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RESEARCH ARTICLE

A checkpoint-independent mechanism delays entry into mitosis after UV irradiation

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ABSTRACT

When cells are exposed to stress they delay entry into mitosis. The most extensively studied mechanism behind this delay is the DNA-damage-induced G2/M checkpoint. Here, we show the existence of an additional stress-response pathway in *Schizosaccharomyces pombe* that is independent of the classic ATR/Rad3-dependent checkpoint. This novel mechanism delays entry mitosis independently of the spindle assembly checkpoint and the mitotic kinases Fin1, Ark1 and Plo1. The pathway delays activation of the mitotic cyclin-dependent kinase (CDK) Cdc2 after UV irradiation. Furthermore, we demonstrate that translation of the mitotic cyclin Cdc13 is selectively downregulated after UV irradiation, and we propose that this downregulation of Cdc13 contributes to the delayed activation of Cdc2 and the delayed mitosis.

KEY WORDS: G2/M, UV, Checkpoint, Cyclin B, Selective translation

INTRODUCTION

Cell cycle progression is tightly regulated and relies on the oscillating activity of the cyclin-dependent kinases (CDKs). Cdc2, the only CDK in the fission yeast *Schizosaccharomyces pombe*, drives the two major cell cycle transitions, from G1 into S phase and from G2 into M phase. For Cdc2 to promote mitotic entry it must be bound to the mitotic B-type cyclin Cdc13 (Nurse, 1990). Through S and G2 phase, the level of Cdc13 increases, leading to increased Cdc2 activity (Creanor and Mitchison, 1996; Fisher and Nurse, 1996; Hayles et al., 1994). Cdc2 is subjected to inhibitory phosphorylation on Tyr15 by the kinases Wee1 and Mik1 (Lundgren et al., 1991; Russell and Nurse, 1987), which keeps the activity of the CDK at a moderate level until the cells enter mitosis. The phosphate is removed by the phosphatase Cdc25 (Millar et al., 1991). A sufficiently high activity of Cdc2–Cdc13 for the cells to enter mitosis is reached when the activity of Cdc25 overcomes the effects of Wee1 and Mik1. The activity of Wee1 and Cdc25 can be modulated by the mitotic kinases Ark1, Plo1 and Fin1, which are required for timely entry into mitosis (Grallert et al., 2004; Mulvihill et al., 1999; Ohkura et al., 1995; Petersen et al., 2001). At mitotic entry, Plo1 is recruited to the spindle pole body (SPB) and is thought to enhance the activity of Cdc25 and to inhibit the activity of Wee1 (MacIver et al., 2003). Fin1 and, at least under stress conditions, also Ark1 are required for the association of Plo1 with the SPB (Grallert and Hagan, 2002; Halova and Petersen, 2011; Petersen and Hagan, 2005).

When cells are exposed to stresses such as DNA-damaging agents, they delay the major cell cycle transitions, namely entry into S phase and entry into mitosis. The most extensively studied of these mechanisms are the DNA-damage-induced cell cycle checkpoints (reviewed in Carr, 2002). Induction of a checkpoint by DNA-damaging agents like ultraviolet light (UV) or ionizing irradiation (IR) leads to activation of the checkpoint sensor, the phosphotidylinositol 3′ kinase-like kinase Rad3, a homologue of mammalian ATR. Rad3, in complex with Rad26, is recruited to DNA damage sites that contain single-stranded DNA (ssDNA) coated with replication protein A (RPA) protein. Another sensory complex, called 9-1-1 and comprising the proteins Rad9, Rad1 and Hus1, is loaded onto damaged DNA independently of Rad3. Activated Rad3 phosphorylates and activates the downstream effector kinases Chk1 or Cds1 in response to damage in G2 or S phase, respectively. These kinases, in turn, target the regulators of Tyr15 phosphorylation on Cdc2 and thus keep the CDK in an inactive state, leading to a delay in mitotic entry (Carr, 2002).

We show here that, in response to UV irradiation, a pathway that is independent of the classic DNA damage checkpoint is activated and delays mitosis. We find that the delay correlates with delayed activation of Cdc2. Furthermore, we demonstrate that Cdc13 translation is selectively downregulated after UV irradiation and propose that this downregulation of the mitotic cyclin Cdc13 contributes to the delayed activation of Cdc2 and delayed mitosis.

RESULTS

Checkpoint-deficient cells delay entry into mitosis when they are UV irradiated in G2

One of the best-characterized mechanisms regulating the cell cycle in response to stress in fission yeast is the classic G2 DNA damage checkpoint. When wild-type cells are irradiated with UV this checkpoint induces a long G2 arrest (Fig. 1A; Al-Khodairy and Carr, 1992). To reveal other stress-response pathways involved in cell cycle regulation in G2, we used a *rad3*Δ mutant, in which the classic checkpoint mechanism is abolished, and monitored cell cycle progression in response to UV irradiation. Early G2 cells were selected by lactose gradient centrifugation from a culture of exponentially growing *rad3*Δ cells and irradiated with UV. Samples were collected at different time points after irradiation (Fig. 1B). The proportion of cells passing into mitosis was scored by counting binucleate cells, which are in anaphase to G1, in a microscope after staining the DNA with 4′,6-diamidino-2-phenylindole (DAPI). Remarkably, in a *rad3*Δ strain, a substantial delay of at least 30 min was observed (Fig. 1B). The delay was dependent on the dose of UV (Fig. S1) and was not a consequence of extremely high UV doses, since the applied dose allows >90% survival in wild-type cells and >10% survival in *rad3*Δ cells (Fig. S1). These results suggest that a Rad3-independent pathway can delay mitosis after UV irradiation.

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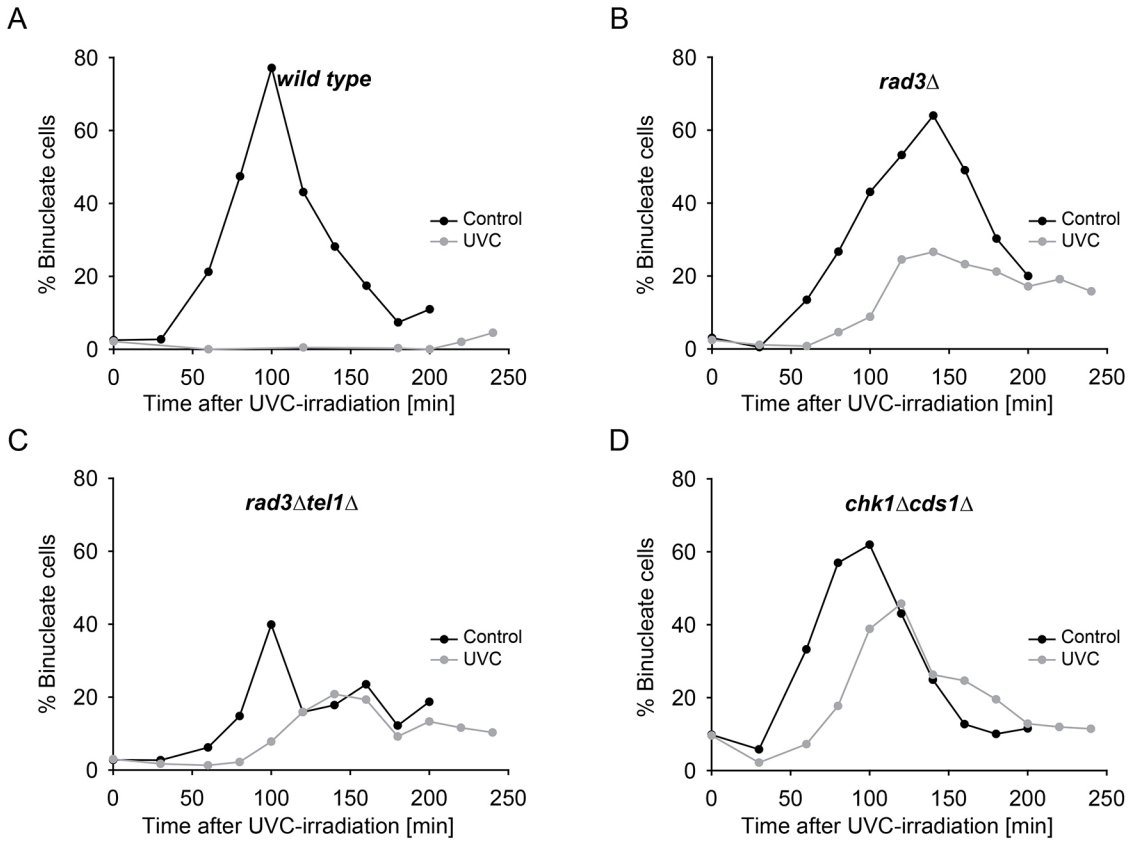

Fig. 1. Cell cycle progression in wild-type cells and checkpoint-deficient mutants after UV irradiation. Wild-type cells or cells of the indicated mutant strains were synchronized in G2 by lactose gradient centrifugation and irradiated with UV. Samples were collected at the indicated time points and the binucleate index (BI) was scored to assess the percentage of cells in mitosis. Experiments were performed three times (A,C,D) or five times (B). Data from a representative experiment are shown.

We considered the possibility that the 30-min delay induced in the absence of Rad3 operates through other members of the classic DNA damage checkpoint. Human cells have two major phosphoinositide 3-kinase (PI3K)-related kinases regulating the DNA damage checkpoint: ATR, the homologue of fission yeast Rad3, which is mainly activated by ssDNA, and ATM, which functions in response to DNA double-strand breaks (DSBs) (Cimprich and Cortez, 2008). The fission yeast homologue of ATM is Tel1 and is mainly involved in telomere maintenance but has also been shown to have a checkpoint function in response to persistent DSBs (Limbo et al., 2011). Upon UV irradiation the *rad3Δtel1Δ* double mutant progressed into mitosis with a 30-min delay as seen in the *rad3Δ* single mutant (Fig. 1C), demonstrating that Tel1 does not have a function that is redundant with Rad3 after UV irradiation.

The effector kinases Chk1 and Cds1 are downstream targets of Rad3 and might be activated in a Rad3-independent manner to bring about the G2 delay. Irradiated *chk1Δcds1Δ* cells entered mitosis 30 min later than non-irradiated cells (Fig. 1D), which is a similar delay to what was observed for the other checkpoint-deficient mutants described above. Taken together, these data demonstrate that UV irradiation of G2-phase cells induces a cell cycle delay that is independent of the classic DNA-damage checkpoint.

The spindle assembly checkpoint is not required for the delay

In our assay, we used the binucleate index (BI; the percentage of cells with two separate nuclei) as an indicator of mitotic cells. The separation of chromosomes is a relatively late event in mitosis, and this separation can be delayed if the spindle assembly checkpoint (SAC) is activated. Thus, a delayed increase in BI in the UV-treated cells could be the result of SAC activation in mitosis. Furthermore, in budding yeast, in the absence of the ATR homologue Mec1, UV induces a delay that is dependent on Tel1 and the SAC protein Mad2 (Clerici et al., 2004). To address the possible involvement of the SAC in the UV-induced delay in fission yeast, we used a mutant in which *mad2* had been deleted in the *rad3Δ* background. Synchronous cells were treated as above. Entry into mitosis, as determined by observing an increase in BI, occurred 30 min later in non-irradiated *rad3Δmad2Δ* cells than in non-irradiated control cells (Fig. 2), arguing that SAC activation is not required for the delay.

rad3Δ cells have a delay in G2 phase after UV irradiation

Since our assay uses BI as a measure of mitotic entry and does not distinguish between cells in G2 phase and in early mitosis, we studied three other events to address where in the cell cycle the arrest occurs. For these experiments, a *rad3Δ* strain was synchronized by elutriation followed by 1 h *cdc25* arrest. First, we monitored formation of the mitotic spindle, which happens early in mitosis. Samples of synchronous *rad3Δ* cells were collected for immunofluorescence and microtubules were visualized with an anti-tubulin antibody (Fig. 3A). Formation of the mitotic spindle was delayed by at least 30 min in response to UV irradiation (Fig. 3B), suggesting that the delay occurs prior to metaphase, either very early in mitosis or in late G2 phase.

Second, we monitored the kinetics of Cdc2 activation. One marker for Cdc2 activation is the removal of the inhibitory phosphate on

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

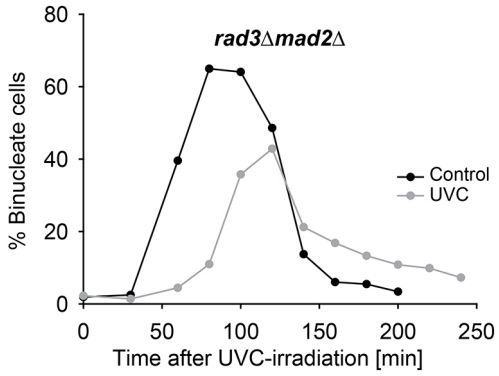

Fig. 2. The spindle-assembly checkpoint is not required for the Rad3-independent delay. The *rad3Δmad2Δ* double mutant was treated as in Fig. 1. The experiment was performed three times. Data from a representative experiment are shown.

Tyr15 (Gould and Nurse, 1989), which can be assessed by using a specific antibody against Cdc2 phosphorylated on Tyr15. Synchronous *rad3Δ* cells were irradiated with UV, and samples were collected for immunoblotting at the indicated time points. Control cells entered mitosis at ∼60 min, as judged by a decrease in Cdc2 phosphorylation (Fig. 3C), whereas in the UV-treated cells Cdc2 was still phosphorylated at this time (Fig. 3C), indicating that the cells arrest in G2. However, in the UV-treated samples, we failed to detect a drop in Cdc2 phosphorylation at later time points (after 80 min), even though the cells start entering mitosis as judged by the BI (*rad3Δ* in Fig. 1B). However, after UV irradiation the increase in BI is slow, suggesting that only a fraction of cells is able to enter mitosis and/or the synchrony is poor. Thus, at any given time, only a small number of cells show a decrease in Cdc2 phosphorylation, which is not detected by our immunoblotting-based assay.

An alternative approach to monitor Cdc2 activation is to investigate the phosphorylation of Cdc2 substrates. For this assay we used an antibody that recognizes phosphorylated Cdc2 substrates based on a common motif (S*PxR/K or PxS*PxR/K). There was a general increase in signal intensity in non-irradiated cells at 80 min (Fig. 3C), indicating phosphorylation of Cdc2 substrates. In addition, one band in particular (arrow on Fig. 3C) appears and disappears with a timing that correlates with the timing of mitotic entry and exit in control cells (as judged from the BI). In UV-irradiated cells, the general increase in signal intensity and the increased phosphorylation of this particular substrate appeared 40 min later than in non-irradiated cells (Fig. 3C), suggesting a delayed Cdc2 activation.

As a third approach, we monitored a mitotic marker that is associated with chromatin condensation. Phosphorylation of histone 3 at serine 10 [H3-P(Ser10)] is involved in chromosome condensation (Petersen et al., 2001; Wei et al., 1998), which occurs right before spindle formation. The same samples as above were subjected to immunoblotting using an antibody against H3-P (Ser10). Phosphorylation of histone 3 occurred 20 min later in UV-irradiated *rad3Δ* cells than in control cells (Fig. 3C). The increase in histone 3 phosphorylation in UV-irradiated cells is not as pronounced as in the control cells, again consistent with the observations above and suggesting that only a fraction of cells is able to enter mitosis and/or the synchrony is poor.

The above results, using different mitotic markers, strongly argue that the Rad3-independent delay takes place in G2 phase rather than in early mitosis, and that Cdc2 activation is delayed. This conclusion is also consistent with the above finding that the SAC is not required for the delay.

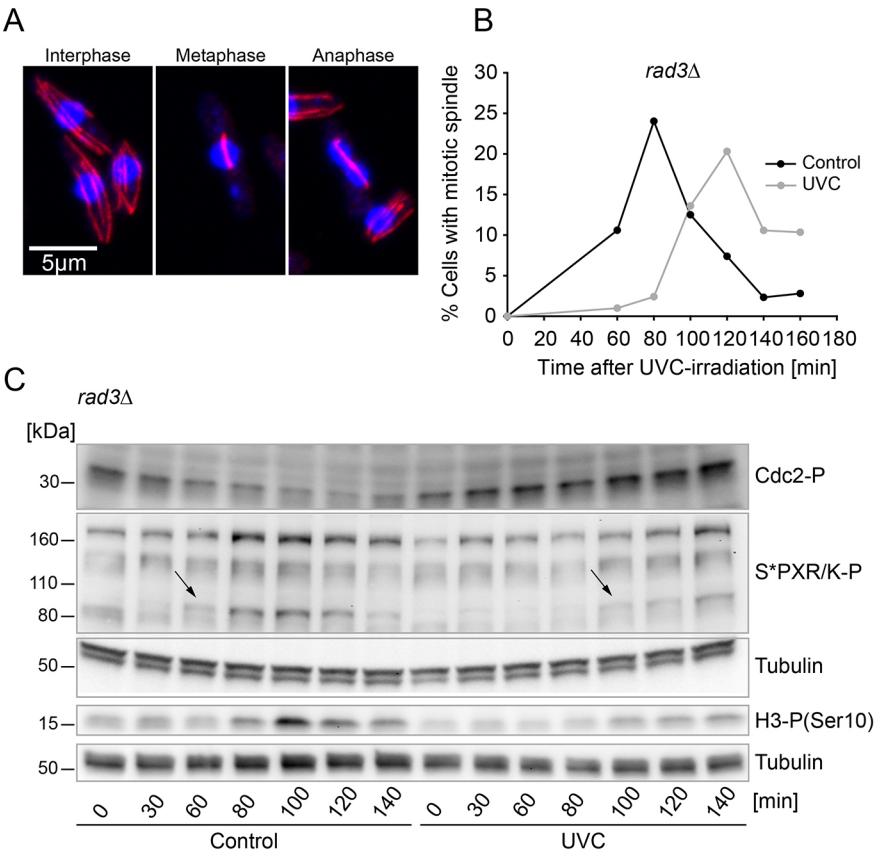

Fig. 3. Rad3-deficient cells delay cell cycle progression in G2 phase after UV irradiation. Cell cycle progression was measured by analysing three different mitotic markers. For all experiments, a *rad3Δ* strain was synchronized by elutriation followed by 1 h *cdc25* arrest and samples were collected at the indicated time points. The experiment was performed three times. Data from a representative experiment are shown. (A) Illustration of cells in interphase and mitosis stained with an anti-tubulin antibody (red) and DAPI (blue). (B) Percentage of cells with a mitotic spindle as quantified through assessing immunofluorescence from an anti-tubulin antibody. (C) Immunoblot showing Cdc2 activity as determined by using an anti-phospho-Cdc2 (Tyr15) antibody (upper panel), an antibody recognizing the S*PxR/K motif in its phosphorylated form (middle panel) or an antibody against phosphorylated histone 3 (Ser10) (lower panel). Tubulin was used as a loading control. Note that the same protein samples are analyzed here as in the experiment reported in Fig. 7B. The same blot was reprobed with the indicated antibodies.

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

The mitotic kinases are not responsible for the delay

The mitotic kinases Plo1, Ark1 and Fin1 are required for timely entry into mitosis. Notably, they can be activated independently of Cdc2. Fin1 and Plo1 contribute to Cdc2 activation (Grallert and Hagan, 2002; Petersen and Hagan, 2005), while Ark1 regulates chromatin condensation and chromatid segregation, as well as Plo1 recruitment to the SPB (Petersen and Hagan, 2003; Petersen et al., 2001), which is the centrosome analogue and the microtubule-organizing centre essential for spindle formation. Here, we set out to investigate whether any of the three kinases is involved in the UV-induced delay.

To address whether Fin1 is required, a *fin1* analogue-sensitive (as) mutant (fin1-M82G G168A; Grallert et al., 2012) was used in the *rad3*Δ background. These analogue-sensitive mutants carry a mutation in the ATP-binding pocket that allows binding of bulky ATP analogues that inhibit their catalytic activity (Bishop et al., 2001). The experiment was performed as described in Fig. 1 except for that the ATP-analogue 3-BrB-PP1 was added to the synchronous cells prior to UV irradiation. Non-irradiated *rad3Δfin1-as* cells (Fig. 4A) enter mitosis later than non-irradiated wild-type cells (Fig. 1), consistent with a previous report (Grallert et al., 2012). The UV-induced delay was not abolished by the loss of Fin1 activity (Fig. 4A), suggesting that Fin1 is not required for the delay. However, we cannot exclude the possibility that the analogue-sensitive mutants retain some activity even in the presence of the ATP analogue, which could conceal a minor effect on the UV-induced delay.

Ark1 is essential in fission yeast (Petersen et al., 2001), and we therefore used an analogue-sensitive *ark1-as* (ark1-L166A S229A; Hauf et al., 2007) mutant in a *rad3*Δ background to study whether the delay could be induced in cells that have a reduced Ark1 activity. The *rad3Δark1-as* strain was treated as described above for *rad3Δfin1-as*. It should be noted that in the absence of Ark1, cells have defects in spindle formation and chromosome segregation, and only a fraction of the non-irradiated cells can form binucleate cells and even fewer can do so after irradiation. However, there is a clear delay in mitotic entry after UV irradiation, similar to that seen in *rad3Δ* cells, (Fig. 4B) suggesting that Ark1 is not involved in the delay.

In order to investigate the possible involvement of Plo1 after UV irradiation, we utilized a phospho-mimicking mutant of Plo1, where Ser402 has been replaced with a glutamic acid residue. Phosphorylation of Ser402 is induced both in an unperturbed cell cycle and in response to stress, and it promotes Plo1 recruitment to SPBs and thus commitment to mitosis (Petersen and Hagan, 2005). We reasoned that a delayed recruitment of Plo1 to the SPB might contribute to the delayed mitotic entry in response to UV. However, after UV irradiation, a 40-min delay was induced in a *rad3Δplo1S402E* double mutant (Fig. 4C), which is similar to that seen in the *rad3Δ* mutant (Fig. 1B). We conclude that regulation of Plo1 Ser402 phosphorylation and delayed recruitment to the SPB is not involved in the mechanism of the UV-induced G2 delay.

Taken together, the above data suggest that the mitotic kinases Fin1, Ark1 and Plo1 are not responsible for the Rad3-independent G2 delay.

The delay is not dependent on Sty1- and Srk1-dependent inhibition of Cdc25

A major responder to various environmental stresses in fission yeast is the stress-activated MAP kinase Sty1, a homologue of mammalian p38 mitogen-activated protein kinases (MAPKs). The

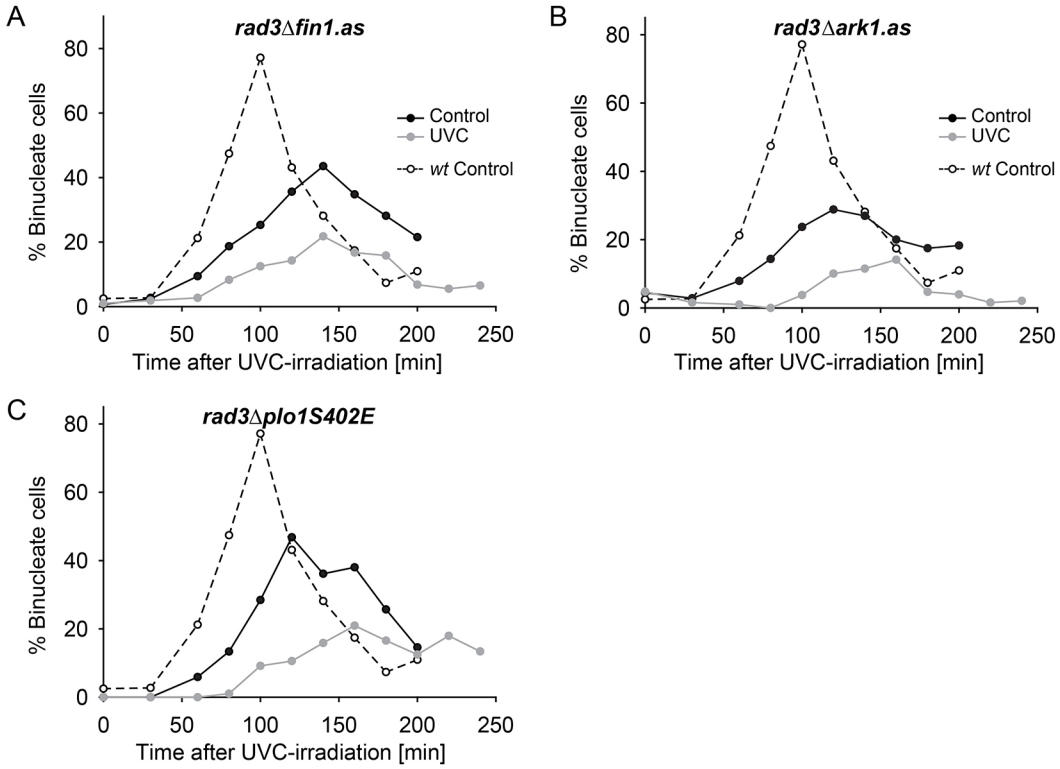

Fig. 4. The mitotic kinases are not involved in the UV-induced delay. *rad3Δark1-as*, *rad3Δfin1-as* and *rad3Δplo1S402E* strains were treated as in Fig. 1 except for addition of the inhibitor 3-BrB-PP1 (final concentration of 20 µM) to the *rad3Δark1-as* and *rad3Δfin1-as* strains prior to UV irradiation. Experiments were performed three times (A,C) or twice (B). Data from a representative experiment are shown. Dashed lines represent the kinetics of binucleate cells in non-irradiated wild-type cells for comparison.

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

*sty1*Δ mutant is sensitive to UV irradiation (Degols and Russell, 1997) and Sty1 delays mitotic entry in response to osmotic stress through activation of the Srk1 kinase and subsequent inhibition of Cdc25 (López-Avilés et al., 2005, 2008). We reasoned that a similar pathway could also induce a G2/M delay after UV irradiation and investigated the involvement of the Sty1 pathway in the UV-induced delay. Because the *rad3*Δ*sty1*Δ mutant grew poorly and was not easily synchronized, we took advantage of an analogue-sensitive allele of *sty1*. To test the analogue sensitivity of the mutant, we exposed the cells to heat shock as described previously (Zuin et al., 2010) and found that, in the presence of the analogue, the *sty1-as* cells survived poorly (Fig. S2) consistent with lack of Sty1 function (Zuin et al., 2010). The experiment was performed using a *rad3*Δ*sty1-as* double mutant, and the ATP-analogue 3-MB-PP1 was added to the synchronous cells prior to UV irradiation. The UV-induced delay in the *rad3*Δ*sty1-as* strain was not abolished but in fact was slightly longer than that seen in *rad3*Δ cells (Fig. 5, compare to Fig. 1B), suggesting that Cdc25 phosphorylation upon Sty1 activation is not the mechanism of the delay.

The G2 delay is not due to size control

UV irradiation leads to a downregulation of global translation (Deng et al., 2002; Iordanov et al., 1998; Knutsen et al., 2015; Tvegard et al., 2007), and lower translation rates lead to slower growth. Cell growth and entry into mitosis are coordinated such that cells do not enter mitosis until they reach the appropriate size, which is determined by the growth conditions. We reasoned that the reduced global translation and thus slower cell growth might be a reason for the UV-induced delay. To address this question, we used a *wee1* mutant, since cells lacking Wee1 are deficient in the G2/M size control (Nurse, 1975; Russell and Nurse, 1987). A temperature-sensitive (ts) *wee1* mutation was used because deletion of *wee1* is synthetically lethal with *rad3*Δ (Al-Khodairy and Carr, 1992; Al-Khodairy et al., 1995; Anda et al., 2016). The strain was synchronized in G2 and incubated at 36°C for 10 min to inactivate Wee1 prior to UV irradiation. Non-irradiated *wee1-50rad3*Δ cells entered mitosis 80 min earlier than *wee1*$^{+}$ *rad3*Δ cells (Fig. 6, compare to Fig. 1B), as would be expected when Wee1 is inactivated. In response to irradiation, *wee1-50rad3*Δ cells delayed entry into mitosis by 40 min (Fig. 6), which is comparable to the delay in *wee1*$^{+}$ *rad3*Δ cells. This result indicates that the reduced size of the cells due to global translational downregulation is not the reason for the G2/M delay.

UV irradiation leads to a reduced Cdc13 protein level

Cdc2 activity is regulated in the cell cycle through the association with the appropriate cyclins. In order to explore whether the availability of the mitotic cyclin is regulated after UV irradiation, we investigated the level of Cdc13 in UV-irradiated cells. Wild-type and *rad3*Δ cells also carrying a *cdc25-22* mutation were synchronized by elutriation followed by a 1 h incubation at 36°C, then irradiated with UV; samples for immunoblotting were then collected at the indicated time points (Fig. 7A,B). In non-irradiated wild-type and *rad3*Δ cells, the level of Cdc13 was increasing slightly for the first 80 min after size selection, but was then sharply reduced at 100 min. The slight increase is consistent with previous reports and the established model that Cdc13 gradually accumulates during the cell cycle (Creanor and Mitchison, 1996). The reduction of the Cdc13 level coincides with anaphase and mitotic exit, as judged from the mitotic index (Fig. 3B). Interestingly, in UV-treated samples of both strains, the level of Cdc13 was low and remained low during the course of the experiment (140 min) (Fig. 7A,B). We compared the level of Cdc13 after UV irradiation with that of another cell-cycle-regulated protein that also accumulates in G2, namely Cdc25. To this end, a strain carrying a PK (V5)-tagged Cdc25 was synchronized by elutriation and UV irradiated. In untreated control cells, the Cdc25 protein level stayed constant throughout the experiment (Fig. 7C). Strikingly, the level of Cdc25 continued to accumulate after UV irradiation, in sharp contrast to the level of Cdc13 (Fig. 7C).

These observations demonstrate that the Cdc13 level is specifically downregulated after UV irradiation. This finding suggests that downregulation of cyclin B and the insufficient amount of active cyclin–CDK complexes is at least partially responsible for the delay observed in *rad3*Δ mutant cells. Furthermore, this downregulation is also observed in *rad3*$^{+}$ cells, suggesting that this mechanism is not specific for checkpoint-deficient cells but also contributes to the UV-induced G2 delay in wild-type cells.

Downregulation of translation leads to reduced Cdc13 after irradiation with UV

The amount of protein present in the cell is determined by the balance between synthesis and degradation. The downregulation of the level of Cdc13 after UV irradiation could result from an inhibition of transcription and/or translation, and/or from increased degradation. In order to investigate the mechanism of downregulation of Cdc13 after UV irradiation, we studied each of these possible mechanisms.

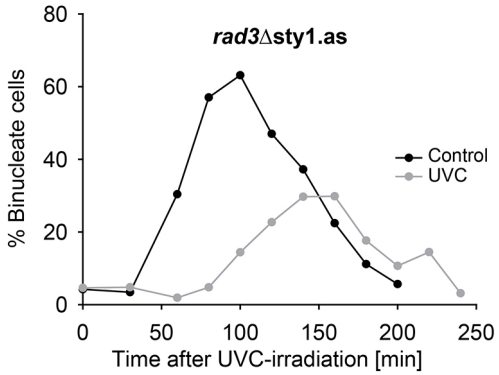

Fig. 5. Sty1 is not responsible for the G2 delay. A *rad3*Δ*sty-as* strain was treated as in Fig. 1 except for addition of the inhibitor 3-MB-PP1 prior to UV irradiation. The experiment was performed three times. Data from a representative experiment are shown.

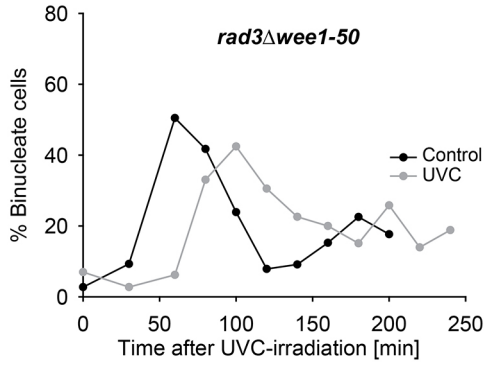

Fig. 6. Wee1 is not required for the delay. The *rad3*Δ*wee1-50* strain was treated as in Fig. 1 except for incubation at 36°C for 10 min prior to UV irradiation to inactivate Wee1. The strain was kept at 36°C for the rest of the experiment. The experiment was performed three times. Data from a representative experiment are shown.

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

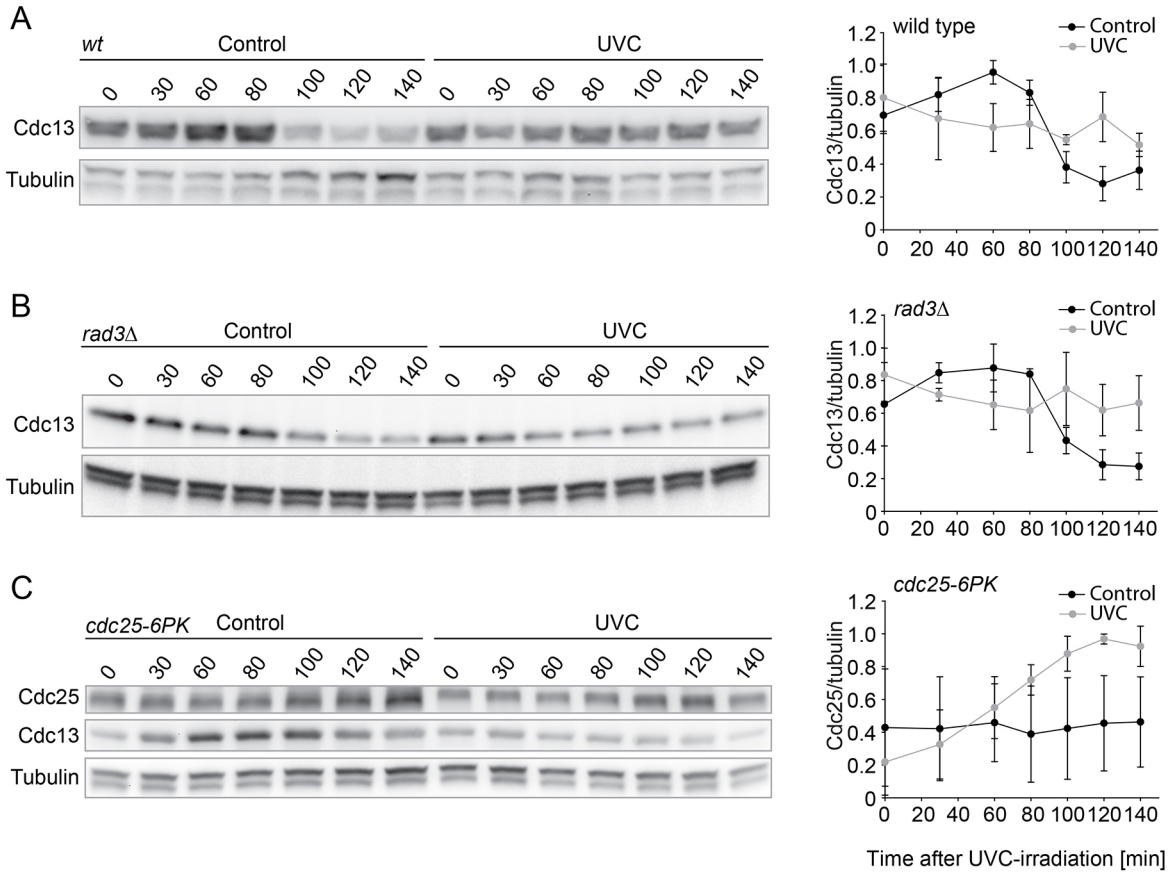

Fig. 7. Cdc13 protein level is specifically downregulated in response to UV. Wild-type (*wt*), *rad3Δ* and *cdc25-6PK* strains were synchronized in G2 phase before irradiation with UV. Samples were collected at the indicated time points. Immunoblots of *wt* (A) and *rad3Δ* (B) strains showing Cdc13 protein level in control and UV-treated samples. Note that the same protein samples are analyzed here as in the experiment reported in Fig. 3C. The same blot was reprobed with the indicated antibodies. An immunoblot of a strain carrying PK (V5; epitope, GKPIPNPLLGLDST)-tagged *cdc25* (C) showing the Cdc25 and Cdc13 protein level in control and UV-treated samples. Tubulin was used as the loading control. Protein levels were quantified in three independent experiments and the average of three experiments with standard deviation is shown to the right of each representative blot.

To see whether the *cdc13* transcript is affected when cells are UV irradiated, cells were synchronized by elutriation followed by a 1 h *cdc25* arrest, and were then irradiated; samples were then taken at different time points and total RNA was isolated. The transcript level of *cdc13* in non-irradiated and UV-irradiated samples was measured by quantitative real-time PCR and normalized to the transcript level of a house-keeping gene. The *cdc13* mRNA levels are not significantly reduced in irradiated samples, rather the peak of expression is somewhat advanced in the treated cells (Fig. 8A), suggesting that the observed downregulation of Cdc13 protein is not due to a downregulation of the level of *cdc13* transcripts.

To investigate whether translation of *cdc13* is regulated, we isolated polysomes from non-irradiated and UV-irradiated cells to estimate how much *cdc13* mRNA is actively translated after UV irradiation. Actively translated mRNAs are associated with polysomes and an increase in monosome-associated mRNA suggests inhibition of translation initiation. In response to UV irradiation, the level of the *cdc13* mRNA was considerably reduced in the polysome fraction and increased in the monosome fraction (Fig. 8B), suggesting decreased translation of *cdc13* mRNA after UV irradiation.

To explore whether increased protein degradation after exposure to UV contributes to the low Cdc13 protein levels, we measured the half-life of Cdc13 after UV irradiation in cells synchronized in G2 phase. At 30 min after irradiation, cycloheximide was added to block protein translation and samples were taken for immunoblotting to assess the Cdc13 protein level (Fig. 8C). The half-life of Cdc13 was not affected by UV irradiation leading us to conclude that increased degradation does not contribute to the reduced Cdc13 protein level after UV irradiation.

Taken together, these results suggest that UV irradiation leads to a reduced amount of Cdc13 protein through selective downregulation of translation.

DISCUSSION

When cells are exposed to DNA damage, stress-response pathways are activated that will alleviate the stress by different means. In order to give the cells time to deal with the stress, checkpoints are activated to delay cell cycle progression. The classic checkpoint pathway operating in G2 phase in fission yeast is dependent on activation of the kinase Rad3, which in turn activates the checkpoint effectors Chk1 and Cds1 leading to inhibition of Cdc2, the mitotic CDK in fission yeast. Here, we show that, in response to UV irradiation in early G2, a Rad3-independent mechanism delays entry into mitosis. This pathway does not involve Tel1 or other classic checkpoint proteins such as Cds1 and Chk1. Furthermore, we find that the mechanism of the delay does not involve the mitotic kinases Fin1, Ark1 and Plo1, nor does it require the SAC. Consistent with

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

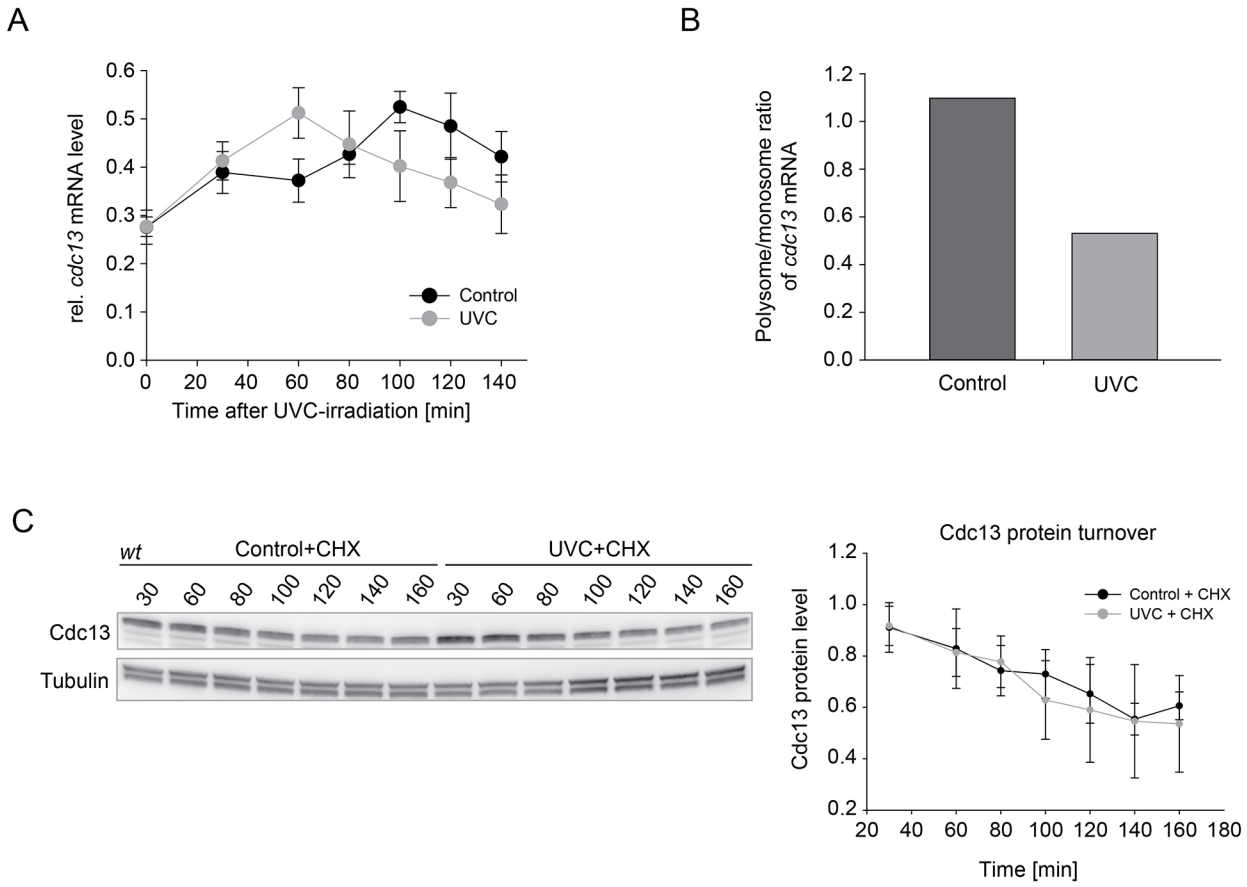

Fig. 8. Cdc13 protein levels are reduced by UV through downregulation of translation. Cells were synchronized in G2 phase and irradiated with UV. Samples were collected at the indicated time points. (A) Total RNA was isolated from non-irradiated and UV-irradiated samples and were then subjected to cDNA synthesis. Quantitative real-time PCR was performed for three independent experiments. The level of the *cdc13* transcript was normalized to that of the *leu1* transcript. The curves represent the mean±s.d. from three independent experiments. (B) The polysome and monosome fractions were isolated from extracts of control and UV-treated samples, and mRNA was isolated from both fractions. Quantitative real-time PCR was performed in four independent experiments with the same results. A representative experiment is shown here, and the other three experiments are shown in Fig. S5. The level of *cdc13* mRNA was normalized to that of *leu1* mRNA. (C) Cycloheximide (CHX) was added 30 min after irradiation to monitor Cdc13 turnover. An immunoblot of the Cdc13 protein level in a wild-type strain after addition of CHX is shown. Tubulin was used as a loading control. A quantification of three blots from three independent experiments is shown to the right (mean±s.d.).

these findings, we show that the delay does not take place in mitosis, but in G2, through delayed activation of Cdc2. We find that the level of the mitotic cyclin Cdc13 is reduced upon UV irradiation due to selective downregulation of translation. We propose that the limited availability of the mitotic cyclin Cdc13 contributes to the delayed Cdc2 activation after UV irradiation.

Previous studies have suggested that the UV-induced cell cycle arrest in G2 is absolutely dependent on Rad3 in fission yeast (Al-Khodairy and Carr, 1992), which is not entirely consistent with the present results. The reason for the discrepancy is not quite clear, but there are important differences between the experimental procedures in the two studies. First, al-Khodairy and Carr compared the UV-induced delay in wild-type and *rad3*Δ cells, where the wild-type cells obviously have a prolonged arrest. As compared to this, a short delay in the checkpoint mutants might have gone unnoticed. Second, they synchronized the cells using a Cdc25 block-and-release approach, which includes an arrest in late G2. When cells are synchronized by Cdc25 inactivation, all other factors required for mitotic entry, including Cdc13, are produced and are ready for action at the time when cells are released from the arrest. In order to test whether employing a *cdc25*-induced arrest can explain the difference in results, we synchronized temperature-sensitive *cdc25-22* cells in G2 phase by elutriation and then incubated them at the restrictive temperature to inactivate Cdc25. Under these conditions, the Cdc13 protein level increased about twofold during the arrest and was at least as high at the time of irradiation as in control cells at entry into mitosis (Fig. S4). However, the delay was clearly observed after a *cdc25* arrest and even after irradiation with a lower dose of UV (Fig. S4). These results indicate that the observed translational regulation of Cdc13 cannot completely account for the Rad3-independent delay at entry into mitosis.

Interestingly, a Rad3-independent mechanism was shown to delay entry into mitosis after irradiation with UVA (see Fig. 3A in Dardalhon-Cuménal et al., 2008). UVA causes a different spectrum of damage to the DNA than UVC; UVA predominantly causes oxidative damage, whereas UVC predominantly induces pyrimidine dimers and 4,6-photoproducts. However, UVC also causes some oxidative damage (Degols and Russell, 1997), although to a lesser extent than does UVA. It is therefore plausible that the delayed mitotic entry seen after UV in a *rad3*Δ strain is caused by oxidative damage and represents the uncharacterized delay mechanism seen after UVA irradiation.

We present several lines of evidence that *rad3*Δ cells arrest for ∼30 min in G2 phase and delay Cdc2 activation. However, the BI of UV-irradiated *rad3*Δ cells never reaches a sharp peak like that of

Journal of Cell Science (2017) 130, 4028-4037 doi:10.1242/jcs.204693

control cells does, but rather reaches a plateau that is maintained during the course of the experiment (240 min). In correlation with these observations, UV-irradiated *rad3*Δ cells do not show a reduction in Cdc2 phosphorylation, indicating that a proportion of the *rad3*Δ cells never enter mitosis and/or that the cells do not recover from the arrest in a synchronized manner.

The classic DNA damage checkpoint affects cell cycle progression by maintaining the inhibitory phosphorylation on Cdc2. Here, we show that cyclin availability is another crucial factor for CDK activity not only during unperturbed cell cycle progression but also after UV irradiation. We observed a distinct downregulation of Cdc13 protein after irradiating cells with UV both in checkpoint-proficient and checkpoint-deficient cells. Interestingly, in HeLa cells the level of the Cdc13 homologue cyclin B1 is reduced after treatment with ionizing radiation due to a reduction in its mRNA level (Kao et al., 1997; Maity et al., 1996). A similar effect on cyclin B1 has also been seen after treatment of brain tumour cells with camptothecin, another DNA-damaging agent (Janss et al., 2001). Overexpression of cyclin B1 reduced the G2 delay in irradiated HeLa cells (Kao et al., 1997), consistent with the model that the reduced cyclin level is responsible for the delay. Whereas HeLa cells regulate cyclin B transcript levels in response to DNA damage, we did not see a significant change in the *cdc13* mRNA level in fission yeast after UV irradiation. Thus, it seems that regulation of cyclin levels upon stress is a conserved strategy even if the exact mechanism might be different. It is not known whether cyclin B translation is selectively regulated upon stress in mammalian cells, although there are several reports of temporally and spatially controlled cyclin translation during development and the cell cycle (Eliscovich et al., 2008; Groisman et al., 2000; Richter, 2001). Here, we show that, in fission yeast, translation regulation is the mechanism that reduces the level of Cdc13 and thereby CDK activity after UV irradiation.

Exposure to UV results in global downregulation of translation (Deng et al., 2002; Iordanov et al., 1998; Tvegard et al., 2007), and we show here that the reduction in Cdc13 protein level is brought about by downregulation of translation. We considered the possibility that the reduced level of Cdc13 is merely a result of global translation being reduced, and that the cell cycle delay is a consequence of slow growth in general and slower cell cycle progression. However, the following observations argue against such a model. First, in the absence of Wee1, which is involved in coordinating cell growth with cell division, the delay is not abolished (Fig. 6). Second, the delay can also be observed in a *rad3*Δ *gcn2*Δ mutant (Fig. S3). Gcn2 is activated by several types of stress including UV (Deng et al., 2002; Krohn et al., 2008; Tvegard et al., 2007) and has been implicated in the regulation of both selective and global translation (Hinnebusch, 1994; Wek et al., 2006). Third, another cell-cycle-regulated protein, Cdc25, is not downregulated by the same treatment (Fig. 7C). Interestingly, it has been reported that the levels of both Cdc25 and Cdc13 are particularly affected in a non-functional *tif1* mutant (Daga and Jimenez, 1999), which is deficient in the eukaryotic translation initiation factor 4A, an RNA helicase important for unwinding of complex secondary structures (Andreou and Klostermeier, 2013; Schmid and Linder, 1991). These results suggest that the translation of the corresponding messages is particularly sensitive to a reduction in global translation rates (Daga and Jimenez, 1999). The *cdc25* 5′ untranslated region (UTR) contains secondary structures and upstream open reading frames (uORFs), which is probably the reason for the reduced protein level of Cdc25 in the *tif1* mutant (Daga and Jimenez, 1999). Furthermore, it has been suggested that the *cdc13* UTR shares this feature (Daga and Jimenez, 1999). The increased sensitivity to reduced global translation is most likely conferred by the unusually long 5′UTR of Cdc13. However, we find that most of the putative regulatory region in *cdc13*, which is similar to that in *cdc25*, is in an intron in *cdc13* (data not shown). Previous work has shown that the translation of the B-type cyclins Cig2 and, to a lesser extent, Cdc13, is particularly dependent on the RNA helicase Ded1 (also known as Sum3) (Grallert et al., 2000), consistent with the idea that the long UTR has an inhibitory effect on translation. Thus, the exact molecular mechanism of the selective translational regulation of Cdc13 remains to be investigated further.

Degradation of Cdc13 in anaphase is mediated by the anaphase-promoting complex/cyclosome (APC/C) and is associated with decreased Cdc2 activity and exit from mitosis. However, in mammalian cells it has also been shown that the APC/C can be activated outside mitosis after DNA damage (Bassermann et al., 2008; Sudo et al., 2001). In fission yeast, we did not observe any difference in turnover of Cdc13 between UV-treated and control samples indicating that the APC/C is not activated by UV irradiation.

Here, we have shown that a mechanism additional to the G2/M checkpoint controls entry into mitosis in fission yeast upon UV irradiation. This mechanism involves selective translational downregulation of the mitotic cyclin Cdc13 to inhibit CDK activity. The mechanism described here might have been the first, primitive defence against stress, before checkpoints evolved. Interestingly, the downregulation of cyclin B in response to DNA damage appears to be conserved, and similar findings have been made in several cancer-derived cell lines (Kao et al., 1997; Maity et al., 1996). Many cancer cells have an impaired checkpoint response, and pathways operating in parallel to the G2/M checkpoint might be particularly important for survival in these cancer cells. The knowledge about such mechanisms can be exploited to design effective therapeutic strategies in cancer treatment.

MATERIALS AND METHODS

Fission yeast strains and media

All strains used were derived from the *Schizosaccharomyces pombe* L972h- strain and are listed in Table S1. The growth conditions and media were as described previously (Moreno et al., 1991). The cells were grown in liquid Edinburgh minimal medium (EMM) (Sunrise Science products) containing the required supplements. Inactivation of Ark1 and Fin1 was achieved by adding the inhibitor 4-amino-1-tert-butyl-3-(3-bromobenzyl)pyrazolo[3,4-d] pyrimidine (3-BrB-PP1) (#DC-001467, Dalton Pharma Services and #ab143756, Abcam), at a final concentration of 20 µM to synchronous analogue-sensitive strains. Sty1 was inactivated adding 20 µM 4-amino-1-tert-butyl-3-(3-methylbenzyl)pyrazolo[3,4-d]pyrimidine (3-MB-PP1) (#529582, Calbiochem/Millipore) to the analogue-sensitive strain. Cycloheximide was used at a final concentration of 0.1 mg/ml.

UV irradiation

Cells suspended in a thin layer (3 mm) of rapidly stirred liquid medium were irradiated at 20–25°C with 254-nm UV light. The incident dose was measured with a radiometer (UV Products), and a dose of 1100 J/m^2 was given at a dose rate of ∼250 J/m^2/min. This dose results in >90% survival for log-phase wild-type cells.

Synchronous cells

In all experiments, cells were selected by size, either through lactose gradient centrifugation (Sabatinos and Forsburg, 2010) or by centrifugal elutriation (Hagan et al., 2016). When indicated, we combined elutriation with a 1 h incubation of *cdc25-22* cells at 36°C to further improve synchrony.

Binucleate index

Cells were fixed in ice-cold 70% ethanol at the time points indicated on the figures. They were resuspended in phosphate-buffered saline, mounted

on microscope slides, dried and stained with 0.2 µg/ml DAPI. Imaging was performed on a Leica CTR DM6000 microscope equipped with a Leica DFC350 FX monochrome digital camera and a 63×/1.4 NA objective.

Immunofluorescence

For tubulin staining, samples were fixed in ice-cold methanol (−20°C), and prepared as described previously (Moreno et al., 1991) using the anti-TAT1 antibody (1:100; kindly provided by Keith Gull, University of Oxford, UK. For analyses by immunofluorescence microscopy, cells were processed and visualized as described above. The mitotic index was determined counting those cells that showed a single mitotic spindle.

Immunoblotting

Samples for immunoblotting were prepared with a trichloroacetic acid protein extraction method (Caspari et al., 2000). Protein extracts were run on 10% SDS-PAGE gels, transferred to a PVDF membrane (#IPVH00010, EMD Millipore Corporation) and probed with the following antibodies: anti-α-tubulin (1:30,000; #T-5168, Sigma-Aldrich), anti-phospho-Cdc2 (Tyr15) (1:300; #9111, Cell Signaling); anti-phospho-MAPK/CDK substrates (1:1000; #2325, Cell Signaling); anti-Cdc13[6F11/2] (1:1000; ab10873, Abcam), anti-V5 (1:5000A190-12A, Bethyl Laboratories), anti-phospho-histone H3(Ser10) (1:1000; #06-570, Millipore) and anti-PSTAIRE (1:5000; sc53, Santa Cruz Biotechnology).

Isolation of total RNA for quantitative real-time PCR

Total RNA was isolated by using the MasterPure™ yeast RNA purification kit (Epicentre Biotechnologies) according to the manufacturer's instructions.

Quantitative real-time PCR

cDNA was synthesized from 1 µg total RNA or 1 µg RNA isolated from ribosome fractions with SuperScript™ III reverse transcriptase (Invitrogen) using oligo(dT) primers according to the manufacturer's instructions. PCR was performed with a 1× Power SYBR® Green PCR Master Mix (Applied Biosystems) on an Applied Biosystems 7500 real-time PCR system (Life Technologies). Primer sequences for *cdc13* and the endogenous control *leu1* are listed in Table S2. Each measurement was performed in duplicate and the results averaged. Starting concentrations of mRNA were calculated with the LinRegPCR computer program (Ramakers et al., 2003; Ruijter et al., 2009). All mRNA levels were normalized to the expression of the endogenous control.

Isolation of ribosome-associated RNA

For isolation of RNA from ribosomes, cycloheximide (0.1 mg/ml) was added 30 min after UV irradiation. Samples were prepared as described previously (Swaminathan et al., 2006).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.L., B.G.; Methodology: C.R., S.A.; Validation: C.R., G.E.R., S.A., V.S.; Investigation: C.R., G.E.R., S.A., V.S., S.L.-A.; Writing - original draft: C.R., G.E.R., B.G.; Writing - review & editing: C.R., S.A., V.S., E.B., S.L.-A., B.G.; Supervision: B.G.; Project administration: B.G.; Funding acquisition: E.B., B.G.

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Supplementary information

Supplementary information available online at
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