osmotic stress (Lopez-Aviles *et al*., 2005; Lopez-Aviles *et al.* 2008). Co-deletion of *srk1*$^+$, however, only partially attenuated the osmotic stress-induced delay in UV irradiated *rad1*Δ mutants (Fig. 3C). The impact of *srk1* deletion on the suppressive effect of osmotic stress on the UV sensitivity of *rad1*Δ mutants was modest (Fig. 3D). Because of the sequence similarity between Srk1/Mkp1 and Cmk2/Mkp2 (Asp and Sunnerhagen, 2003), we wanted to also investigate a possible effect of deleting *cmk2*$^+$. However, even a *srk1*Δ-*cmk2*Δ-*rad1*Δ triple mutant showed no additional effect (Fig. 3D). Furthermore, osmotic stress suppressed the UV (*rad24*Δ) or HU (*cdc2-3w* and *cdc2-3w cdc25Δ*) sensitivity and delayed cell cycle progression in mutants refractory to the effects of Srk1 overexpression (Lopez-Aviles *et al*., 2005; 2008) (Fig. 3E and F). Indeed, despite our finding that Srk1 and Cmk2 accumulate in wt cells exposed to osmotic stress (Fig. S1C and E), FACS analyses demonstrated such cells do undergo cell division (Fig. 3A). Srk1 thus


A
(i)
wt
(ii)
rad3Δ
(iii)
rad1Δ
(iv)
Septation index (%)
- KCl
+ KCl
rad1Δ
sty1Δ rad1Δ
Time (min)


B
Time: 0h 2h 4h
HU
KCl+ HU
wt
rad3Δ
rad1Δ
1C 2C 4C


C
cdc2-3w
chromosome missegregation (%)
Control
HU
HU+ KCl

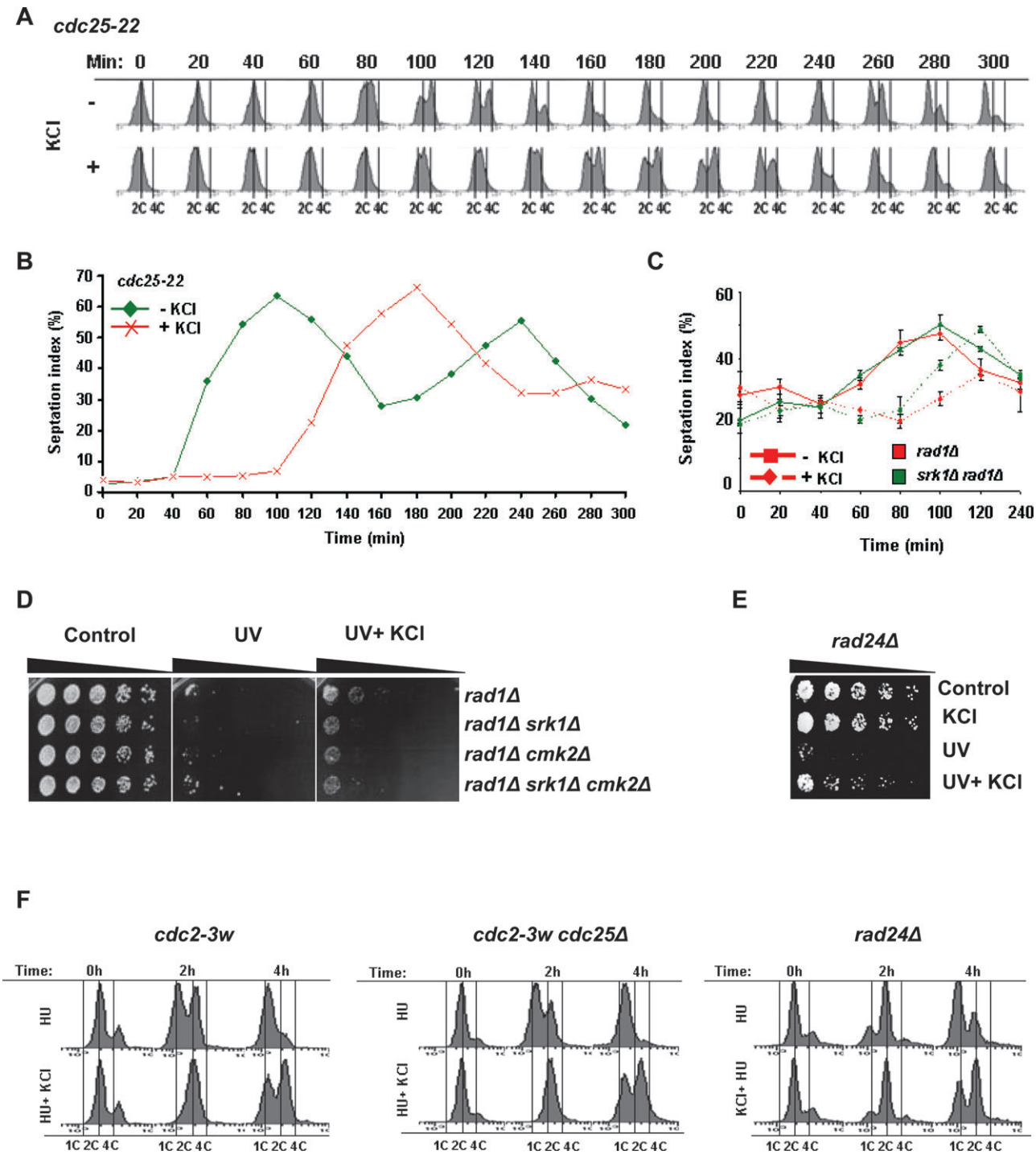


Fig. 3. Srk1-independent delay of cell cycle progression by osmotic stress.
A. The *cdc25-22* mutant was synchronized by incubation at 36°C for 4 h. Cells were downshifted to 25°C in YES medium with or without 0.6 M KCl. Samples harvested at the indicated time points, fixed and analysed by FACS.
B. Samples were treated as in (A), stained with aniline blue and the septation index determined by fluorescence microscopy. Results represent mean values from two independent experiments.
C. *rad1*Δ and *srk1*Δ *rad1*Δ mutants were exposed to 120 Jm^{-2} UV and incubated with or without 0.6 M KCl. Samples were harvested at the indicated time points and the septation index determined as in (B).
D. The indicated strains were spotted on YES plates with or without 0.6 M KCl and 120 Jm^{-2} UV. Plates were incubated at 30°C for 3 days.
E. *rad24*Δ cells were exposed to 180 Jm^{-2} UV and incubated with or without 0.6 M KCl for 4 h. Equal cell numbers were spotted unto YES plates and incubated at 30°C for 3 days.
F. The indicated strains were cultured in YES medium containing 20 mM HU with or without 0.6 M KCl and samples harvested at the indicated time points and fixed in 70% ethanol prior to FACS analysis.

plays a limited role in regulating cell cycle delays in asynchronous populations of *S. pombe* cells exposed to osmotic stress.

Osmotic stress sustains the spindle checkpoint

Our findings suggested that Srk1 alone does not account for the osmotic stress-induced delay in cell cycle progression. We thus investigated if osmotic stress delays progression through mitosis. Osmotic stress delayed mitotic progression in cold sensitive *nda3-KM311* (β-tubulin) mutants, synchronized in prometaphase, by about 40 min (Fig. 4A and B). Mad2 activity is required to maintain prometaphase arrest in *nda3-KM311* mutants unable to form cytoplasmic microtubules and mitotic spindles (Hiraoka *et al*., 1984; He *et al*., 1997). Our observations suggest that osmotic stress sustains Mad2 activation in *S. pombe* cells and thus delays progression through mitosis. Consistent with this, osmotic stress induced stabilization of both Cdc13 and Cut2 in asynchronous wt *S. pombe* cells (Fig. 4C). *mad2*Δ and *mad2*Δ *rad1*Δ mutants were advanced through mitosis relative to *wt* and *rad1*Δ single mutants in the presence of 0.6 M KCl with or without HU (Fig. 4D, Fig. S3B and C). Furthermore, deletion of *mad2*$^+$ attenuated the osmotic stress-mediated cell cycle delay and suppression of HU sensitivity in *rad1*Δ mutants (Fig. 4E, Fig. S3A and C). In contrast to its effect on chromosome segregation in *rad1*Δ mutants, HU did not induce chromosome mis-segregation in *mad2*Δ mutants alone or in the presence of KCl (Fig. S3D). We examined spindle formation in *nda3-KM311* mutants released into media at the permissive temperature in the absence or presence of 0.6 M KCl. By 30 min after release into normal medium, all cells either displayed anaphase spindles or were undergoing cytokinesis (Fig. 4A, B and F, Fig. S4A). In marked contrast, cells released into media containing KCl remained predominantly mononuclear with cytoplasmic microtubule arrays (Fig. 4B and F, Fig. S4A). Osmotic stress inhibits microtubule dynamics (Tatebe *et al*., 2005; Robertson and Hagan, 2008). The *nda3-KM311* mutation alone did not suppress UV sensitivity when *rad3*$^+$ was deleted in this background (Fig. S4B). Our observations suggest that osmotic stress may delay progression through mitosis by interfering with mitotic spindle formation.

We also noted that osmotic stress increased the length at division of *rad1*Δ mutants exposed to HU, indicative of cell cycle arrest within G2 (Fig. S3E). The osmotic stress-induced length increase was dependent on Cdc25 and Srk1, as *srk1*Δ *rad1*Δ and *cdc2-3w cdc25*Δ mutants delayed cell cycle progression in the absence of cell elongation under these conditions (Fig. S3E), indicating that the remaining, Mad2-dependent, delay in mitosis confers no cell length increase. By contrast, the osmotic stress-induced cell cycle delay in a *mad2*Δ *rad1*Δ double mutant was associated with an increase in cell length at division (Fig. S3D). Our findings further support the notion that osmotic stress induces cell cycle delays in distinct phases of the cell cycle: prior to (G2) and subsequent to (mitosis) the attainment of maximal cell length.

Osmotic stress-induced delay of cytokinesis suppresses DNA damage sensitivity

Osmotic stress induces accumulation of septated cells (Kishimoto and Yamashita, 2000) (Fig. S3B) and has been shown to perturb cytokinesis (Robertson and Hagan, 2008). As shown in Fig. 2A panel i, osmotic stress abolished the decline in the number of septated cells normally observed in wt cells exposed to UV. In order to delineate the mechanisms underlying this phenomenon, we performed microscopy and FACS analyses of synchronous *cdc25-22* and *nda3-KM311* cultures, which demonstrated that they delayed cytokinesis when released into the cell cycle in the presence of KCl. Both mutants accumulate cell populations with a $>2C$ DNA content for an extended period under these conditions (Figs 3A and B and 4A and B). Asynchronous populations of wt and *rad3*Δ mutants underwent cell division with substantially elevated levels of Cdc25 (Fig. 5A) and Cdc13 (Fig. 4C) under conditions of osmotic stress. Previous studies have shown that the timely execution of exit from mitosis and cytokinesis is delayed in mutants unable to properly inactivate Cdc25 activity (Trautmann *et al*., 2001; Esteban *et al*., 2008). We thus investigated if delaying cytokinesis is sufficient to suppress the sensitivity of checkpoint mutants exposed to HU and UV. The actin-depolymerizing drug latrunculin B (Lat B) inhibits septum formation and cytokinesis in *S. pombe* (Gachet *et al*., 2001). Co-treatment of *rad3*Δ mutants with HU and Lat B suppressed sensitivity more efficiently than KCl (Fig. 5B). Furthermore, Lat B completely abolished the chromosome mis-segregation ('cut' phenotype) normally observed in *rad3*Δ mutants exposed to HU (Jimenez *et al*., 1992) (Fig. 5D and E). Lat B also partially suppressed the UV sensitivity of *rad1*Δ mutants (Fig. 5C). Actin depolymerization by Lat B has been shown to activate a MAP kinase-dependent actin checkpoint that delays mitosis in *S. pombe* (Gachet *et al*., 2001). We observed, however, that KCl delayed cell cycle progression in asynchronous and synchronous cultures co-treated with Lat B. KCl thus delays cell cycle progression independently of the MAP kinase-dependent actin checkpoint activated by Lat B (Fig. S2). Cytochalasin D, an inhibitor of actin polymerization, suppressed the HU sensitivity of *rad3*Δ mutants similarly to Lat B (Fig. 5F and G). By contrast, 2,3-butanedione-2-monoxime, which transiently delays progression through mitosis and

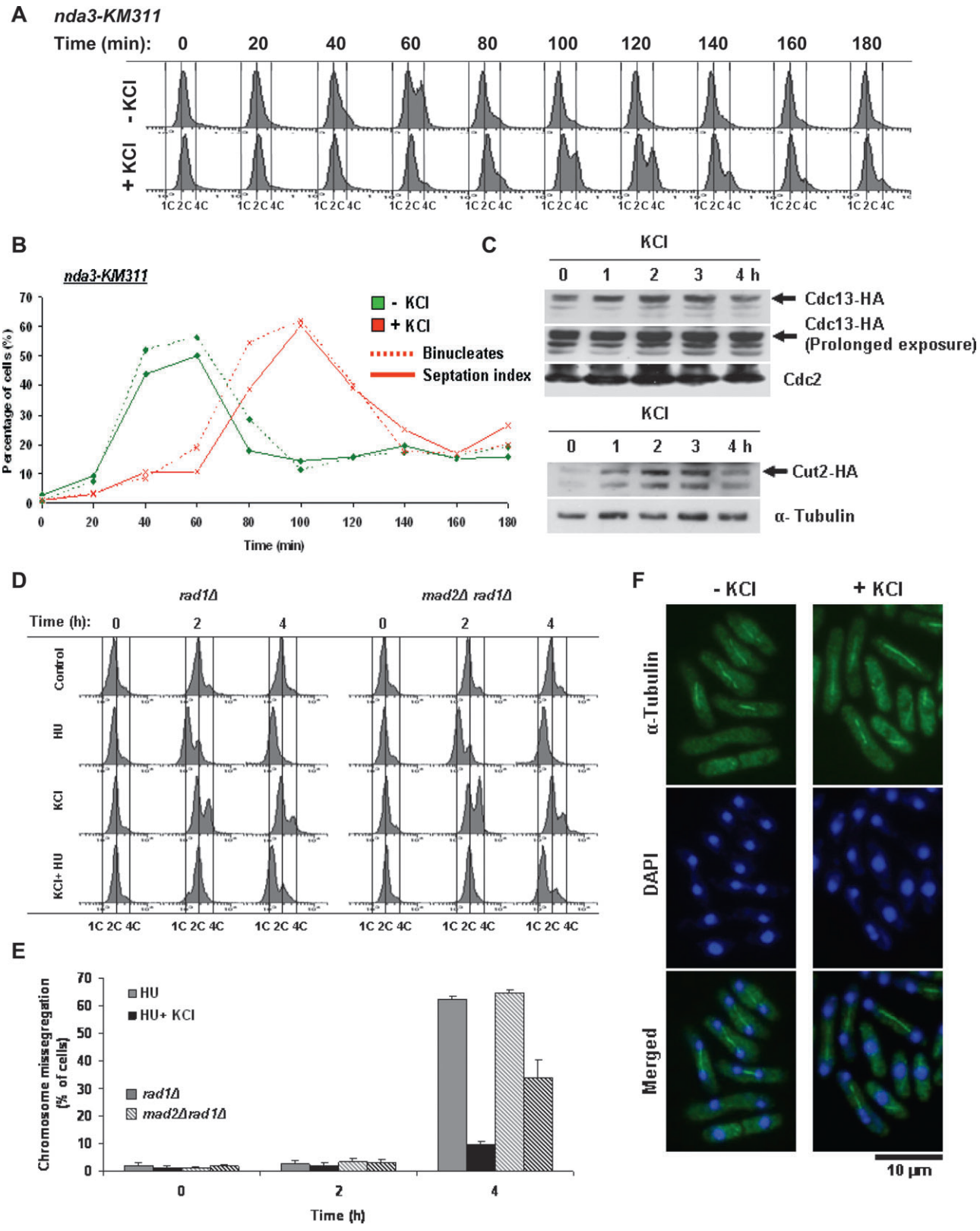

A
nda3-KM311
Time (min): 0 20 40 60 80 100 120 140 160 180
- KCl
+ KCl
1C 2C 4C
B
nda3-KM311
Percentage of cells (%)
- KCl
+ KCl
Binucleates
Septation index
Time (min)
C
KCl
0 1 2 3 4 h
Cdc13-HA
Cdc13-HA (Prolonged exposure)
Cdc2
KCl
0 1 2 3 4 h
Cut2-HA
α- Tubulin
D
rad1Δ
mad2Δ rad1Δ
Time (h): 0 2 4 0 2 4
Control
HU
KCl
KCl+ HU
1C 2C 4C
E
Chromosome missegregation (% of cells)
HU
HU+ KCl
rad1Δ
mad2Δrad1Δ
Time (h)
F
- KCl
+ KCl
α-Tubulin
DAPI
Merged
10 μm

Fig. 4. Osmotic stress influences spindle checkpoint activation.
A. The *nda3-KM311* mutant was synchronized by incubation at 18°C for 4 h. Cells were upshifted to 30°C in YES medium with or without 0.6 M KCl. Samples harvested at the indicated time points, fixed and analysed by FACS.
B. Samples were treated as in (A), stained with aniline blue or DAPI. The septation index and number of binucleate cells was determined by fluorescence microscopy. Results represent mean values from two independent experiments.
C. wt strains expressing HA-tagged Cdc13 or Cut2 were cultured in YES medium containing 0.6 M KCl for the indicated time points. Whole cell extracts were subjected to immunoblot analyses using monoclonal antibodies directed against HA. Antibodies directed against α-tubulin or Cdc2 were used for loading controls.
D. *rad1*Δ and *mad2*Δ *rad1*Δ mutants were cultured in YES medium as indicated. Cultures contained 20 mM HU and/or 0.6 M KCl. Samples were harvested at the indicated time points, fixed in 70% ethanol and analysed by FACS.
E. *rad1*Δ and *mad2*Δ *rad1*Δ mutants were exposed to 20 mM HU in 0.6 M KCl and harvested at the indicated time points. Cells were stained with aniline blue and the septation index determined by fluorescence microscopy. Results are representative of the means from three independent experiments ± SE.
F. *nda3-KM311* cells were synchronized by incubation at 20°C for 4 h. Cells were upshifted to 30°C in YES medium with or without 0.6 M KCl. Cells were harvested after 30 min, fixed in –20°C methanol and stained with antibodies directed against α-tubulin as described in *Experimental procedures*. Cells were counterstained with DAPI to visualize nuclei.

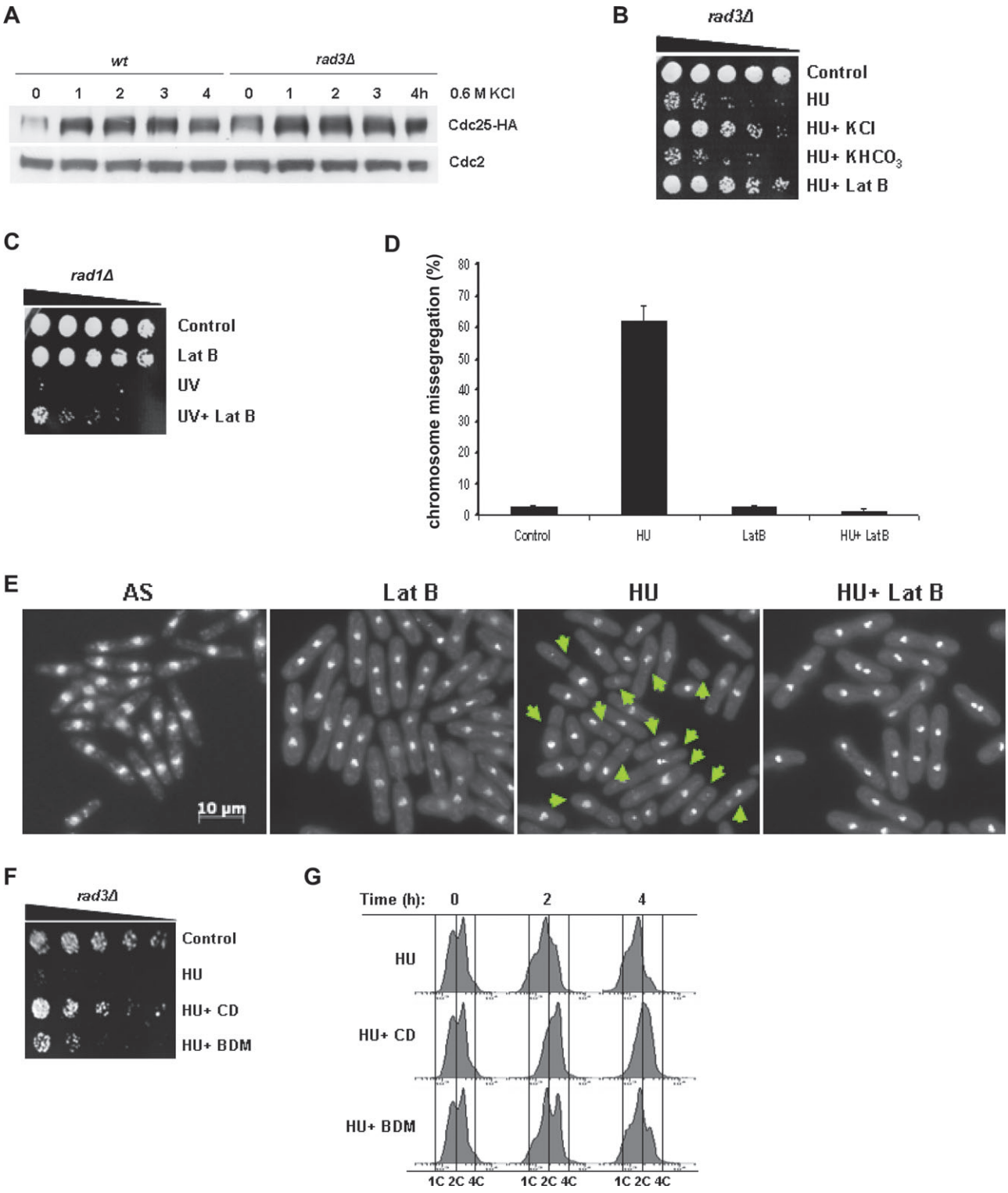
cytokinesis (May *et al*., 1998), only partially suppressed the HU sensitivity of this mutant (Fig. 5F and G). Together, our observations suggest that KCl delays cytokinesis and this activity in part, contributes to its suppressive effect on DNA damage sensitivity.

Discussion

We have demonstrated that osmotic stress suppresses the DNA damage sensitivity of *S. pombe* checkpoint mutants. This observation has allowed us to further characterize the effects of osmotic stress on cell cycle progression. Although frequently activated simultaneously, the stress response and DNA damage response pathways exert opposing effects on cell cycle progression in *S. pombe*. It has thus been of interest to determine how the signalling of these two pathways is integrated. We demonstrate that Srk1 alone is insufficient to account for the cell cycle delay(s) observed when *S. pombe* cells are exposed to osmotic stress. We have identified a novel role for the Mad2-dependent spindle checkpoint in regulating progression through mitosis under these conditions. Additionally, we find that cell division under osmotic stress conditions occurs in the presence of elevated Cdc25 levels and this delays progression through cytokinesis. Osmotic stress thus delays cell cycle progression in *S. pombe* cells via multiple mechanisms that result from the activation of Srk1 and Mad2, as well as delayed cytokinesis. Together, these cell cycle delays provide sufficient time for the repair of DNA damage in the absence of a functional DNA damage checkpoint pathway.

In our study, exposure to osmotic stress, which strongly activates Sty1, suppressed the sensitivity of DNA damage checkpoint mutants to UV and HU. Osmotic stress suppressed the sensitivity of *chk1*Δ and *wee1*Δ mutants that specifically lack a functional G2 checkpoint (reviewed in Humphrey, 2000). Furthermore, exposure to osmotic stress did not suppress the UV sensitivity of NER *rad13*Δ mutants or the HU sensitivity of *cds1*Δ mutants. Activation of the stress response pathway thus compensates specifically for the loss of checkpoint activation. *S. pombe* checkpoint mutants not only fail to activate a functional G2 checkpoint following exposure to UV or HU but appear to be advanced into mitosis (as determined by the sharp increase in septating cells under these conditions) (Jimenez *et al*., 1992; Kanter-Smoler *et al*., 1995; Alao *et al*., 2009). Checkpoint mutants exposed to osmotic stress following UV irradiation delayed entry into mitosis. Previous studies have demonstrated that the suppression of Sty1-mediated cell cycle progression enhances the resistance of checkpoint mutants to HU (Humphrey and Enoch, 1998). In contrast to *rad1*Δ mutants, *sty1*Δ *rad1*Δ double mutants activated a checkpoint response following irradiation with UV. The sensitivity of the *sty1*Δ *rad1*Δ mutant was however significantly enhanced relative to that of *rad1*Δ mutants. Our findings indicate that although Sty1 drives progression through mitosis in UV-irradiated *rad1*Δ mutants, its transcriptional activity nevertheless enhances the survival of these mutants. This is consistent with the previously reported requirement for Sty1 in the resumption of cell division following exposure to UV and various other environmental stresses (Degols *et al*., 1996; Degols and Russell, 1997; Marchetti *et al*., 2006; Robertson and Hagan, 2008).

More recent studies suggest that exposure to osmotic stress delays rather than advances entry into mitosis (Lopez-Aviles *et al*., 2005; 2008; Kawasaki *et al*., 2006). In our experiments, osmotic stress significantly delayed the cell cycle progression of both wt and checkpoint mutants exposed to HU or UV. Srk1 has previously been shown to delay entry into mitosis following exposure to osmotic stress, by negatively regulating Cdc25 activity in a Sty1-dependent manner (Lopez-Aviles *et al*., 2005; 2008). Compared with its effect on UV-irradiated *rad1*Δ mutants, osmotic stress was less effective at delaying cell cycle progression in *srk1*Δ *rad1*Δ double mutants in our experiments. Accordingly, osmotic stress was also less effective at suppressing the UV sensitivity of *srk1*Δ *rad1*Δ double mutants relative to *rad1*Δ mutant cells. Osmotic stress also delayed the progression of G2 synchronized



cdc25-22 mutants through mitosis following a shift to the permissive temperature. Our findings suggest that osmotic stress suppresses the DNA damage sensitivity of checkpoint mutants, by delaying entry into mitosis in a manner that is partially dependent on Srk1. We noted, however, that osmotic stress significantly delayed the cell cycle progression and suppressed the DNA damage sensitivity of *cdc2-3w*, *cdc2-3w cdc25Δ* and *rad24Δ* mutants. These mutants have previously been shown to be refractory to the cell cycle delay induced by Srk1 activation or

Fig. 5. The suppression of cytokinesis enhances resistance to DNA damage.
A. Wt and *rad3*Δ strains expressing HA-tagged Cdc25 were cultured in YES medium containing 0.6 M KCl for the indicated time points. Whole cell extracts were subjected to immunoblot analyses using monoclonal antibodies directed against HA. Antibodies directed against Cdc2 were used for loading controls.
B. *rad3*Δ mutants were cultured in YES medium containing 20 mM HU alone or together with 0.6 M KCl, 0.2 M $KHCO_3$ or 10 μM Lat B for 4 h at 30°C. YES medium alone served as an untreated control. Equal numbers of cells were spotted onto YES plates and incubated at 30°C for 3 days.
C. *rad1*Δ cells were exposed to 120 Jm^{-2} UV and incubated with or without 10 μM Lat B for 4 h at 30°C. Equal cell numbers were spotted onto YES plates and incubated at 30°C for 3 days.
D, E. *rad3*Δ mutants were cultured in YES medium containing 20 mM HU alone or together with 10 μM Lat B for 4 h at 30°C. Cells were fixed, stained with DAPI and examined by fluorescence microscopy. AS indicates untreated cells from an asynchronous culture. Arrows indicate cells with chromosome mis-segregation. Results in D are representative of the means from three independent experiments ± SE.
F. *rad3*Δ mutants were cultured in YES medium containing 20 mM HU alone or together with 10 μg ml^{-1} cytochalasin D (CD) or 30 mM 2,3-butanedione-2-monoxime (BDM) for 4 h at 30°C. Samples were then treated as in (C).
G. Samples from (F) were harvested at the indicated time points, fixed and analysed by FACS.

overexpression (Lopez-Aviles *et al*., 2005; 2008). Indeed, both wt and checkpoint defective *S. pombe* cells underwent cell division despite the sustained accumulation of Srk1. Hence, the Srk1-mediated inhibition of Cdc25 is insufficient to account for the entire cell cycle delay observed in *S. pombe* cells exposed to osmotic stress.

We have shown a role for the Mad2-dependent spindle checkpoint in mitotic delay during osmotic stress. A recent study demonstrated that exposure to osmotic stress induces the accumulation of Cut2 (securin), a target of the anaphase promoting complex (APC) (Kawasaki *et al*., 2006). The improper bi-orientation of chromosomes on the metaphase spindle activates the spindle checkpoint pathway that inhibits APC activity. Inhibition of APC prevents it from targeting Cut2 and Cdc13 for ubiquitin-dependent degradation, thus delaying anaphase and cytokinesis (reviewed in Peters, 2006). Furthermore, osmotic stress has been shown to inhibit or 'freeze' microtubule dynamics in *S. pombe* (Tatebe *et al*., 2005; Robertson and Hagan, 2008). We thus investigated if osmotic stress activates the spindle checkpoint in *S. pombe*. The cold sensitive *nda3-KM311* (β-tubulin) mutant is unable to form microtubules at the restrictive temperature and can thus be synchronized in prometaphase in a Mad2-dependent fashion (Hiraoka *et al*., 1984; He *et al*., 1997). Upon shift-up to the permissive temperature, the *nda3-KM311* mutant cells rapidly form mitotic spindles and proceed through mitosis in a highly synchronous manner (Hiraoka *et al*., 1984). In our study, we present four lines of evidence for a previously unappreciated role for a Mad2-dependent spindle checkpoint in regulating progression through mitosis in *S. pombe* cells exposed to osmotic stress. First, progression through mitosis was considerably delayed when this mutant was exposed to osmotic stress at the temperature shift-up (80–100 min vs. 40–60 min under normal conditions). Second, osmotic stress induced the accumulation of both Cdc13 and Cut2. Third, FACS and microscopic analyses demonstrated that *mad2*Δ *rad1*Δ mutants are advanced through mitosis relative to *rad1*Δ mutants following exposure to osmotic stress. Fourth, osmotic stress was less efficient at suppressing the HU sensitivity of *mad2*Δ *rad1*Δ mutants relative to *rad1*Δ mutants (Fig. S3B and C). Upon shift-up to the permissive temperature, *nda3-KM311* mutants rapidly form spindles, inactivate the spindle checkpoint and proceed through mitosis. Our studies suggest that exposure to osmotic stress sustains Mad2 activity and thus spindle checkpoint activation in *nda3-KM311* mutants following a temperature shift-up. Exposure to osmotic stress has been reported to inhibit microtubule dynamics for approximately 40 min (Robertson and Hagan, 2008). This time period correlates closely with the mitotic delay we have observed when *nda3-KM311* mutants are shifted to the permissive temperature under osmotic stress conditions. Under normal conditions, the majority of *nda3-KM311* mutants had proceeded through anaphase and/or cytokinesis within 30 min of the temperature shift-up. Under osmotic stress conditions, however, only a small percentage of cells had proceeded through anaphase at this time point. Intriguingly, a large percentage of cells displayed cytoplasmic microtubule arrays even at 30 min after the temperature shift-up. Together with previous observations (Tatebe *et al*., 2005; Robertson and Hagan, 2008), our findings suggest that osmotic stress interferes with proper spindle formation and may thus sustain the Mad2-dependent maintenance of the spindle checkpoint.

We show that cytokinesis is delayed in hyperosmosis, and that delayed cytokinesis induced by Lat B and cytochalasin B also provides protection against radiation and HU. In the absence of a functional DNA damage checkpoint, *S. pombe* cells proceed through mitosis with unreplicated or damaged DNA resulting in chromosome mis-segregation and a loss of viability. Under these conditions, unsegregated sister chromosomes are frequently cleaved by the newly formed septum during cytokinesis resulting in a characteristic 'cut' phenotype (Hirano *et al*., 1986). In theory, therefore, delayed septum formation and/or cytokinesis should partially compensate for any loss in checkpoint function by providing extra time for DNA repair. Wt cells exposed to osmotic stress following UV irradiation maintained a high septation index. This suggested that cytokinesis is protracted under these

conditions. Our study also demonstrates that osmotic stress induces the accumulation of the mitotic regulators Cdc13 and Cdc25. The timely execution of cytokinesis depends on the rapid inhibition of Cdc2 activity at the end of mitosis. Consequently, cytokinesis is delayed in *S. pombe* mutants defective in their ability to target Cdc25 for ubiquitin-dependent degradation (Trautmann *et al*., 2001; Esteban *et al*., 2008). Our findings indicate that Cdc2 activity is similarly deregulated when *S. pombe* cells are exposed to osmotic stress. Indeed, both wt and checkpoint defective strains accumulate a large population of septated cells with a > 2C DNA content under these conditions. Furthermore, the cell cycle kinetics of synchronized *cdc25-22* mutants under osmotic stress conditions bears similarity to that of *cdc25-22 clp1*Δ mutants defective in their ability to negatively regulate Cdc25 (Trautmann *et al*., 2001). Abolition of septum formation and cytokinesis with Lat B, an inhibitor of actin polymerization, suppressed the HU sensitivity of *rad3*Δ mutants. Lat B also suppressed the UV sensitivity of *rad1*Δ mutants. The osmotic stress-induced delay during cytokinesis likely contributes to its suppressive effect on DNA damage sensitivity in *S. pombe* checkpoint mutants.

In summary, we have investigated the mechanisms that underlie the suppression of DNA damage sensitivity by osmotic stress in *S. pombe* checkpoint mutants. The suppressive effect of osmotic stress on DNA damage sensitivity provides a useful model system for investigating the role of the stress response pathway in regulating cell cycle progression. Our studies demonstrate that the Srk1-mediated inhibition of Cdc25 activity only partly accounts for the cell cycle delay observed when *S. pombe* cells are exposed to osmotic stress. We have identified a novel role for Mad2 in regulating progression through mitosis under these conditions. Our findings also indicate that osmotic stress interferes with the timely execution of cytokinesis. Osmotic stress thus induces cell cycle delays during at least three distinct phases of the cell division cycle between G2 and cytokinesis.

In view of the increased DNA damage resistance of fission yeast checkpoint mutants under osmotic stress, it is worth considering if the hyperosmotic conditions experienced, e.g. by mammalian renal medulla cells would lead to complications for cancer treatment with DNA-damaging agents in such tissues. Our findings highlight the special challenges imposed by osmotic stress on the cellular checkpoint pathways and spindle dynamics, and provide a first step towards a mechanistic understanding of these interactions.

Experimental procedures

All strains (Table 1) were cultured on YES agar or in YES broth (0.5% yeast extract, 3% glucose, 225 mg l^{-1} each of adenine, histidine, leucine, lysine and uracil) at 30°C except where indicated. DNA constructs and chromosomal disruptions were performed as previously described (Alao *et al*., 2009). Stock solutions of HU (1 M; Sigma Aldrich) in water were stored at –20°C. Stock solutions of Lat B (10 mM; Calbiochem, VWR International AB) in dimethylsulphoxide were stored at –20°C. Cytochalasin D and 2,3-Butanedione-2-Monoxime were from Sigma. Unless otherwise stated, experiments were performed on cells in early to mid-log phase.

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 0.6 M KCl for 4 h. The cells were then equilibrated to an OD_{600nm} of 0.2–0.3, serially diluted, spotted on plates and incubated for 2–3 days at the appropriate temperature. Alternatively, cells were grown to stationary phase and then diluted into fresh medium at an OD_{600nm} of 0.2–0.3. The cells were then cultured at 30°C for 4–5 h to allow re-entry into the cell cycle, serially diluted and spotted on YES agar plates with or without 0.6 M KCl. The spotted cultures were allowed to air dry and were then exposed to the appropriate dose of UV.

Measurement of survival after exposure to HU

Early- to mid-log phase cultures were treated with 20 mM HU alone or with 0.6 M KCl and incubated for the indicated times with shaking at 30°C. Following incubation, the cultures were equilibrated to an OD_{600nm} of 0.2–0.3, serially diluted, spotted and incubated for 2–3 days at the appropriate 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 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.2% Triton X-100 and 1% NP-40] supplemented with Complete protease inhibitor and phosphatase inhibitor cocktails (Roche), using a FastPrep FP120 apparatus (Savant) with a speed setting of 5.0 for 20 s. Lysates were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes using a semi-dry blotting apparatus. Proteins were detected using antibodies directed against HA (Santa Cruz Biotechnology), phospho-Sty1 (Cell Signaling Technology), α tubulin (Sigma) or Cdc2 (Abcam). HRP-conjugated secondary antibodies and the ECL Western blotting system (General Electric Bio-Sciences) were used for detection.

4,6-diamidino-2-phenylindole (DAPI) and aniline blue staining for fluorescence microscopy

Cells were washed, fixed in 70% ethanol and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories) to counterstain DNA. Aniline blue staining of septa

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*	Dahlkvist *et al.* (1995)
hus1Δ	*hus1::LEU2*	Anthony Carr
cds1Δ	*cds1::ura4⁺*	Hiroto Okayama
chk1Δ	*h⁻ chk1::kanMX6*	This study
wee1Δ	*h⁻ wee1::ura4⁺ leu1-32 ura4-D18*	YGRC
sty1Δ	*h⁺ sty1::ura4 ura4D-18 leu1-32*	Jonathan Millar
sty1Δ rad1Δ	*h⁺ sty1::ura4 ura4D-18 leu1-32 rad1::kanMX6*	This study
srk1Δ	*h⁻ leu1 ura4 his3 srk1::ura4⁺*	Asp and Sunnerhagen (2003)
srk1Δ rad1Δ	*h⁻ leu1 ura4 his3 srk1::ura4⁺ rad1::hphMX6*	This study
cmk2Δ rad1Δ	*h⁻ cmk2::kanMX rad1::hphMX6*	This study
srk1Δ cmk2Δ rad1Δ	*h⁻ leu1 ura4 his3 srk1::ura4⁺ cmk2::kanMX6 rad1::hphMX6*	This study
srk1-HA	*h⁻ srk1-HA$_{(3)}$:kanMX6 leu1-32*	Asp and Sunnerhagen (2003)
cmk2-HA	*h⁻ cmk2-HA$_{(3)}$:kanMX6 leu1-32*	Asp and Sunnerhagen (2003)
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
rad24Δ	*h⁻ rad24::ura4⁺ leu1 ura4-D18 ade6-M210*	YGRC
cdc13-HA	*h⁻ cdc13-HA (ura4⁺) leu1-32 ura4-D18*	YGRC
cdc25-HA	*h⁺ cdc25-HA$_{(6)}$ (ura4⁺) leu1 ura4-D18*	YGRC
cut2-HA	*h⁻ leu1 ura4 cut2HA:LEU2*	YGRC
cut2-364	*h⁻ leu1 cut2-364*	YGRC
cdc25-22	*h⁻ cdc25-22 leu1-32*	Sergio Moreno
cdc2-3w	*h⁻ cdc2-3w*	YGRC
cdc2-3w cdc25Δ	*h⁻ cdc2-3w cdc25::ura4⁺ leu1-32 ura4-D18*	YGRC
nda3-KM311	*h⁻ leu1 nda3-KM311*	YGRC
nda3-KM311 rad3Δ	*h⁻ ade6 leu1-32 ura4-D18 rad3::ura4⁺ nda3-KM311*	YGRC

YGRC, Yeast Genetic Resource Centre, Osaka, Japan.

was carried out as described (Alao *et al.*, 2009). Stained cells were examined by fluorescence microscopy and a minimum of 300 cells were scored for septation assays.

Indirect immunofluorescence

Indirect immunofluorescence was carried out as previously described (Hagan and Hyams, 1988) (http://www.biotwiki.org/bin/view/Pombe/SectionFour#4-2_Indirect_Immuno fluorescence) with slight modifications. Cells were fixed and stored in methanol at –20°C. Cell walls were digested with 1.0–1.5 mg ml^{-1} of Zymolyase (Zymo Research) for 40 min at 37°C. Microtubules were detected using mouse antibodies directed against α tubulin (Sigma) (1:200 dilution) and fluorescein (FITC)-conjugated goat anti-mouse secondary antibody (1:50 dilution) (Jackson Immunoresearch, Fisher Scientific AB).

FACS

Approximately, 10^7 cells were harvested at the desired time points, resuspended in 70% ethanol and stored at 4°C until use. FACS analyses were performed as described (Sazer and Sherwood, 1990), using propidium iodide (32 μg ml^{-1}) as outlined on the Forsburg lab page (http://www-rcf.usc.edu/~forsburg/yeast-flow-protocol.html). Flow cytometry was performed with a BD FACSAria™ cell sorting system (Becton Dickinson AB).

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Supporting information

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